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Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria*

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ABSTRACT: Quahog Parasite Unknown (QPX) is a protistan parasite that causes disease and mortality in the hard clam *Mercenaria mercenaria*. PCR primers and DNA oligonucleotide probes were designed and evaluated for sensitivity and specificity for the QPX organism specifically and for the phylum Labyrinthulomycota in general. The best performing QPX-specific primer pair amplified a 665 bp region of the QPX small-subunit ribosomal DNA (SSU rDNA) and detected as little as 1 fg cloned QPX SSU rDNA and 20 fg QPX genomic DNA. The primers did not amplify DNA of uninfected hard clams *M. mercenaria* or of the thraustochytrids *Schizochytrium aggregatum*, *Thraustochytrium aureum*, and *T. striatum*. The general labyrinthulomycete primers, which were designed to offer broader specificity than the QPX primers, amplified a 435 bp region of SSU rDNA from QPX, and a 436 to 437 bp region of SSU rDNA from *S. aggregatum*, *T. aureum*, and *T. striatum*, but did not amplify that of the clam *M. mercenaria*. Field validation of the QPX-specific primer pair, through comparative sampling of 224 clams collected over a 16 mo period from a QPX endemic site in Virginia, USA, indicated that the PCR assay is equivalent to histological diagnosis if initially negative PCR products are reamplified. Oligonucleotide DNA probes specific for QPX and the phylum Labyrinthulomycota were evaluated for *in situ* hybridization assays of cell smears or paraffin-embedded tissues. Two DNA probes for QPX offered limited sensitivity when used independently; however, when used together as a probe cocktail, sensitivity was greatly enhanced. The probe cocktail hybridized to putative QPX organisms in tissues of hard clams collected from Virginia, New Jersey, Massachusetts and Canada, suggesting that the QPX organisms in these areas are either very closely related or the same species. The QPX probe cocktail did not hybridize with clam tissue or with the thraustochytrids *S. aggregatum*, *T. aureum*, and *T. striatum*. The labyrinthulomycete DNA probe hybridized with QPX and the 3 thraustochytrids, with no background hybridization to clam tissue. SSU rDNA sequences were obtained for the putative QPX organisms from geographically distinct sites. Phylogenetic analyses based on the QPX and Labyrinthulomycota sequences confirmed earlier reports that QPX is a member of this phylum, but could not definitively demonstrate that all of the QPX organisms were the same species.

KEY WORDS: Hard clams · Disease · QPX · Molecular techniques · PCR · Probe

INTRODUCTION

QPX (Quahog Parasite Unknown) causes a disease of hard clams that has been documented to be present in New Brunswick, Nova Scotia, and Prince Edward Island, Canada, and at various locations in Massachusetts, New Jersey, and Virginia, USA (Drinnan & Henderson 1963, Whyte et al. 1994, Ragone Calvo et al. 1998, Smolowitz et al. 1998, MacCallum & McGladdery 2000). The pathogen has been most problematic in populations of cultured hard clams. Although first

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observed in the late 1950s in New Brunswick, Canada, where it caused high mortalities of wild hard clams (Drinnan & Henderson 1963), it did not become a recognized threat to hard clam aquaculture until the late 1980s and early 1990s. Morphological and molecular evidence suggests that the organism is a member of the phylum Labyrinthulomycota (Whyte et al. 1994, Smolowitz et al. 1998, Maas et al. 1999, Ragan et al. 2000). Members of this phylum are most commonly associated with sediments, vascular plants, benthic algae and detritus in marine and estuarine environments (Porter 1989). A few parasitic species have been identified within this group (McLean & Porter 1982, Jones & O'Dor 1983, Bower 1987).

Based on our present understanding, QPX is an opportunistic facultative parasite that is widespread in high salinity coastal environments. QPX-associated mortalities have been limited to market and near-market sized clams; the pathogen has not been detected in hatchery-produced seed clams (Ford et al. 1997). Outbreaks of QPX disease can be sudden and catastrophic; hard clam crop losses exceeding 80% have been documented in Massachusetts, New Jersey and Virginia (Smolowitz et al. 1998, Ragone Calvo unpubl. data).

The swiftness by which epizootics can emerge, and their potentially devastating impact, necessitates the development of effective disease management strategies for both traditional fisheries and aquaculture. This requires both an understanding of the epizootiology of a particular disease and the ability to perform rapid, sensitive, and accurate diagnosis of disease agents. In recent years DNA-based techniques have enhanced our ability to diagnose marine bivalve diseases and provided a suite of new tools for conducting novel research. The objectives of this study were: (1) to develop sensitive and specific molecular tools, PCR primers and a DNA probe, for the diagnosis of QPX in hard clams; (2) to field validate the PCR QPX diagnostic assay relative to standard histological diagnostic methods; and (3) to utilize the molecular tools to determine the relationship of geographically distinct putative QPX organisms from the United States and Canada.

MATERIALS AND METHODS

Isolation and sequencing of QPX small-subunit ribosomal DNA. *In vitro* cultures of QPX were obtained from Dr. Roxanna Smolowitz (Marine Biological Laboratory, Woods Hole, MA). The cultures were established from QPX cells that were isolated from infected hard clams from Duxbury, Massachusetts, as described by Kleinschuster et al. (1998). QPX genomic DNA was extracted from an aliquot of the culture as previously described for *Perkinsus marinus* cultured cells (Reece et al. 1997). The small subunit ribosomal DNA (SSU rDNA) was amplified by PCR from the QPX genomic DNA using universal SSU primers for eukaryotic organisms (Medlin et al. 1988). PCR reaction mixtures contained reaction buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 10 µg ml^{-1} gelatin), 400 µg ml^{-1} bovine serum albumin, 25 pmol of each primer, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.6 units Ampli*Taq* DNA polymerase (Perkin-Elmer), and template DNA, to a total volume of 25 µl. The reaction mixtures were cycled in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) 35 times at 94°C for 30 s, 45°C for 30 s, and 65°C for 2 min, with a final extension at 65°C for 5 min.

PCR products from 3 separate reactions were pooled, purified by spin column (PCR Select III, 5 Prime-3 Prime), ligated into the plasmid vector pCR2.1 (Invitrogen), and transformed into *Escherichia coli* INVαF′ cells. Plasmid DNA was prepared from clones containing inserts using the Perfect Prep kit (5 Prime-3 Prime). These were cycle sequenced via simultaneous bidirectional sequencing using M13 forward and reverse primers labeled with the fluorescent dyes IRD-700 and IRD-800 (LI-COR). Sequencing reactions were electrophoresed on 66 cm 4% polyacrylamide gels in a LI-COR Model 4200 automated sequencer. A consensus sequence for nearly full-length QPX SSU rDNA was obtained from 12 separate clonal sequences using the MacVector 7.0 DNA sequence analysis software package (Oxford Molecular) and deposited into GenBank (accession no. AY052644).

