NUCLEAR SMALL-SUBUNIT RIBOSOMAL RNA GENE-BASED CHARACTERIZATION, MOLECULAR PHYLOGENY AND PCR DETECTION OF THE NEOPARAMOEBA FROM WESTERN LONG ISLAND SOUND LOBSTER

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ABSTRACT Western Long Island Sound (LIS) lobsters collected by trawl surveys, lobstermen and coastal residents during 2000 to 2002 were identified histologically as infected with a parasite-containing amoeba. Primers to conserved SSU rRNA sequences of parasite-containing amoebae and their nonparasome-containing relatives were used to amplify overlapping SSU rRNA fragments of the presumptive parasite from gill, antenna, antennal gland and ventral nerve cord of infected lobsters. The consensus sequence constructed from these fragments had 98% or greater nucleotide sequence identity with SSU rRNA gene sequences of strains of Neoparamoeba pemaquidensis and associated with high confidence in distance- and parsimony-based phylogenetic analyses with strains of Neoparamoeba pemaquidensis and not members of the family Paramoebidae, e.g., Paramoeba elحت. Primers designed to SSU rRNA sequences of the lobster amoeba and other paramoebid/vexilliferid amoebae were used in a nested polymerase chain reaction (PCR) protocol to test DNA extracted from formalin-fixed paraffin-embedded tissues of lobsters collected during the 1999 die-off, when this amoeba initially was identified by light and electron microscopy and reported to be a paramoeba of the genera Paramoeba or Neoparamoeba (Mullen et al. 2004). All sequences amplified from 1999 lobsters, with the exception of one, had 98% to 99% identity to each other, and the 1999 PCR product consensus had 98% identity to Neoparamoeba pemaquidensis strains CCAP 1560/4 (AF371960.1) and 1560/5 (AF371970.1). Molecular characterization of the amoeba from western LIS lobsters by direct amplification circumvents a collective inability to culture the organism in vitro, provides insight into the molecular epidemiology of neoparamoebiasis in American lobster, and allows for PCR-based detection of infected lobsters for future research and diagnostics.

KEY WORDS: Homarus americanus, lobster, molecular phylogeny, Neoparamoeba pemaquidensis, paramoebiasis, PCR, small-subunit rRNA

INTRODUCTION

A few species of naked lobose amoebae (gymnamoebae) are considered to be parasitic to shellfish. Gymnamoebae belonging to the paramoebid/vexilliferid (PV) lineage (Peglar et al. 2003); families Paramoebidae and Vexilliferidae as defined by Page (1987) and Page & Siemensa (1991) have been associated with “grey crab disease” of blue crabs, Callinectes sapidus (Johnson 1977) and with a wasting disease of green sea urchins, Strongylocentrotus droebachiensis (Jones & Scheibling 1985). Both diseases have caused significant, often catastrophic, economic losses. In addition, one PV lineage species, Neoparamoeba pemaquidensis, is the etiologic agent of amoebic gill disease, a commercially important disease of aquaculture-reared finfish (Munday 1986, Munday et al. 2001) including Atlantic salmon, Salmo salar (Roubal et al. 1989, Zilberg et al. 2001, Adams & Nowak 2003, Adams & Nowak 2004, Butler & Nowak 2004), cohoh salmon, Oncorhynchus kisutch (Kent et al. 1988), turbot, Scophthalmus maximus (Dyková et al. 1995, 1998, Fiala & Dyková 2003), European sea bass, Dicentrarchus labrax (Dyková et al. 2000) and sea bream, Sparus auratus (Athanassopoulou et al. 2002, as S. aurata).

Though infective gymnamoebae have previously been reported in tissues of the American lobster, Homarus americanus (H. Milne Edwards, 1837) (Sawyer 1976, Sawyer & MacLean 1978), they were not associated with either demonstrable pathology or epizootic disease. This changed in 1999, with the mass lobster die-off and subsequent collapse of the natural lobster fishery in western Long Island Sound (LIS). The lobster die-off has been attributed, in part, to infection by a parasite-containing amoeba (Mullen et al. 2004). The identity of this amoeba is unknown, yet knowledge of its identity is essential in determining its origin in LIS and in lobsters.

The identity of the amoebae infecting western LIS lobster is unknown because criteria previously used to identify these amoebae have now been shown to be inadequate. The parasite, sometimes referred to as a “secondary nucleus” because it contains DNA and superficially resembles the authentic cell nucleus (Grell 1961, Grell & Benwitz 1970, Perkins & Castagna 1971), has been considered diagnostic for the genera Paramoeba and Neoparamoeba (Page 1987). Both genera belong to the PV lineage (Peglar et al. 2003), and both genera contain species known or strongly suspected to be pathogens of fish and shellfish. However, the parasite has been shown to be a parasitic protozoon, genus Perkinsella (Hollandse 1980, Dyková et al. 2003), and species of this parasite are present in amoebae that are unlikely to belong to the PV lineage (Hollandse 1980). Moreover, species of Paramoeba and Neoparamoeba have been differentiated on the basis of submicroscopic structures on their cell surfaces (Page 1987, Page & Siemensa 1991): scales on Paramoeba species (Grell & Benwitz 1970), glycostyles on Neoparamoeba species (Cann & Page 1982, Page 1987). The amoeba from lobster, however, has neither scales nor glycostyles on its cell surface (Mullen et al. 2004), a feature that it shares with tissue-borne forms of the pathogenic amoebae from blue crab (Perkins & Castagna 1971) and urchin (Jones 1985). Therefore, available characters from neither light nor electron microscopy are sufficient to identify the lobster-borne amoeba or, for that matter, the crab- and urchin-borne amoebae.
Identifying the amoeba from lobster, comparing it with other amoebae responsible for diseases of fish and shellfish and understanding its pathogenicity are inhibited by the episodic nature of disease outbreaks, a lack of cultures and a lack of knowledge about the fundamental biology of marine gymnamoebae in general and of members of the PV clade in particular. To date, efforts to isolate the lobster amoeba into in vitro culture have not been successful (Mullen et al. 2004). Efforts to culture the blue crab pathogen have likewise been unsuccessful (Johnson 1977). Cultures of the urchin pathogen were achieved (Jones 1985, Jellett & Scheibling 1988a, Jellett & Scheibling 1988b) but not archived. The only cultures of Paramoeba and Neoparamoeba species available when this study began were of free-living amoebae isolated from water and sediment samples (e.g., Grell 1961, Cann & Page 1982) or from the gills of finfish (e.g., Kent et al. 1988).