Primer and probe development. The QPX SSU rDNA sequence was aligned with SSU rDNA sequences downloaded from GenBank for hard clam *Mercenaria mercenaria* (accession no. AF106073) and members of the phylum Labyrinthulomycota. Sequences for the latter available at the time were: *Aplanochytrium kerguelense* (AB022103), *Japonochytrium* sp. (AB022104), *Labyrinthula* sp. (AB022105), *Labyrinthuloides haliotidis* (U21338), *L. minuta* (L27634), *Schizochytrium aggregatum* (AB022106), *S. limacinum* (AB022107), *S. minutum* (AB022108), *Thraustochytrium aggregatum* (AB022109), *T. aureum* (AB022110), *T. kinnei* (L34668), *T. multirudimentale* (AB022111), *T. pachydermum* (AB022113), *T. striatum* (AB022112), *Ulkenia profunda* (L34054, AB022114), *U. radiata* (AB022115), and *U. visurgensis* (AB022116). Regions that appeared to be specific for either QPX or the labyrinthulomycetes in general were identified. Sequences appropriate for use as PCR primers and/or DNA probes were checked for specificity and applicability by BLAST analysis (Altschul et al. 1997) and MacVector software. Probe and primer candidates

were commercially synthesized (Genosys Biotechnologies); DNA probes were 5′ end-labeled with digoxigenin.

Evaluation of primer sensitivity and specificity. Sequences of QPX and labyrinthulomycete PCR primers are listed in Table 1. Initial PCR reactions were conducted with plasmid QPX SSU rDNA to optimize amplification conditions (i.e. annealing temperature, $MgCl_2$ concentration). All candidate primers were then tested for specificity to determine the best primer pairs. Primer pairs QPX-F + QPX-R2 and LABY-A + QPX-R2 were tested for QPX specificity and LABY-A + LABY-Y were tested for labyrinthulomycete specificity. PCR reaction mixtures for QPX-F + QPX-R2 and for LABY-A + QPX-R2 contained reaction buffer (60 mM Tris, pH 8.5; 15 mM $[NH_4]_2SO_4$; 3.5 mM $MgCl_2$), 400 µg ml^{-1} bovine serum albumin, 25 pmol of each primer, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.6 units Ampli*Taq* DNA polymerase (Perkin-Elmer), and template DNA, to a total volume of 25 µl. PCR reaction mixtures for LABY-A + LABY-Y were identical except that the reaction buffer contained 2 mM $MgCl_2$. The QPX-F + QPX-R2 reaction mixtures were cycled in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) 35 times at 94°C for 30 s, 56°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. The LABY-A + QPX-R2 and LABY-A + LABY-Y reaction mixtures had identical cycling conditions except that the annealing temperature was 50°C. An aliquot of each reaction (10% of reaction volume) was checked by agarose gel electrophoresis for the expected product sizes of 665 bp for QPX-F + QPX-R2, ca. 435 bp for LABY-A + LABY-Y, and 396 bp for LABY-A + QPX-R2 reactions.

Specificity of the PCR assays was determined using SSU rDNA plasmids for QPX and clam, and genomic DNA of cultured QPX cells, QPX-infected and uninfected clams (diagnosed by histological examination), and the thraustochytrids *Schizochytrium aggregatum* (ATCC 28209), *Thraustochytrium aureum* (ATCC 34304), and *T. striatum* (ATCC 24473). Thraustochytrids were chosen for specificity testing since previous work showed that QPX is morphologically and phylogenetically closest to this group (Whyte et al. 1994, Smolowitz et al. 1998, Maas et al. 1999, Ragan et al. 2000). QPX-infected and uninfected clams were collected from Burton Bay and Black Narrows, Chincoteague Bay, Virginia, in spring 1997 and 1998. Clams were considered uninfected when no QPX cells were detected by histological examination of the tissue section. Clam SSU rDNA plasmids were prepared as described above for QPX. Total genomic clam DNA was isolated from approximately 150 mg mantle tissue of the clam samples by guanidine thiocyanate lysis (GTC) (Yarnall et al. 2000). Cultured cells of the 3 thraustochytrids were purchased from ATCC (Manasses, VA) and maintained in ATCC culture medium 790 (1 g yeast extract, 1 g peptone, 5 g D+-glucose in 1 l seawater). Cells were washed in phosphate buffered saline, resuspended in extraction buffer (100 mM Tris, pH 8; 100 mM EDTA; 250 mM NaCl), and disrupted by grinding in sterile glass homogenizers. DNA was then extracted using the DNeasy Plant Kit (Qiagen) as per manufacturer's instructions. Each PCR reaction contained 1 pg cloned QPX SSU rDNA, 10 ng cloned SSU rDNA from uninfected clams, 20 pg genomic DNA of cultured QPX cells, 30 to 40 ng genomic DNA from infected and uninfected clams, or 100 pg genomic DNA of cultured thraustochytrid cells. All PCR reactions that did not generate product were re-amplified; 1 µl of unpurified PCR reaction was added to a fresh reaction mixture and cycled as described above.

Sensitivity of the QPX-F + QPX-R2 PCR assay was determined using cloned SSU rDNA and genomic DNA from cultured QPX cells. PCR reactions contained 10-fold serial dilutions of template, ranging from 100 pg to 0.1 fg for the cloned SSU rDNA and 2 ng to 2 fg for the genomic DNA. DNA concentrations were determined using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech).

Evaluation of probe sensitivity and specificity. Sequences of QPX and labyrinthulomycete DNA probes are listed in Table 1. Initial evaluations of the sensitivity and specificity of the candidate QPX probes were conducted using archived paraffin-embedded clam tissues that were collected from

Table 1. Sequences and target regions of PCR primers and DNA probes. QPX primers and probes were designed to be specific for QPX, LABY primers and probes were designed to offer broader specificity for members of the phylum Labyrinthulomycota. SSU rDNA: small-subunit ribosomal DNA

Primer/probe name	Sequence (5′→3′)	Target bases in QPX SSU rDNA
PCR primers		
QPX-F	ATCCTCGGCCTGCTTTTAGTAG	674–695
QPX-R2	GAAGTCTCTACCTTTCTTGCGA	1318–1339
LABY-A	GGGATCGAAGATGATTAG	944–961
LABY-Y	CWCRAACTTCCTTCCGGT	1361–1378
DNA probes		
QPX641	GATGACACACAGCAAACATTGACATT	641–666
QPX674	CTACTAAAAGCAGGCCGAGGAT	674–695
QPX1318	GAAGTCTCTACCTTTCTTGCGA	1318–1339
LABY1336	AACCCGAAATGTCCCTCTAAGAAG	1336–1359

Black Narrows, Chincoteague Bay, Virginia (VA) in fall 1996 (C-477) and from Burton Bay, VA, in spring 1998 (C-1895). Subsequent evaluations incorporated clam samples histologically diagnosed as QPX-infected from New Jersey (NJ), Massachusetts (MA), and Canada (CAN). MA clams were collected from Duxbury (n = 4), NJ clams were collected from Great Bay (n = 6) and CAN clams were collected from St. Andrews, New Brunswick (n = 2). Fixation of tissues varied with location: VA and NJ clams were fixed in Davidsons' AFA; MA clams were fixed in 10% neutral buffered formalin; and CAN clams were fixed in glutaraldehyde.

Paraffin-embedded tissues were sectioned at 5 µm using a microtome. The sections were kept in order as they were cut and the consecutive sections numbered on the slides. Selected sections were stained with Harris hematoxylin and eosin (H&E) and coverslipped. Adjacent sections were prepared for *in situ* hybridizations (ISH). Tissue sections for ISH were mounted on positively charged slides (Fisher Scientific) and treated as described below. The microtome blade and forceps were cleaned with xylenes between samples in order to prevent DNA carry-over contamination. ISH assays for specific detection of QPX were performed with DNA probes QPX641, QPX674, and QPX1318 (see Table 1). All probes were tested separately, and QPX641 and QPX1318 were later combined and used as a hybridization cocktail. When applied separately, hybridization solutions contained 5 ng μl^{-1} of probe. The hybridization cocktail contained 4 ng μl^{-1} of each probe. ISH for general detection of labyrinthulomycetes was conducted with DNA probe LABY1336 at 5 ng μl^{-1}. ISH was conducted as described previously (Stokes & Burreson 1995), with Bismarck Brown Y counter-stain and coverslip modifications (Stokes & Burreson 2001).

Additional specificity evaluations were conducted with cell smears of the thraustochytrids *Schizochytrium aggregatum*, *Thraustochytrium aureum*, and *T. striatum*. Cells were applied to positively charged slides, allowed to dry for 15 min, then soaked in 8% paraformaldehyde overnight in a humid chamber. The fixative was decanted, and the slides were rinsed in TE buffer 3 times, air-dried, and subjected to ISH as described above.

Sequencing of geographically distinct samples. QPX SSU rDNA from selected VA, NJ, and MA infected clams was sequenced to examine molecular similarities. Two 5 µm thick tissue sections per clam were scraped off the slides into a microcentrifuge tube using a sterile razor blade. Total genomic DNA was isolated from the paraffin-embedded tissues after deparaffinization and extended lysis in a Proteinase K detergent solution, as previously described (Vachot & Monnerot 1996). Genomic DNA from each sample was PCR amplified with the 3 primer pairs, QPX-F + QPX-R2, LABY-A + QPX-R2, and LABY-A + LABY-Y, as described above. PCR products from the LABY-A + LABY-Y reactions were cloned and at least 5 clones per sample were sequenced, as described for QPX SSU rDNA.

Phylogenetic analyses. Both distance and parsimony analyses were done to examine taxonomic affinities based on DNA sequences from the organisms identified as QPX. Nearly full-length SSU rDNA sequence data were available for 3 QPX isolates. Two sequences were previously deposited in GenBank. One sequence was from a MA QPX isolate used in a previous study (Maas et al. 1999, AF155209) and another was from a CAN QPX isolate (Ragan et al. 2000, AF261664). The third nearly full-length SSU rDNA sequence was from the MA cultured QPX isolate used in this study. The ~435 bp SSU rDNA fragment resulting from the LABY-A + LABY-Y amplifications of QPX DNA samples from histological sections of infected clams from VA, NJ and MA was used in phylogenetic analyses with homologous fragments from other taxa (A-Y fragments) where data were available. Pairwise distances were calculated with PAUP* 4b8.0 (Swofford 2001) for the members of the Labyrinthulomycota with both DNA datasets. These distances were used to examine the relationships among sequences from the same species, species within a single genus and, for the A-Y fragments, the sequences from the geographically distinct isolates identified as QPX.

Phylogenetic analyses were performed with SSU rDNA sequences from the QPX samples, other members of the Labyrinthulomycota listed above and recently deposited sequences, which included sequences for new species and additional distinct SSU sequences for species already in GenBank. Recently deposited sequences for *Thraustochytrium motivum* (AF265337), *Thraustochytrium* sp. (AB052556), *T. striatum* (AF265338), *Labyrinthula yorkensis* (AF265333), *L. zosterae* (AF265334, AF265335), *Labyrinthula* spp. (AF265330, AF265332), *Labyrinthuloides minuta* (AF265339), *Schizochytrium aggregatum* (AF265336), and several sequences simply designated *Thraustochytriidae* spp. (AF2577314, AF257315, AF257316, AF257317) were included in the analyses. Two other stramenopiles, *Achlya bisexualis* (M32705) and *Ochromonas danica* (M32704) and the alveolates *Oxytricha nova* (X03948) and *Prorocentrum minimum* (Y16238) were used as outgroup taxa.

Sequences were aligned using the CLUSTAL-W algorithm (Thompson et al. 1994) in the MacVector 7.0 DNA Sequence Analysis Software package. Neighbor joining and parsimony jackknife analysis

were performed using PAUP* 4b8.0 (Swofford 2001). Neighbor joining trees were constructed based on uncorrected 'p' distances. Parsimony jackknife was done with 100 bootstrap resamplings of 100 random addition replicates with gaps treated either as missing data or as a 5th base. Taxa with only partial sequences for the region of the SSU rDNA being examined were excluded from the distance analyses and any parsimony analyses treating gaps as a 5th base.

Field validation of QPX PCR assay. Cultured hard clams (n = 25) were collected from planted beds of mixed stocks (2 to 4 yr old) located in Burton Bay, Virginia, on 9 occasions between February 2000 and June 2001 (see Table 3). Each clam was diagnosed for QPX using both standard histological techniques and PCR. Clams were shucked, and approximately 0.125 g of mantle tissue was carefully dissected using a flame-sterilized scalpel and placed in TE buffer for DNA extraction and PCR. Then a cross section of each clam, which included pericardial cavity, intestine, stomach, digestive diverticula, gill, mantle, and foot, was fixed in 10% neutral-buffered formalin for histological examination.

Histological processing followed standard protocols, paraffin-embedded tissues were sectioned at 5 µm, stained with H&E, and examined with a light microscope for the presence of QPX. The presence and distribution of QPX in each tissue or organ was characterized as focal, multifocal, or diffuse. The infection intensity, or number of viable parasites per tissue section, was estimated as rare (1–10), light (11–100), moderate (100–1000), or heavy (>1000).

For PCR, total genomic clam DNA was isolated from approximately 125 mg clam mantle tissue by GTC (Yarnall et al. 2000) for all 9 samplings. For the last collection date, an additional 50 mg mantle tissue was dissected and total genomic DNA isolated using the QIAamp® DNA Mini Kit (Qiagen). Tissue was lysed overnight at 56°C with gentle shaking and DNA purified as per manufacturer's instructions. Genomic DNA from both methods was PCR-amplified using primers QPX-F + QPX-R2 as described above. An aliquot of each reaction (10% of reaction volume) was checked by agarose gel electrophoresis for the expected product size of 665 bp. Samples that were negative for QPX were reamplified by PCR. For reamplification, 1 µl of the reaction mix from the initial PCR reaction served as the template DNA.

RESULTS

Molecular diagnostics

Genomic DNA was extracted from *in vitro* cultured QPX cells and the SSUr DNA was amplified by PCR using universal SSU primers for eukaryotic organisms. The amplicon was cloned, and multiple clones were sequenced. The derived QPX SSU rDNA sequence was 1745 bp.

Several sequence regions were identified for the development of probes and primers (Table 1). Two sets of primer pairs were designed. The first set, QPX-F and QPX-R2, was designed to be specific for QPX and amplified a 665 bp region of the QPX SSU rDNA. It detected as low as 1 fg cloned QPX SSU rDNA and 20 fg QPX genomic DNA in a single amplification (Fig. 1). Primer pair QPX-F and QPX-R2 was specific in that it did not amplify DNA of uninfected host or any of the 3 thraustochytrids tested—*Schizochytrium aggregatum*, *Thraustochytrium aureum*, and *T. striatum* (Fig. 1).

The second primer pair, LABY-A and LABY-Y, was designed to offer broader specificity for the phylum Labyrinthulomycota and amplified approximately 435 bp of SSU rDNA (A-Y fragment) from QPX and the 3 thraustochytrids, but not host DNA (Fig. 2). Pairing one of the QPX-specific primers with one of the labyrinthulomycete primers, LABY-A and QPX-R2, maintained specificity for QPX while targeting a smaller region of 395 bp.