Efforts to characterize gymnamoebae at the molecular level are in their infancy, especially for marine species. Phylogenetic studies, mostly based on sequences of the nuclear-encoded small-subunit ribosomal RNA (SSU rRNA) gene, have discovered new lineages and significantly rearranged existing ones (Sims et al. 1999, 2002, Amaral-Zettler et al. 2000, 2001, Bolivar et al. 2001). Peglar et al. (2003) were the first to demonstrate the existence of the PV lineage, phylogenetically distinct from all other groups of amoebae. Molecular tools are beginning to be used to probe Neoparamoeba strains associated with amoebic gill disease (Fiala & Dyková 2003, Wong et al. 2004), but otherwise, the data and procedures that would permit DNA-based identifications of gymnamoebae in the environment, in mixed cultures or in infected host tissues, are still lacking or insufficient.

This article reports efforts to characterize the amoeba present in the tissues of western LIS lobsters based on direct amplification and sequence analysis of the amoebal nuclear SSU rRNA gene. In addition to presenting the first epidemiologic data of this emerging disease of LIS lobster, this report communicates the identification of a consensus SSU rRNA gene sequence representative of the amoeba infecting western LIS lobsters, its molecular phylogenetic characterization and the development and application of a nested PCR protocol to detect this and similar SSU rRNA gene sequences in lobster tissue.

MATERIALS AND METHODS

Sample Collection

Lobsters were collected during trawl surveys conducted by Connecticut Department of Environmental Protection officers as part of the State’s Long Island Sound Zoning Project (including only zones 1 and 2), or by independent submission of dead and dying, “limp,” lobsters by fishermen and biologists from autumn 2000 through autumn 2002. Animals were euthanized using 100 mg of KCl/100 g of body weight as previously described (Battison et al. 2000). Tissues including antenna, antennal gland, appendages, carapace, compound eye, gill, gonad, heart, hepatopancreas, intestine, mandibular apparatus and tail muscle were collected for histopathologic examination, while antenna, antennal gland, gill and ventral nerve cord were subsampled for molecular biologic analysis.

Tissue samples of lobsters from the 1999 die-off were obtained from paraffin blocks prepared as described by Mullen et al. (2004). Briefly, dead and moribund lobsters from western LIS (zone 1) were collected from late October through early December 1999, and tissues including antenna, antennal gland, compound eye, hepatopancreas, gonad, stomach, intestine, gill, carapace and ventral nerve cord were fixed in 10% neutral buffered formalin, decalcified using Bouin’s fixative and processed routinely for paraffin embedding.

Histopathology

Soft tissues for histopathologic examination were fixed by immersion in 10% neutral buffered formalin for 24 to 48 h. Hard tissues (e.g., antennae and eye) were placed in 5% trichloroacetic acid solution overnight then returned to formalin. Tissues were routinely processed for paraffin embedding, sectioned at 4 μm, mounted on glass slides and stained using hematoxylin and eosin (H&E). Slides were examined using bright field microscopy, and lobsters were identified as being infected with paramoebae based on published morphologic criteria describing paramoebiasis in western LIS lobsters (Mullen et al. 2004).

DNA Isolation

Tissues for nucleic acid studies were placed into 800 μL DEPC-treated water (ResGen, Carlsbad, California) at necropsy and snap frozen in liquid nitrogen for storage. Tissue samples from specimens diagnosed as infected with paramoebae were thawed on ice, minced, and approximately 25–50 mg of tissue placed into 400 μL ATL buffer (Qiagen Inc., Chatsworth, California) containing 40 μL of 20 mg/ml (≥600 mU/ml) proteinase K (Qiagen Inc., Chatsworth, California). Gills received more rigorous mincing and often sterile, plastic mortar and pestles (Fisher Scientific, Hampton, New Hampshire) were used to mechanically break down this tissue and facilitate digestion. Lysates were incubated at 55°C in a dry bath overnight and, if necessary, additional proteinase K was added and longer times were used to complete digestion. To improve the quality and quantity of DNA extracted from gill and antennae, these tissues were commonly allowed longer digestion times, typically 48 h. Genomic DNA was isolated using silica-gel spin-column technology (DNeasy DNA Extraction System, Qiagen, Inc., Chatsworth, California), and total DNA was eluted from spin columns in 100–200 μL volumes. Total genomic DNA was quantified by either spectrophotometry using a Hoechst 33258 spectrophotometer (Bio-Rad Laboratories, Hercules, California) or by fluorometry using a Bio-Rad VersaFlour Fluorometer (Bio-Rad, Hercules, California).

Genomic DNA Quality Control PCR Amplification

To determine the efficacy of genomic DNA extraction in isolating amplifiable DNA for downstream experimentation, all lobster tissues were tested for positive PCR amplification using universal primers to eukaryotic small-subunit ribosomal RNA gene sequences. Primers 18e and 18h were used to amplify an approximating amplifiable DNA for downstream experimentation, all lob-
positive control. Products were separated on either 1.5% or 2% agarose gels and visualized by ethidium bromide staining and UV transillumination. Samples that resulted in no amplification were eliminated from the study.