Several DNA probes were evaluated for use in *in situ* hybridization (ISH) assays of cell smears and paraffin-embedded tissues. Probes QPX674 and QPX1318

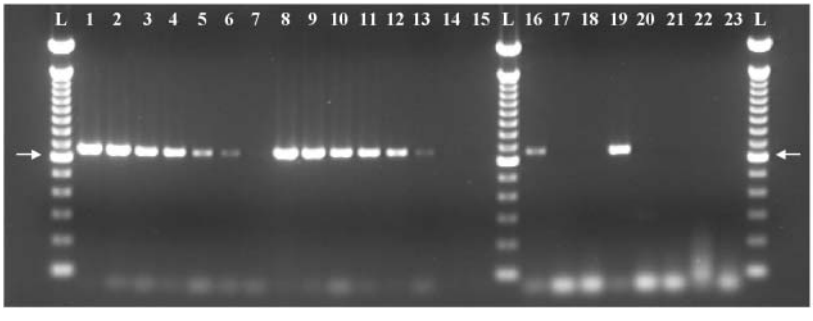

Fig. 1. PCR amplification with primers QPX-F and QPX-R2. Lanes 1 to 14 demonstrate sensitivity and lanes 16 to 23 demonstrate specificity of the PCR assay. Lanes L: 100 bp ladder, arrows indicate 600 bp; lanes 1 to 7: serial dilutions of cloned QPX SSU rDNA: 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg template DNA, respectively; lanes 8 to 14: serial dilutions of genomic DNA from QPX-cultured cells: 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg template DNA, respectively; lane 15: control (no DNA); lane 16: QPX-infected clam genomic DNA; lane 17: uninfected clam genomic DNA; lane 18: cloned clam small-subunit ribosomal DNA (SSU rDNA); lane 19: QPX-cultured cells genomic DNA; lane 20: *Schizochytrium aggregatum* genomic DNA; lane 21: *Thraustochytrium aureum* genomic DNA; lane 22: *T. striatum* genomic DNA; lane 23: control (no DNA). PCR reactions loaded in lanes 17, 18, 20, 21, 22, and 23 were reamplifications

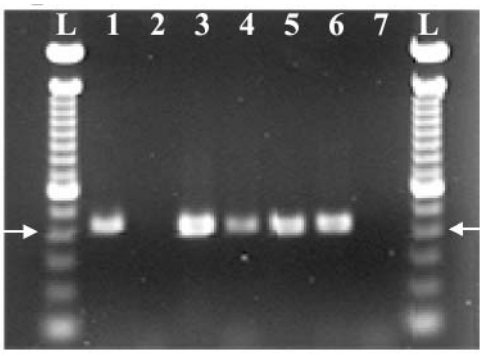

Fig. 2. PCR amplification with primers LABY-A and LABY-Y. Lanes L: 100 bp ladder, arrows indicate 400 bp; lane 1: QPX-infected clam genomic DNA; lane 2: uninfected clam genomic DNA; lane 3: QPX cultured cells genomic DNA; lane 4: *Schizochytrium aggregatum* genomic DNA; lane 5: *Thraustochytrium aureum* genomic DNA; lane 6: *T. striatum* genomic DNA; lane 7: control (no DNA). PCR reactions loaded in lanes 2 and 7 were reamplifications

(same target regions as primers QPX-F and QPX-R2, respectively), as well as probe QPX641, were tested for QPX specificity (Table 1). Probe LABY1336, which was designed to offer broader specificity to hybridize with any member of the phylum Labyrinthulomycota, was evaluated for hybridization with QPX, *Schizochytrium aggregatum*, *Thraustochytrium aureum*, and *T. striatum* (Table 1).

DNA probe QPX674 was quickly discarded as it did not hybridize with QPX cells. Initial trials of the other 2 QPX DNA probes, QPX641 and QPX1318, produced variable results, as the probes failed to hybridize with all infected clam samples tested, and sometimes failed to hybridize with all QPX cells within a particular tissue section (Table 2). Various steps of the ISH protocol (e.g. probe concentration, type and concentration of protease, hybridization temperature) were altered in an attempt to improve hybridization; however, these alterations did not significantly enhance the results. This problem was finally overcome by utilizing a hybridization cocktail that contained both probes QPX641 and QPX1318. Utilization of the probe cocktail greatly enhanced the technique's sensitivity. The probe cocktail consistently hybridized to QPX in tissues of hard clams collected from VA, NJ, MA, and CAN (Table 2, Fig. 3). ISH labeling of the QPX cells in clams from VA, MA and CAN was very intense when the probe cocktail was applied. QPX cells in the NJ clams hybridized with the probe cocktail, but not as intensely as those in samples from the other locations. Regardless of collection location, hybridization was absent or less intense when probes QPX641 and QPX1318 were used independently than when the probe cocktail was used (Table 2). The QPX probe cocktail did not hybridize with clam tissue or with cell smears of the 3 thraustochytrids (Figs. 3 & 4). The general labyrinthulomycete DNA probe LABY1336 hybridized with QPX in histological sections of infected clams and with cell smears of *Schizochytrium aggregatum*, *Thraustochytrium aureum*, and *T. striatum* (Fig. 4).

In order to examine the molecular similarity of the QPX organisms originating from VA, NJ, and MA, genomic DNA was isolated from paraffin-embedded tissues of infected clam samples, amplified using LABY-A and LABY-Y primers, cloned, and sequenced. The resultant sequences of the targeted region (bases 944 to 1378) were identical to the QPX SSU rDNA sequence initially generated in this study, except for a transition at base 1072 in the NJ sample. The base change from A to G was in all the clones sequenced for this sample location and was not within the annealing site of any QPX probe or primer.

Table 2. Origin and identification of samples tested for QPX by *in situ* hybridization (ISH). ISH results using a single QPX probe and QPX probe cocktail are contrasted. Samples originated from Virginia (VA), New Jersey (NJ), Massachusetts (MA), and New Brunswick, Canada (CAN). Hybridization signal intensities denoted as negative (–), weak (+/–), moderate (+), and strong (++); ND: not done

Sample identification	Origin	Hybridization using probe QPX641	Hybridization using probe QPX1318	Hybridization using QPX probe cocktail
C-477A	VA	+	++	++
C-1048	VA	–	–	++
C-1895	VA	ND	+	++
9844-8	NJ	–	–	+/–
9844-29	NJ	–	–	+
9847-25	NJ	ND	–	+/–
9545-5	MA	+	+	++
9545-6	MA	ND	++	++
9545-8	MA	ND	+	++
9545-19	MA	–	–	+/–
20	CAN	ND	++	++
24A	CAN	ND	++	++

Phylogenetic analyses

Results of phylogenetic analyses with both the nearly full-length and truncated (A-Y fragment) rDNA sequence datasets supported earlier studies that had employed fewer taxa (Maas et al. 1999, Ragan et al. 2000), which placed QPX in the phylum Labyrinthulomycota as a sister taxon to *Thraustochytrium pachydermum* (Ragan et al.

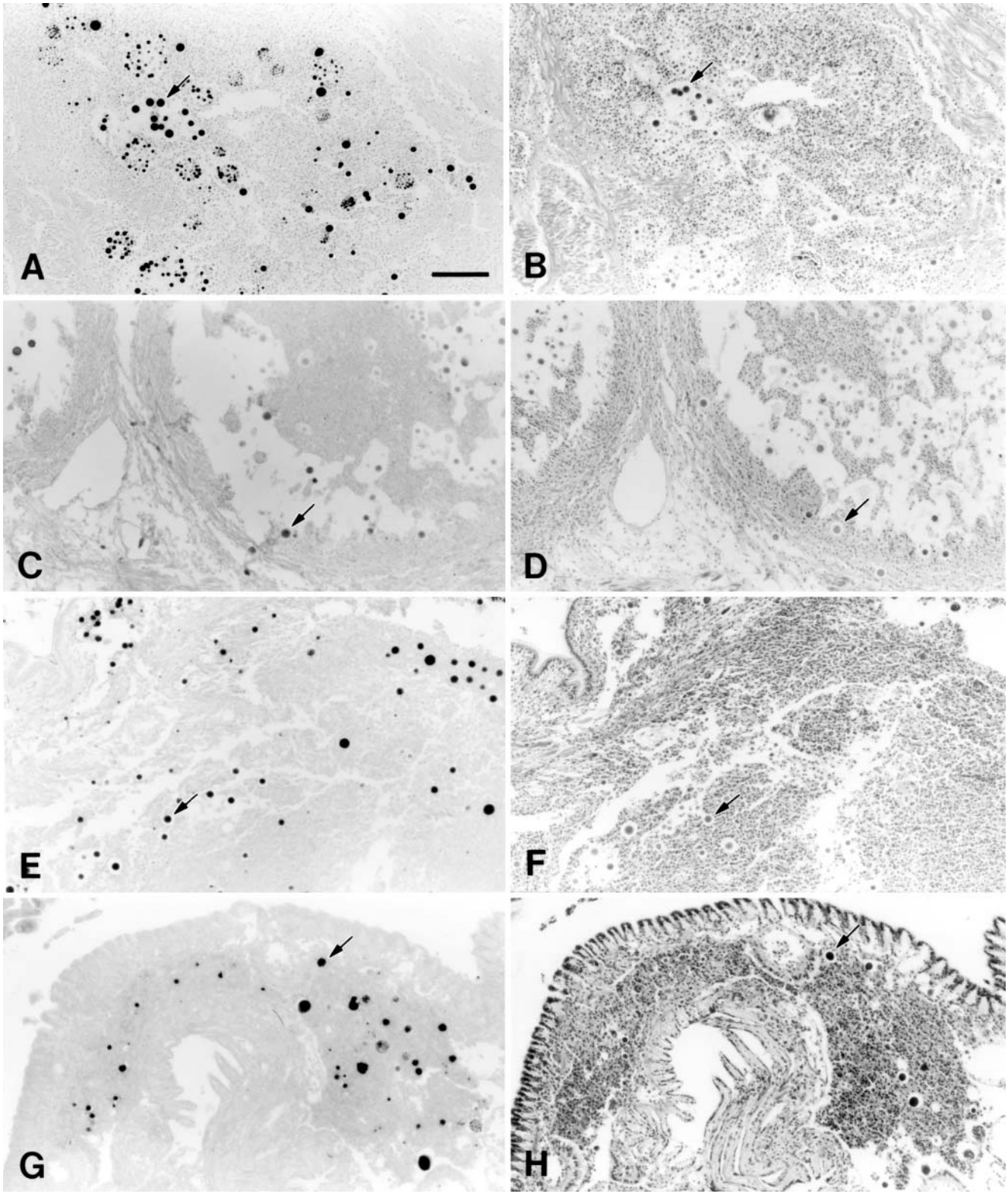

Fig. 3. Detection of QPX cells in consecutive histological sections of clams from various geographic sites using *in situ* hybridization (ISH) with QPX641 and QPX1318 probe cocktail (A,C,E,G) and hematoxylin and eosin (H&E) stain (B,D,F,H). Clams were collected from (A,B) Virginia, (C,D) New Jersey, (E,F) Massachusetts, and (G,H) New Brunswick. Arrows indicate QPX cells. Scale bar = 100 µm (all panels)

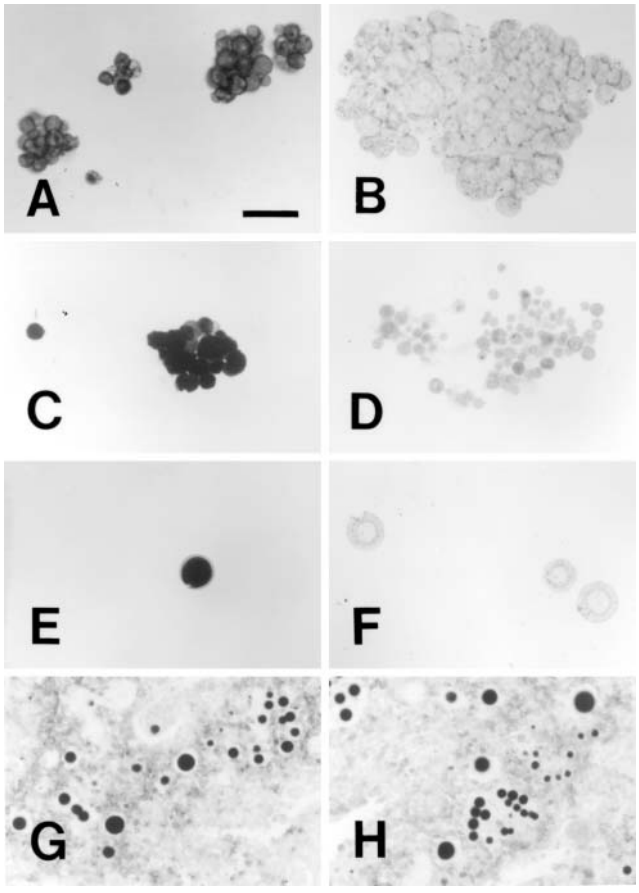

Fig. 4. ISH of various thraustochytrids with the general labyrinthulomycete probe LABY1336 (A,C,E,G) and the QPX DNA probe cocktail (B,D,F,H). Cell smears of (A,B) *Schizochytrium aggregatum*, (C,D) *Thraustochytrium aureum*, and (E,F) *T. striatum* and (G,H) histological section of QPX-infected clam from Virginia. Scale bar = 50 µm (all panels)

2000) (Figs. 5 & 6). With a reduced taxonomic dataset, consisting of only taxa with nearly full-length SSU rRNA gene sequences, all QPX sequences grouped together with parsimony jackknife support of 100. Using either the alveolates or other stramenopiles as outgroup taxa, the phylum was monophyletic (trees with alveolates not shown).