**Direct Amplification of Paramoeba SSU rRNA Gene Sequences**

Small-subunit rRNA gene sequences of the paramoeba infecting western LIS lobsters were amplified directly from tissues of lobsters identified by histopathologic examination as infected with paramoebae. SSU rRNA gene sequences from 8 strains of paramoebid/vexilliferid amoebae, *Korotnevella hemistylolepis*, O’Kelly et al. 2001 (ATCC 50804); *Korotnevella stella* (Schaeffer 1926) Goodkov 1988 (CCAP 1547/6); *Neoparamoeba aestivalina* (Page 1970) Page 1987 (CCAP 1560/7); *N. pemaquidensis* (Page 1970) Page 1987 (CCAP 1560/4, ATCC 30735, ATCC 50172); *Paramoeba eilhardi* Schaudinn 1896 (CCAP 1560/2) and Pseudoparamoeba pagei (Sawyer 1975) Page 1979 (CCAP 1560/2), (Table 1), were aligned with each other and with the SSU rRNA gene sequence of the American lobster, *Homarus americanus*, (GenBank AF235971; Ragan et al. 1996), using the multiple sequence alignment functions of DNAMAN (Lynnon Biosoft, Quebec, Canada) and Vector NTI (InfoMax Inc., Frederick, Maryland). Primer sequences were designed to hybridize with variable and conserved regions of SSU rRNA gene sequences from the amoebae that did not share significant nucleotide identity with the lobster SSU rRNA gene (Table 2). Three sets of nested primer pairs were designed in this manner to amplify overlapping regions of amoebal SSU rRNA genes that included a 5’, an internal 1.3-kb and a 3’ region (Table 2).

To avoid cross-reactivity with genomic DNA of actual and potential hosts, and to confirm amplification from representative amoebae, specificity was assessed by testing first and second-round primer pairs separately and then in tandem in PCR protocols using genomic DNA from lobster (*Homarus americanus*), blue crab (*Callinectes sapidus*) and sea urchin (*Strongylocentrotus droebachiensis*), along with genomic DNA from *Neoparamoeba pemaquidensis* (ATCC 50172), *Korotnevella stella* (CCAP 1560/6) and *Paramoeba eilhardi* (CCAP 1560/2). Sensitivity was assessed by using serial 10-fold (10−2–10−6) dilutions of cloned *Neoparamoeba pemaquidensis* (CCAP 1560/4) SSU rRNA gene added to infection-negative genomic lobster DNA.

Direct amplification of SSU rRNA gene sequences of parasitic paramoebae used total genomic DNA isolated from lobsters determined to be infected in one or more tissues by histopathologic analysis and tested for DNA of sufficient quality to support host SSU rRNA gene amplification. Tissue samples were tested in triplicate using the three sets of nested primer pairs. Because the internal 1.3-kb region accounted for approximately 60% of the SSU rRNA gene sequence of the amoeba, and because it also

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**TABLE 1.**

Amoebae representative of paramoebid/vexilliferid and vannellid clades used in small-subunit rRNA gene sequence comparisons for primer design (*) and molecular phylogenetic analyses. SSU rRNA gene sequences of the amoebae in boldface were determined in preliminary investigations in support of this study. Authorities are provided beneath the first-appearing entry for each species.

<table>
<thead>
<tr>
<th>Genus and Species</th>
<th>Source Designation</th>
<th>GenBank Number</th>
</tr>
</thead>
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<td><em>Neoparamoeba aestivalina</em></td>
<td>ATCC 50805</td>
<td>AY121851.1</td>
</tr>
<tr>
<td><em>Neoparamoeba aestivalina</em></td>
<td>ATCC 50806</td>
<td>AY121852.1</td>
</tr>
<tr>
<td><em>Neoparamoeba aestivalina</em></td>
<td>CCAP 1560/7</td>
<td>AY686574</td>
</tr>
<tr>
<td><em>Neoparamoeba pemaquidensis</em></td>
<td>ATCC 50172</td>
<td>AF371971.1</td>
</tr>
<tr>
<td><em>Neoparamoeba pemaquidensis</em></td>
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<td>AF371972.1</td>
</tr>
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<td><em>Neoparamoeba pemaquidensis</em></td>
<td>ATCC 30735</td>
<td>AF371972.1</td>
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<tr>
<td><em>Neoparamoeba pemaquidensis</em></td>
<td>ATCC 30735</td>
<td>AF371972.1</td>
</tr>
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<td>AF371970.1</td>
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<tr>
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<td><em>Paramoeba eilhardi</em> Schaudinn, 1896</td>
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<td><em>Pseudoparamoeba pagei</em> (Sawyer, 1975) Page, 1979</td>
<td>CCAP 1566/2</td>
<td>AY686576</td>
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<td><em>Korotnevella hemistylolepis</em> O’Kelly et al., 2001</td>
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<td>ATCC 50819</td>
<td>AY121854.1</td>
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<tr>
<td><em>Korotnevella stella</em> (Schaeffer, 1926)</td>
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<td>AY686573</td>
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<td><em>Vannella aberdonica</em> Page, 1980</td>
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<td><em>Vexillifer armata</em> Page, 1979</td>
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<tr>
<td><em>Clydonella</em> sp.</td>
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<tr>
<td><em>Clydonella</em> sp.</td>
<td>ATCC 50816</td>
<td>AY183890.1</td>
</tr>
</tbody>
</table>

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**TABLE 2.**

Forward (-F) and reverse (-R) primer sequences and combinations used in nested PCR to amplify SSU rRNA gene sequences from paramoebae infecting western LIS lobster.