Topologies of the distance and parsimony trees were similar (Figs. 5 & 6). In parsimony analysis with the complete taxonomic dataset, which included some taxa for which approximately two-thirds (~1200 bp) of the SSU rRNA gene sequence was available and those taxa with nearly full-length SSU rDNA sequences, gaps were treated as missing, and 1452 of 1954 sites were parsimony informative (Fig. 5A). With the reduced taxonomic dataset, which included only taxa for which nearly full-length SSU rDNA sequences were available, gaps were treated as a 5th base, and 738 of 1829 sites were parsimony informative (Fig. 5B). Ninety-seven of 410 sites were found to be parsimony informative with the A-Y fragment dataset (Fig. 6A). The Labyrinthulomycota taxa fell into 2 major phylogenetic groups in the neighbor joining analysis (Figs. 5C & 6B). This result was consistent with the 2 major phylogenetic groupings within the Labyrinthulomycota, the thraustochytrid phylogenetic group (TPG) and the labyrinthulid phylogenetic group (LPG), described by Honda et al. (1999). Parsimony jackknife support was relatively strong for the TPG and LPG groups with both the nearly full-length SSU rDNA (Fig. 5A,B) and the A-Y fragment (Fig. 6A) datasets. Analysis using the nearly full-length SSU rDNA dataset with the more inclusive taxonomic composition, which included some taxa with shorter SSU rDNA sequences, also supported the TPG and LPG but placed several species of the *Labyrinthula* genus into a third group (the *Labyrinthula* species group) that was sister to the other members of the phylum. The parsimony jackknife support value for this third grouping was relatively high (88, Fig. 5A). Interestingly, in both distance and parsimony analyses, QPX, *Thraustochytrium pachydermum* and often 2 of the Thraustochytriidae spp. sequences fell into a clade that was separate, but sister to, the other taxa in the TPG (Figs. 5B,C & 6). Sequences that were presumably from the same species often did not group together in these phylogenetic analyses. In addition, species currently placed in the same genus (e.g. *Labyrinthuloides minuta* and *L. haliotidis*, Fig. 5A) could often be found in 2 distinct phylogenetic groupings (Figs. 5 & 6).

Pairwise distances were calculated for the labyrinthulomycete taxa to determine if there was reasonable support from the SSU rDNA sequence data for the QPX samples from geographically distinct sites being from the same, or at least closely related, species by comparison to pairwise distances within and among the other species and genera in the Labyrinthulomycota. A-Y fragment data were available for all of the QPX samples, while for other taxa in the phylum more complete sequence data were available and pairwise ('p') distances were calculated for overlapping regions that could be aligned. QPX A-Y fragments had uncorrected 'p' distances ranging from 0–0.00501, which corresponded to 0–2 nucleotide differences across a total of 411 characters. The 2 *Ulkenia profunda* sequences, however, showed a 'p' distance of 0.08613 in the A-Y fragment region, corresponding to more nucleotide differences. On the other hand, a 'p' distance of only 0.00767 was observed for the A-Y fragment between 2 species that are currently placed in different genera, *Japonochytrium* sp. and *U. visurgensis*. The pairwise distances among species of the genus *Ulkenia* ranged from 0.00251 to 0.09084, while among *Thraustochytrium* species distances were generally

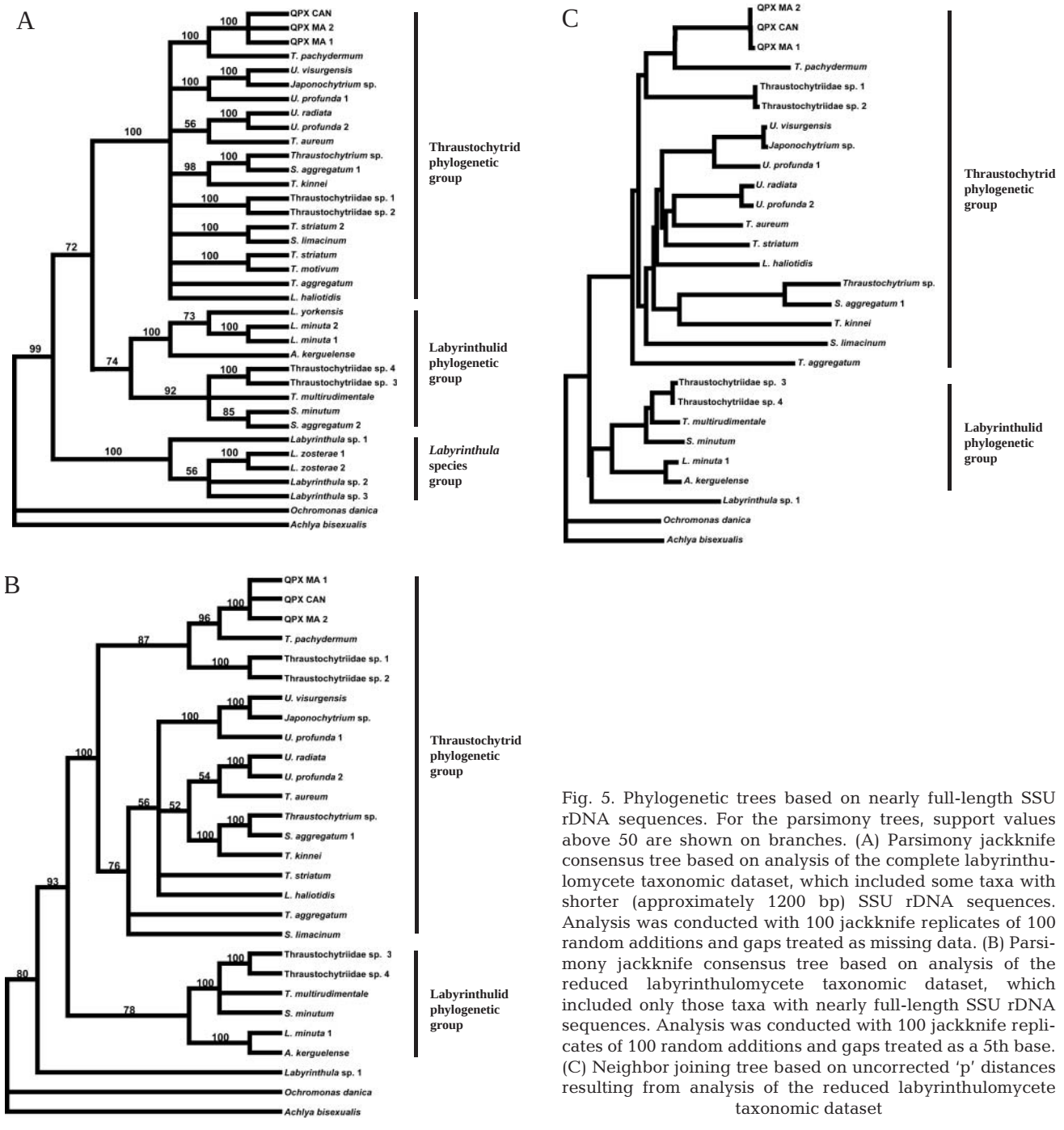

Fig. 5. Phylogenetic trees based on nearly full-length SSU rDNA sequences. For the parsimony trees, support values above 50 are shown on branches. (A) Parsimony jackknife consensus tree based on analysis of the complete labyrinthulomycete taxonomic dataset, which included some taxa with shorter (approximately 1200 bp) SSU rDNA sequences. Analysis was conducted with 100 jackknife replicates of 100 random additions and gaps treated as missing data. (B) Parsimony jackknife consensus tree based on analysis of the reduced labyrinthulomycete taxonomic dataset, which included only those taxa with nearly full-length SSU rDNA sequences. Analysis was conducted with 100 jackknife replicates of 100 random additions and gaps treated as a 5th base. (C) Neighbor joining tree based on uncorrected 'p' distances resulting from analysis of the reduced labyrinthulomycete taxonomic dataset

greater, ranging between 0.06516 and 0.13393 for the A-Y fragment. For the nearly full-length SSU rDNA QPX sequences from the CAN and MA isolates, 'p' distances ranged from 0 to 0.00062, demonstrating very few nucleotide differences among the sequences. The uncorrected 'p' distance between *Japonochytrium* sp. and *U. visurgensis* across this nearly full-length gene sequence was 0.00246, while the distances among *Ulkenia* species were much greater, ranging from 0.06196 to 0.12568 and among *Thraustochytrium* species there were very high 'p' distances, ranging from 0.15425 to 0.23224. (Complete pairwise distance data available on request.)

Field validation

Clams (n = 224) were examined for QPX in field validation trials using both standard histology and PCR of guanidine thiocyanate lysis (GTC) DNA. Standard his-

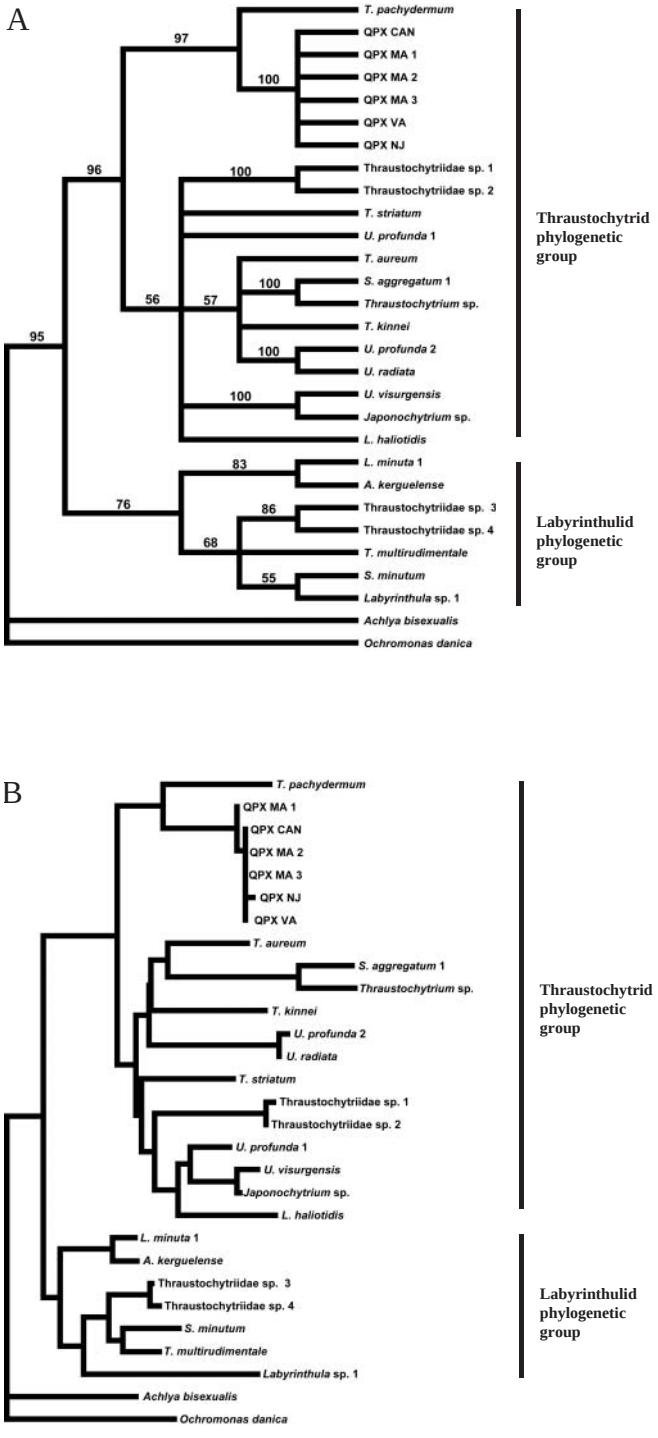

Fig. 6. Phylogenetic trees generated based on the A-Y fragment of the SSU rDNA sequences. For the parsimony tree, support values above 50 are shown on branches. (A) Parsimony jackknife consensus tree based on analysis with 100 jackknife replicates of 100 random additions and gaps treated as a 5th base. (b) Neighbor joining tree resulting from analysis based on uncorrected 'p' distances

tology detected QPX in 35/224 (16%) of the clams while PCR detected QPX in 22/224 (10%) (Table 3). Sixty percent (21/35) of clams diagnosed as positive by histology were falsely diagnosed as negative by initial PCR assays. In most instances false negative PCR diagnoses occurred in clams with infections that were diagnosed as focal, and rare to light in intensity by histology (Fig. 7). PCR failed to detect 81% of the rare infections and 53% of the light infections. Eight of the 22 (36%) clams that tested positive by PCR were negative by histology. The frequency of positives and negatives did not significantly differ ($\chi^2 = 0.069$, $p = 0.7923$, $df = 1$) between the 2 diagnostic methods.

Reamplification of the PCR products that were initially determined to be negative for QPX improved the sensitivity of the assay. Upon reamplification PCR detected 7 additional infections that were also positive by histology and 4 that were negative by histology (Table 4). The infections that were positive by histology were ranked as focal and rare (Fig. 7). The increased detection efficacy that was brought about by reamplification resulted in nearly equivalent determinations of QPX prevalence between the 2 methods—35/224 (15.6%) by histology and 33/224 (14.7%) by PCR (Table 4). PCR detection of infections, scored as focal by histology, increased from 20 to 48%, and PCR detection of infections, scored as rare by histology, increased from 19 to 63% (Fig. 7). There was no increase in the detection by PCR of infections ranked as light by histology. Interestingly, there were still numerous cases in which infections were detected by one method and not the other (Table 4).

The effect of DNA isolation technique on PCR diagnosis was tested on the last group of clams collected for the field validation study. These clams were experiencing mortalities, presumably due to QPX. Genomic DNA was isolated from clam tissue using GTC, in the same manner as for the previous 8 samplings, and the QIAamp® DNA Mini Kit (QIA). Standard histology detected QPX in 10/25 (40%) of the clams while initial PCR amplification detected QPX from GTC DNA in 8/25 (32%) and from QIA DNA in 11/25 (44%) (Table 5). Reamplification of the PCR-negative samples increased the number of QPX-positive clams for both DNA isolation techniques: GTC DNA detected 4 additional infections and QIA DNA detected 5 additional infections, for a combined PCR detection of QPX in 48 and 64% of the clams, respectively. Two infections positive by histology were negative by PCR regardless of DNA preparation; these infections were ranked as focal light and focal rare (1 cell). Comparison of PCR results from the 2 DNA isolation methods suggest that QIA may be more sensitive, since PCR with this DNA detected more QPX infections overall as well as more of the infections diagnosed by histology, especially before reamplification; however,

Table 3. Comparison of observed frequencies of positive and negative diagnoses of QPX made by PCR and histology (Histo). PCR diagnosis based on a single amplification reaction of genomic DNA prepared by guanidine thiocyanate lysis

Sample date	PCR negative Histo negative	PCR negative Histo positive	PCR positive Histo negative	PCR positive Histo positive
16 Feb 00	25	0	0	0
4 May 00	12	5	0	8
19 Jul 00	25	0	0	0
1 Aug 00	18	6	1	0
12 Sep 00	23	1	0	1
1 Nov 00	20	1	1	3
1 Feb 01	24	0	0	0
21 May 01	25	0	0	0
5 Jun 01	9	8	6	2
Total	181	21	8	14

Table 4. Comparison of observed frequencies of positive and negative diagnoses of QPX made by PCR and histology (Histo) after PCR reamplification of samples initially diagnosed as negative by PCR