<table>
<thead>
<tr>
<th>Target Region</th>
<th>Primer Name</th>
<th>Primer Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-terminus 1st round</td>
<td>733-F</td>
<td>CTGGTGTGTCCTGCAAGTATGTC</td>
</tr>
<tr>
<td>5’-terminus 1st round</td>
<td>733-R</td>
<td>CATGAAAGTCTAGTAAGGAGAWG</td>
</tr>
<tr>
<td>5’-terminus 2nd round</td>
<td>673-F</td>
<td>GCTTGCTTAAGACTAAGCC</td>
</tr>
<tr>
<td>5’-terminus 2nd round</td>
<td>673-R</td>
<td>CAAACATACCTTGTACAAGGCAGT</td>
</tr>
<tr>
<td>Internal 1st round</td>
<td>1609-F</td>
<td>ATRAACACTTGTACTTGTG</td>
</tr>
<tr>
<td>Internal 1st round</td>
<td>1609-R</td>
<td>GCCCTAAACTTCCCTGTGAAAAAC</td>
</tr>
<tr>
<td>Internal 2nd round</td>
<td>1277-F</td>
<td>GAGAGGGAGCCTTGAGAAA</td>
</tr>
<tr>
<td>Internal 2nd round</td>
<td>1277-R</td>
<td>CCCCCATAGGTCTTATCG</td>
</tr>
<tr>
<td>3’-terminus 1st round</td>
<td>570-F</td>
<td>AAAGCTTTGATGTCGTCTT</td>
</tr>
<tr>
<td>3’-terminus 1st round</td>
<td>570-R</td>
<td>CGGATAACCTTTGACCATTT</td>
</tr>
<tr>
<td>3’-terminus 2nd round</td>
<td>405-F</td>
<td>GTTATATTTGATCTTTCG</td>
</tr>
<tr>
<td>3’-terminus 2nd round</td>
<td>405-R</td>
<td>GCTTACCCGAGGAAACAAATCG</td>
</tr>
</tbody>
</table>
included the majority of variable sites within the gene, this target was amplified first. DNA samples were amplified in 50-µL reactions containing 100–300 ng sample DNA, 1× Qiagen PCR Buffer (Tris-HCl, KCl, (NH₄)₂SO₄, 1.5 mM MgCl₂, pH 8.7; Qiagen Inc., Chatsworth, California), 200 µM dNTPs, 10 pmol each forward and reverse primer and 0.2 U HotStar Taq DNA polymerase (Qiagen Inc., Chatsworth, California) using an initial 15-min heat activation step followed by cycling protocols individually optimized for each nested set of primer pairs. The following conditions for both first and second round reactions were used: (1) for primer pairs 733-F/733-R and 673-F/673-R, 40 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min; (2) for primer pairs 1609-F/1609-R and 1277-F/1277-R, 40 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 2 min; (3) for primer pairs 570-F/570-R and 405-F/405-R, 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, extension at 72°C for 45 sec. A final extension step at 72°C for 7 min concluded each PCR protocol. Two microliters of the first round amplification was carried over to the second round. From the second round amplification, 18 µL of amplified product was electrophoresed through a 10% polyacrylamide gel, detected by means of ethidium bromide staining and UV transillumination, then photodocumented. Genomic DNA from lobster,urchin and/or blue crab served as negative controls, whereas purified plasmid DNA containing the complete SSU rRNA gene from Neoparamoeba pemaquidensis (CCAP 1560/4) served as positive control.

**Cloning, Sequencing and Assembly**

Amplicons were purified into 10 µL volumes using commercially available DNA purification reagents (QIAquick Mini-Elute PCR purification/gel extraction kit, Qiagen Inc., Chatsworth, California). Purified products were ligated independently of one another into the pCR II-TOPO vector, transformed, and cloned using the TOPO TA cloning kit with dual promoter (Invitrogen Corp., Carlsbad, California). Multiple clones from each independent cloning reaction were grown for at least 24 h in LB broth containing 75 mg/mL ampicillin using a rotating (200 rpm) 37°C incubator. Plasmids were isolated using the QIaprep Spin Miniprep kit (Qiagen Inc., Chatsworth, California), screened by PCR for confirmation amplification and length polymorphism analysis of a single product insert, then one clone representing each independently cloned PCR product was submitted for bidirectional sequencing by primer walking (HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine, New Haven, Connecticut). Nucleotide sequences were determined by oligonucleotide-directed dideoxynucleotide chain-termination sequencing reactions utilizing 300 ng of template DNA, forward (M13F) and reverse (M-13R) primers, fluorescently-labeled dideoxynucleotides and AmpliTaq FS DNA polymerase in a cycling sequencing method. The resultant fragments were subjected to electrophoresis and analyzed using an automated Applied Biosystems 377 DNA sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, California).

Sense and antisense sequence ABI files from each clone were assembled using Sequencher 4.1.1 for Macintosh (Gene Codes Corp., Ann Arbor, Michigan). A sequence for each clone from each tissue of each host lobster was constructed, and contigs were assembled to yield consensus SSU rRNA gene sequences from each host lobster. Additionally, contigs from each target region were assembled from all host lobster yielding products to construct an overall consensus SSU rRNA gene sequence of the paramoeba from western LIS lobster. This overall consensus SSU rRNA gene sequence, as well as one near-full-length SSU rRNA gene sequence obtained by contig alignment of products from one tissue source of one host lobster, were used in nucleotide-nucleotide BLAST searches of the National Center for Biotechnology Information (NCBI) GenBank database and in molecular phylogenetic analyses.

**Nucleotide Sequence Accession Numbers**

SSU rRNA gene sequences representing the amoeba infecting western LIS lobster derived from single-source, lobster 01-9828-7 gill, or multisource, overall consensus, origins are available in the GenBank database under accession numbers AY686577 and AY686578, respectively. SSU rRNA gene sequences of *Paramoeba eilhardi* CCAP 1566/2, *Pseudoparamoeba pagei* CCAP 1566/2, *Neoparamoeba aestuaria* CCAP 1560/7, *Corotonevella stella* CCAP 1547/6 were determined previously in support of this study and are available in the GenBank database under accession numbers AY686575, AY686576, AY686574 and AY686573, respectively (Table 1).

**Molecular Phylogenetic Analysis**

Both the single-source and overall consensus SSU rRNA gene sequences representing the paramoeba from western LIS lobster were included in multiple sequence alignments constructed using ClustalX v1.81 (Thompson et al. 1997) and comprising the SSU rRNA gene sequences of representative gymnamoebae of the amoeboid/vexilliferid and vannellid clades (Peglar et al. 2003), (Table 1). Phylogenetic trees were inferred by distance and parsimony algorithms using PAUP* 4.0b10 (Swofford 2002). The data set consisted of 25 taxa with 2,537 total characters, of which 893 were phylogenetically informative in parsimony analysis, and 244 were variable but parsimony-uninformative. A bootstrapped maximum parsimony tree was generated using heuristic search parameters. The confidence of branching was assessed using 1,000 bootstrap resamplings of the data set with a starting tree obtained via random stepwise addition and tree-bisection-reconnection branch-swapping. A distance-based phylogenetic tree was reconstructed by 1,000 bootstrap resamplings of the dataset using the minimum evolution optimality criterion and the HKY85 substitution model (Hasegawa et al. 1985) with among site rate variation assumed to follow a gamma distribution of 0.5. Missing data (gaps) were ignored for sites affected in pairwise comparison, and negative branch lengths were allowed, but collapsed to zero. Heuristic search parameters were used and starting tree(s) were obtained by neighbor-joining followed by tree-bisection-reconnection branch-swapping.

**PCR Detection and Characterization of Paramoeba from 1999 Lobsters**

Primers for PCR were designed to incorporate or span variable regions of SSU rRNA sequences to detect known PV clade amoebae and distinguish them by sequence analysis of amplicons. Comparative sequence alignments were performed using the SSU rRNA gene sequences representing the paramoeba from western LIS lobster along with the following PV clade amoebae, *Neoparamoeba pemaquidensis* (ATCC 50172), *Neoparamoeba pemaquidensis* (CCAP 1560/4), *Neoparamoeba pemaquidensis*
(ATCC 30735), *Paramoeba eilhardi* (CCAP 1560/2), *Pseudoparamoeba pagel* (CCAP 1566/2), *Korotnevella hemistylolepis* (ATCC 50804), *Korotnevella stella* (CCAP 1547/6), *Neoparamoeba aestuarina* (CCAP 1560/7), (Table 1), and the SSU rRNA gene sequence of lobster. SSU rRNA gene sequences were aligned using the multiple sequence alignment functions of DNAMAN (Lynnon Biosoft, Quebec, Canada) and Vector NTI (InfoMax Inc., Frederick, Maryland). Primers were designed to sequences that included or spanned selected variable regions. A nested PCR approach was adopted to accommodate density and distribution of paramoebae in tissue section. First and second round primer pairs were selected based on the following criteria: (1) ability to amplify SSU rRNA genetic sequences from total genomic DNA of PV clade amoebae; (2) inability to amplify genomic DNA of lobster, blue crab and sea urchin and (3) amplicon size less than 500-bp to accommodate amplification from formalin-fixed paraffin-embedded (FFPE) tissues.

The study examined 170 paraffin-embedded tissue blocks from 77 lobsters collected during the die-off of 1999; of these 170 paraffin blocks, 144 blocks were identified by histopathologic examination as having one or more tissues infected with paramoebae.

Fifty-micron tissue sections were obtained from each paraffin block under study. DNA was extracted using the DNeasy DNA Extraction System (Qiagen Inc., Chatsworth, California) according to DNA isolation methods previously described for fresh tissues, with the following amendments to accommodate paraffin-embedded samples. FFPE tissues were deparaffinized using three 800-µL washes of xylene, followed by three 800-µL washes of 100% ethanol, then allowed to air dry in a biologic safety cabinet until chalky white. After drying, tissues were digested using lysis buffer and proteinase K, and DNA was extracted using silica gel spin columns according to the same protocol used for DNA isolation from fresh tissues. Extracted DNA from each paraffin block was individually quality-control tested by positive PCR amplification from fresh tissues. Extracted DNA from each paraffin block was individually quality-control tested by positive PCR amplification of a 440-bp eukaryotic SSU rRNA genetic sequence using primers 18e and 18h (Hillis & Dixon 1991) in 50-µL reactions as previously described. DNA samples that failed quality control amplification were eliminated from the study.

DNA samples from 1999 FFPE tissues that passed quality control assessment were tested for presence of paramoeba DNA using a nested PCR protocol that used Para A (5’-AACCTGCTTGTGA-CAAGGTATGTTTG-3’) and Para C (5’-CGCCACTCCAAAGAAGTGACT-3’) as first round primers and Para B (5’-CATCCTGTTACTAGTCTTATC-3’) and Mullen B2 (5’-GAAGCTAGATTCTARTGAATA-3’) as second round primers. DNA samples were tested in triplicate using 50-µL reactions containing an estimated 10-50 ng sample DNA, 1X Qiagen PCR Buffer (Tris-HCl, KCl, (NH4)2SO4, 1.5 mM MgCl2, pH 8.7; Qiagen Inc., Chatsworth, California), 200 µM dNTPs, 10 pmol each forward and reverse primer and 0.2 U HotStar Taq DNA polymerase (Qiagen Inc., Chatsworth, California). Reactions were initiated by a 15-min heat activation step followed by 40 cycles optimized for each round. First round reactions were cycled at 30 sec denaturation at 94°C, 45 sec annealing at 54°C, and 45 sec extension at 72°C; second round reactions were cycled at 30 sec denaturation at 94°C, 45 sec annealing at 50°C, and 45 sec extension at 72°C. Each round was completed by a 7-min extension at 72°C. Two microliters of the completed first round reaction mixture was transferred to the second round PCR using the stated reaction conditions. A 12% polyacrylamide gel was used to separate the second round PCR products, and bands were visualized using ethidium bromide staining and UV transillumination, then photodocumented.

PCR products were purified, cloned using the previously described protocol, then submitted for oligonucleotide-directed dideoxynucleotide chain-termination sequencing reactions initiated by M13 forward and reverse primers (HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine, New Haven, Connecticut). Nucleotide sequences were determined by sense and antisense ABI sequence files using Sequencher 4.1.1 for Macintosh (Gene Codes Corporation, Ann Arbor, Michigan) and aligned using Clustal X v1.81 (Thompson et al. 1997).

**RESULTS**

**Sample Analysis**

A total of 240 lobsters were examined from autumn 2000 through autumn 2002. The disease during this period had a low prevalence, and in histopathologic terms was pauci-organismal and multifocal (i.e., a small number of amoebal cells in few and scattered foci).

Histologic examination resulted in the identification of 13 animals positive for the presence of parasome-containing amoebae in tissues, providing a prevalence estimate of 5% for the 3-y period. Three additional lobsters had probable infections, in which amoebal cells had distinctive nuclei, but clear demonstration of parasites could not be made. Two more lobsters had suspect infections, in which amoeba-like bodies were found, but clear demonstration that these bodies contained either nucleus-like or parasome-like organelles could not be made. Paramoeba-positive lobsters were identified in collections from both offshore and inshore waters. Seven of the 13 infection-positive lobsters were collected in autumn months (September to November), whereas the remainder were collected in late winter and spring (February to May; Table 3). Paramoebae were identified most often in antennae (11 of 13 lobsters), followed by eye (5 of 13), gill (3 of 13), ventral nerve cord (2 of 13) and antennal gland (1 of 13; Table 3).

Quantification of total genomic DNA and amplification of host SSU rRNA gene sequences for quality control assessment were performed on all extracted lobster tissues. Most tissue extractions yielded 5 to 20 µg (100–400 ng/µl) in 50 µL of genomic DNA, and most DNA extractions (approximately 90%) amplified using the eukaryotic SSU rRNA gene amplification protocol. However, it was observed early in the study that DNA extractions from gill and antenna yielded low quantities of total genomic DNA, and some gill extractions resulted in no SSU rRNA gene amplification. The quality and quantity of DNA extracts from gill and antennae were improved by longer digestion times with added protease K. However, these samples and any others that resulted in no amplification were eliminated from the study.

**Direct Amplification of Paramoeba SSU rRNA Gene Sequences**

Three nested PCR primer sets were targeted to three overlapping regions of the SSU rRNA gene of PV clade amoebae, a 673-bp fragment from the 5’-terminus, an internal 1277-bp fragment, and a 405-bp fragment from the 3’-terminus (Fig. 1). The nested PCR procedure targeted to the internal 1.3-kb region successfully amplified SSU rRNA sequences from the several PV clade amoebae tested (e.g., *Korotnevella stella, Paramoeba eilhardi* and *Neoparamoeba pemaquidensis*). Initial sequence analy-
ses of PCR products from this region suggested the paramoeba from western LIS lobster was a *Neoparamoeba* species. The nested PCR procedures targeted to the 5’ and 3’ termini of the SSU rRNA gene specifically amplified SSU rRNA sequences from *Neoparamoeba pemaguidensis*; however, some PV clade amoebae were not available for testing (e.g., *P. eilhardi*). None of the nested PCR procedures amplified regions from the total genomic DNA of lobster, sea urchin or blue crab.

**SSU rRNA Gene Sequence Assembly and Characterization**

Ribosomal RNA gene sequences referable to the three target regions of amoebal DNA were amplified from 6 of the 13 lobsters determined by histopathology to be infected with paramoebae. Sequences were obtained from antenna, antennal gland, gill and nerve cord tissues, with individual animals yielding sequences from one or two of these four tissues. Fifteen sequences were obtained, 3 from the 5’ fragment, 5 from the internal fragment, and 7 from the 3’ fragment. One lobster of the six, identified as 01-9828-7, yielded amplicons for each of the three target regions from a single gill sample (Fig. 2), and a near-full-length SSU rRNA gene sequence was assembled from these amplicons. An overall consensus SSU rRNA gene sequence (GenBank AY686578) was constructed from an alignment of all 15 sequence fragments, including the three overlapping fragments from lobster 01-9828-7 gill tissue, which in turn were also assembled and treated as a single sequence from a single-source (GenBank AY686577).

**Molecular Phylogenetic Analyses**

The single-source and overall consensus sequences representing the SSU rRNA gene sequence of the amoebae from western...
LIS lobsters shared 99% nucleotide sequence identity to each other and demonstrated 98% or greater nucleotide sequence identity in nucleotide BLAST searches with SSU rRNA gene sequences of *Neoparamoeba pemaquidensis* strains. Phylogenetic analyses (Fig. 3) also supported this result, robustly placing the two sequences as sister taxa to each other and in a clade consisting exclusively of *Neoparamoeba pemaquidensis* sequences. Within this clade, sequences were very closely related but nonidentical. In fact, two nonidentical sequences were isolated, by different investigators, for each of three *N. pemaquidensis* strains, CCAP 1560/4, ATCC 30735 and ATCC 50172. The sequences from the ATCC strains are sister taxa, but the CCAP 1560/4 isolates are not (Fig. 3). Relationships among the strains of *Neoparamoeba pemaquidensis* were generally unresolved except for a clade that included the WLIS sequences plus those from strains CCAP 1560/4, CCAP 1560/5, and ATCC 50172. A clade composed of strains identified as *N. aestuarina* was also consistently recovered in the analysis.

Distance and parsimony analyses, moreover, recovered a monophyletic PV clade, within which the *Neoparamoeba* sequences were nested. *Paramoeba eilhardi* branched as a sister taxon to the neoparamoebae. Two other subclades were also reconstructed, one consisting of *Korotnevella* species, the other of the species *Vexillifera armata* and *Pseudoparamoeba pagei*.

**PCR Detection and Characterization of Paramoeba from 1999 Lobsters**

Primer pairs were selected based on comparative sequence alignments of PV amoebae and tested separately against genomic DNA of PV amoebae and hosts (lobster, blue crab and sea urchin) to determine possible combinations for nested PCR (Fig. 4A). The first round primer pair, Para A & Para C, amplified all the PV amoebae tested with the exception of *Pseudoparamoeba pagei* CCAP 1566/2 and *Neoparamoeba pemaquidensis* ATCC 50172, whereas second round primer pair, Para B & Mullen B2, amplified all PV amoebae tested with the exception of *Korotnevella hemistylolepis* ATCC 50804. Neither of these first or second round primers amplified genomic DNA from lobster, blue crab and sea urchin, which had been quality control tested for its ability to support PCR by successful amplification of SSU rRNA gene sequences in preliminary PCRs. The resultant nested PCR protocol utilizing Para A/Para C and Para B/Mullen B2 generated a 165-bp product from the PV amoebae tested without cross-reactivity with genomic DNA of lobster, blue crab or sea urchin.

Of the 170 paraffin blocks that underwent DNA extraction, representing 77 lobsters collected during the 1999 die-off, 39 supported PCR amplification of a 440-bp eukaryotic SSU rRNA gene sequence during quality control testing. Of these 39, representing 30 lobsters, 11 yielded 165-bp amplicons using the nested PCR protocol and 28 did not (Fig. 4B). All 39 paraffin blocks contained tissues that were histologically positive for parasome-containing amoebae.

Nucleotide sequences of the 11 amplicons were determined individually and independently of each other. Single nucleotide differences at several sites allowed separation of the 11 nested PCR product sequences into 6 groups. The nucleotide sequence identity between the groups was 98% to 99%, with the exclusion of one sequence that was 94% to 96% identical with the other 1999 nested products (Fig. 5). The consensus nucleotide sequence, generated by alignment of all 11 samples, and the sequences of all six “groups,” from which the consensus was constructed, were most
closely related on the basis of BLAST search scores and percent nucleotide identity with strains of *N. pemaquidensis* (93% to 98% identity), the next nearest matches being with strains of *N. aestuarina* (<90% identity). The closest matches (98% identity) were to sequences from CCAP strains 1560/4 and 1560/5, the sequences most closely related to the consensus sequence from 2001 to 2002 lobsters (Fig. 3). The consensus nucleotide sequence from the 1999 samples had 95% identity with the single- and multi-source SSU rRNA gene sequences from the 2001 to 2002 lobsters; however, the sequence of the exceptional group, group 4 (Fig. 5), from
Figure 4. Paramoebid-Vexilliferid (PV) nested PCR primer alignments and PCR products from archival 1999 formalin-fixed paraffin-embedded (FFPE) lobster tissues. A. Comparative sequence alignments used to design PV primers. Dots (.) represent sites wherein there is exact nucleotide identity with the lobster parasite and spaces (-) represent gaps due to the absence of nucleotides at those sites. B. PCR products typical of FFPE tissues of histologically infection-positive lobsters. Lanes 1&2, 99-8438-2B; Lanes 3–5, 99-8438-3A; Lane 6, 50 bp marker; Lane 7, reagent blank; Lane 8, negative control (lobster); Lane 9, positive control (N. pemaquidensis CCAP 1560/4 plasmid cloned SSU rRNA gene); Lanes 10–12, 99-8438-5B; Lanes 13–15, 99-7374-21H; Lanes 16–18, 99-8438-3B; Lanes 19&20, 99-8438-4B.
the 1999 PCR products had 98% identity with the 2001 to 2002 amoebal SSU rRNA sequences.

**DISCUSSION**

**Identity of the Lobster Amoeba**

All DNA sequences obtained, whether from paraffin-embedded tissues collected from 1999 lobsters or from fresh tissues obtained from lobsters collected in 2001 to 2002, indicate that the amoebae present in western LIS lobsters during and after the 1999 die-off belong to the species *Neoparamoeba pemaquidensis*, a species already known to be pathogenic to other shellfish species and to certain marine finfish. No sequences belonging to other species of amoebae were obtained, even though the protocols used were capable of amplifying at least other members of the amoebal PV clade. Instead, partial SSU rRNA sequences from 1999 and 2001 to 2002 lobsters had the highest degree of nucleotide identity with the same subset of *N. pemaquidensis* strains, CCAP strains 1560/4 and 1560/5. The occurrence of *Neoparamoeba* SSU rRNA sequences in 1999 and 2001 to 2002 lobsters, and their relatively high
nucleotide identity (95% to 98%), is evidence of continued, endemic, infection of a subgroup of western LIS lobster in the years after the die-off with one or more similar strains of *N. pemaquidensis*.

The Neoparamoeba sequences isolated from lobster tissues are closely related but nonidentical. The most straightforward interpretation of this result is that several closely related *N. pemaquidensis* strains have participated in the lobster infection, as many as six in the 1999 samples. This interpretation, however, assumes that all populations of nuclear-encoded small-subunit ribosomal RNA genes in a clonal isolate of *N. pemaquidensis* are identical in primary sequence. If sequence microheterogeneity exists, possibly because SSU rDNA loci have not evolved in concert, as is the case in certain other protists (e.g., Reddy et al. 1991, Scholin et al. 1993), then the observed sequence variation will overstate the number of strains present. The isolation of two nonidentical SSU rRNA gene sequences from each of three cultured *N. pemaquidensis* strains suggests that rRNA gene sequence microheterogeneity is commonplace in this species, and ongoing work (O’Kelly & collaborators, unpublished) is consistent with this interpretation. The number of amoebal strains infecting western LIS lobster during the 1999 die-off, and thereafter, may have been as many as 6 or as few as one.

Species of Neoparamoeba, including *N. pemaquidensis*, have been characterized at the ultrastructural level by the presence of glycostyles on the cell surface (Cann & Page 1982, Page 1987). Glycostyles are absent from the surface of the lobster-borne amoeba (Mullen et al. 2004), as they are from the blue crab-borne (Perkins & Castagna 1971) andurchin-borne (Jones 1985) amoebae. This finding would appear to be inconsistent with the molecular results. However, O’Kelly (unpublished) isolated two strains of amoebae from moribund sea urchins in the Gulf of Maine in the autumn of 2002. Partial SSU rRNA gene sequences from these amoebae belong to *N. pemaquidensis*, with high (98% to 99%) BLAST similarity scores to a sequence obtained from strain PA027, a Tasmanian strain isolated from salmon gill lesions. These amoebae produce glycostyles in monoprotist culture. This result suggests that Neoparamoeba amoebae can suppress the production of glycostyles when they are pathogenic within tissues, and therefore the glycostyle character is not reliable for taxonomic or clinical diagnosis of tissue-invasive stages. It also suggests that the species name *Paramoeba invadens* Jones 1985, created for the pathogen of sea urchins, is a junior synonym of *N. pemaquidensis*.

**Origin of the Amoeba in Western Long Island Sound**

*Neoparamoeba pemaquidensis* is considered an amphiizoic protozoan, one that is normally free living but becomes pathogenic under certain circumstances (Scholz 1999). The species was initially isolated as a free-living bacterivorous amoeba from surface sediments (Page 1970), and it is considered to be among the most ubiquitous of marine gymnamoebae (Page 1983). In an examination of the exoskeletal microbiota of lobsters in the Gulf of Maine during July and August 2003, *N. pemaquidensis* strains were isolated frequently (O’Kelly, unpublished). It is possible that *N. pemaquidensis* strains were not only common in western LIS during 1999 but were intimately associated with lobsters.

Sequences of western LIS lobsters were most closely related to sequences from two strains of *N. pemaquidensis* isolated from sediment samples in Wales. It is likely, however, that this circumstance reflects undersampling of this species in sequence datasets, and unlikely that it represents strain migration from one locality to another.

**Infectivity of the Amoeba**

Only in the case of amoebic gill disease of salmon has disease been recreated through experimental exposure to cultured *Neoparamoeba pemaquidensis* (Zilberg et al. 2001); however, Mullen et al. (2004) induced paramoebiosis in healthy Maine lobsters through cohabitation with moribund, “limp,” lobsters obtained from western LIS, thus providing circumstantial evidence for the pathogenicity of one or more LIS strains of *N. pemaquidensis* to lobsters and the ability of the disease to be transmitted from one lobster to another. From the results of this study, and examination of *N. pemaquidensis* infections in other animals, some additional inferences are possible.

It is unlikely that strains of *N. pemaquidensis* can be separated into “pathogenic” and “nonpathogenic” cohorts. In the phylogenetic trees reported here, strains identified as having been isolated from animals with paramoebiosis do not cluster together, but rather are scattered within the clade. Moreover, in both the sea urchin (Jellett & Scheibling 1988b, as *Paramoeba invadens*) and the finfish (e.g., Adams & Nowak 2004) systems, virulence of cultured amoebae is lost over time. Adams and Nowak (2004) speculated that virulence factors are downregulated in the amoeba when they are no longer exposed to target host cells/tissues. Jellett and Scheibling (1988b) reported that the loss of virulence was in part dependent on the bacterial food source used to maintain the cultures and that virulence could be restored by passing the amoebae through sea urchins. In this view, pathogenesis of *N. pemaquidensis* is an inducible phenomenon, and any strain of *N. pemaquidensis* is potentially pathogenic to any susceptible host.

**Progression of Paramoebiosis in Western Long Island Sound**

During the mass mortality event of 1999, paramoebae were identified in neural hemocytic infiltrates in 29 of 31 animals (94%) examined from October 27 to November 10, 1999, and 11 of 38 (29%) animals examined from November 18 to December 3, 1999 (Mullen et al. 2004). However, only approximately 5% of lobsters examined during the 2000 to 2002 sampling period for this study were identified as infected with paramoebae by histologic examination. Furthermore, although qualitative and subjective in its assessment, densities of paramoeba infections in 2000 to 2002 lobster appear to have been much less than those described in 1999 (Mullen et al. 2004). Nevertheless, the observation that 5% of lobsters sampled after the die-off were infected supports the preliminary proposition that this parasite represents an emerging disease in American lobster. Low prevalence and reduced density of paramoeba infections in 2000 to 2002 lobster were factors that limited intersample diversity in generation of single-source PV SSU rRNA gene sequences and were the principal reasons for adopting a nested PCR approach to direct amplification from infected lobster tissues. An accurate prevalence of paramoebiosis in western LIS after the 1999 mortality is not known and may be precluded by an inability to properly assess subpopulations of infected lobster not reliably sampled by conventional trawl survey (e.g., dead or dying lobster in burrows).

**Preliminary Model for the Paramoebiosis Outbreak in Western Long Island Sound**

Douglas-Helders et al. (2003) examined environmental factors associated with amoebic gill disease in salmonids. They found that *Neoparamoeba* densities increased with temperature and with numbers of bacteria in the water column, and that the numbers of bacteria in the water column also increased with temperature.
Summer water temperatures in western LIS in 1999 were well above the 30-y-mean, providing ideal conditions for Neoparmaoeba growth (cf. Scheibling & Hennigar 1997) and borderline conditions for survival and growth of lobsters. Moreover, lobster populations in 1999 were at an historic maximum, suggesting that lobsters were not only heat-stressed but also crowded.

Based on this evidence, one possible model accounting for the occurrence of paramoebiasis as a contributing factor to the 1999 lobster die-off is the following. Under conditions of maximum crowding, maximum temperature stress and maximum Neoparmaoeba pemaquidensis growth experienced by western LIS lobsters in the summer of 1999, one or more strains or groups of strains of the amoeba, perhaps those already associated with the lobster exoskeleton, were able to establish competent infections in stressed lobsters. Further environmental insults, such as those circumstantially associated with the major rain events of August and September 1999, contributed to a larger percentage of stressed and crowded lobsters susceptible to paramoebal infection and presumably, a subpopulation of lobsters that died during the mass mortality died with paramoebiasis (Mullen et al. 2004). The exact percentages of lobsters with paramoebiasis during the 1999 die-off will never accurately be known, given the practical discrepancy between the real numbers of lobsters sampled and the theoretical numbers necessary to achieve significant prevalence and incidence values relative to the geographic area of LIS and the number of dead lobsters reported, which was in the millions. Lower levels of paramoebiasis in subsequent years, 2000 to 2002, reflect declining transmission due to a combination of factors. First, reduced lobster population densities after the die-off have presumably resulted in fewer encounters between lobsters and fewer opportunities for transmission. Second, the relative reduction in host animals may have progressively altered survival pressures on strains of the amoeba, leading to a reduction in the expression of virulence-associated genetic traits. This model, however, supports the assertion that Neoparmaeoeba pemaquidensis is the proximate cause of death of a subpopulation of lobsters in western LIS during the 1999 die-off, and that N. pemaquidensis is present in western LIS lobster at low levels after the die-off. Still to be determined are the identification of virulence factors in pathogenic Neoparmaoeba, the transcriptional regulation of putative virulence-associated genes and the full spectrum of immunomodulatory environmental conditions leading to the inability of lobsters to cope with Neoparmaoeba infection.

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LITERATURE CITED


Characterization and Detection of Lobster Paramoeba