Sample date	PCR negative Histo negative	PCR negative Histo positive	PCR positive Histo negative	PCR positive Histo positive
16 Feb 00	25	0	0	0
4 May 00	9	2	3	11
19 Jul 00	25	0	0	0
1 Aug 00	17	6	2	0
12 Sep 00	23	1	0	1
1 Nov 00	20	1	1	3
1 Feb 01	24	0	0	0
21 May 01	25	0	0	0
5 Jun 01	9	4	6	6
Total	177	14	12	21

Table 5. Comparison of observed frequencies of positive and negative PCR diagnoses of QPX as influenced by DNA isolation method for the 5 June 2001 samples. Samples diagnosed by guanidine thiocyanate DNA isolation and PCR amplification (GTC), QIAamp® kit DNA isolation and PCR amplification (QIA), and histology (Histo). GTC-1 and QIA-1: PCR diagnoses based on single amplification reaction; GTC-2 and QIA-2: PCR diagnoses after reamplification of initially negative samples

DNA isolation method	PCR negative Histo negative	PCR negative Histo positive	PCR positive Histo negative	PCR positive Histo positive
GTC-1	9	8	6	2
QIA-1	10	4	5	6
GTC-2	9	4	6	6
QIA-2	6	3	9	7
	GTC negative QIA negative	GTC negative QIA positive	GTC positive QIA negative	GTC positive QIA positive
1st amplification	12	5	2	6
Reamplification	7	6	2	10

the difference between the 2 methods was not significant (χ^2 = 1.299, p = 0.2545, df = 1).

DISCUSSION

The PCR primers QPX-F and QPX-R2 demonstrated both sensitivity and specificity for the QPX organism. The primer pair readily detected 1 fg cloned QPX SSU rDNA or 20 fg QPX genomic DNA in a single amplification. The sensitivity of the amplification reaction to as little as 20 fg of genomic DNA suggests that this technique could detect a very small number of QPX cells. PCR amplification of specific numbers of cells was not conducted because it has not been possible to accurately enumerate cells due to copious amounts of mucus released by QPX cells in culture. The primers did not amplify DNA of the host, *Mercenaria mercenaria*, or any of the 3 thraustochytrids, *Schizochytrium aggregatum*, *Thraustochytrium aureum*, and *T. striatum*, suggesting that they are specific for the QPX organism.

The more general PCR primers, LABY-A and LABY-Y, amplified DNA from the QPX organism and from the 3 thraustochytrids. The primer pair did not amplify hard clam DNA. Pairing one of the QPX specific primers (QPX-R2) with one of the labyrinthulomycete primers (LABY-A) maintained specificity for QPX while targeting a smaller region of 395 bp, rather than the 665 bp targeted by QPX-F and QPX-R2. Primer pairs that target relatively small regions of DNA are generally more efficient in amplifying DNA from formalin-preserved material; hence the LABY-A and QPX-R2 primer pair may be useful for this purpose.

Three DNA probes for ISH were developed and tested for QPX sensitivity and specificity. The first probe, QPX674 and PCR primer QPX-F, targeted QPX SSU rDNA bases 674 to 695. This region worked well for PCR amplification of the nuclear rDNA but not for ISH, which primarily targets the SSU rRNA molecules. Presumably secondary structure within the SSU rRNA

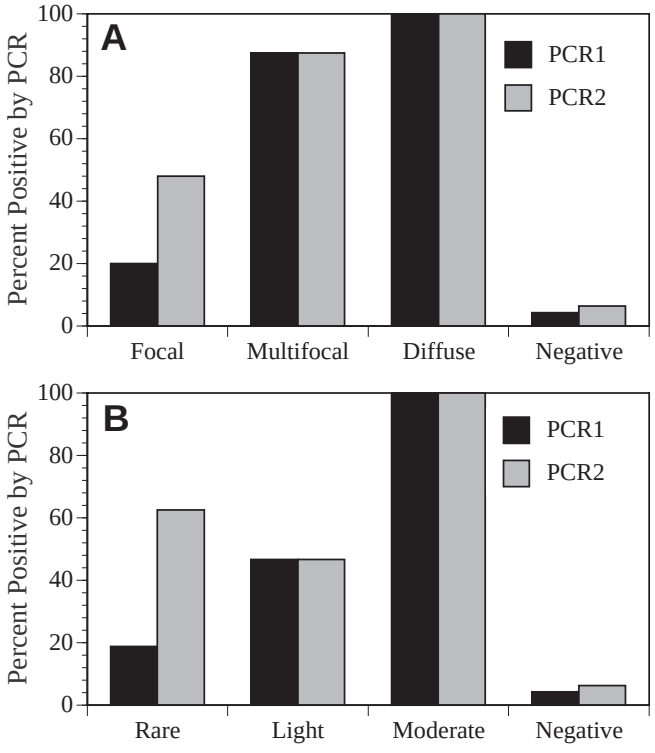

Fig. 7. Percent of QPX infections having (A) tissue distributions classified as focal, multifocal, diffuse, and negative and (B) intensities classified as rare, light, moderate, and negative by histological examination of field validation samples that were determined to be positive by initial (PCR 1) and reamplification (PCR 2) QPX PCR assays

may have limited access to the probe binding site. Highly variable results were obtained with the other 2 DNA probes designed to detect the QPX organism. However, a cocktail of both probes greatly improved detection in ISH assays, resulting in enhancement of the hybridization signal as reported by Trembleau & Bloom (1995). The probe cocktail did not hybridize with host tissue or with 3 thraustochytrids tested, suggesting that the probe cocktail is specific for the QPX organism. Reasons for the signal variability associated with the individual probes are not clear. It is possible that the 2 probes hybridized with 2 different strains or species of histologically indistinguishable thraustochytrids within the samples. One or both of the general labyrinthulomycete primers may not be able to amplify the DNA of one of these histologically identical thraustochytrids. This would result in a PCR product representing only one of the strains or species of putative QPX organisms observed in the histological section. This is highly unlikely, however, given that these primers target conserved regions within the SSU rDNA of both labyrinthulids and thraustochytrids. Reduced variability associated with the probe cocktail, as opposed to the individual probes, is probably due to increased destabilization of SSU rRNA secondary structure by the presence of more than one oligonucleotide bound to the target molecule. The enhanced ISH sensitivity by multiple, non-overlapping probes yielded an additive signal with no increase in background for Trembleau & Bloom (1995), and the QPX probe cocktail generated the same result in this study.

In recent years tremendous progress has been made in the development of molecular diagnostic techniques. Numerous PCR assays and oligonucleotide probes are now available for a variety of bivalve mollusc disease agents including *Haplosporidium nelsoni* (Fong et al. 1993, Stokes & Burreson 1995, Stokes et al. 1995a, Ko et al. 1999, Day et al. 2000), *H. costale* (Ko et al. 1995, Stokes & Burreson 2001), *Minchinia teredinis* (Stokes et al. 1995b), *Mikrocytos roughleyi* (Adlard & Lester 1995), *Marteilia sydneyi* (Anderson et al. 1995, Kleeman & Adlard 2000), *Marteilia refringens* (Le Roux et al. 1999), *Perkinsus marinus* (Marsh et al. 1995, Yarnall et al. 2000, Reece et al. 2001), and *Bonamia ostreae* (Carnegie et al. 2000, Cochennec et al. 2000). Discussions regarding the value of these novel molecular diagnostic tools generally cite high sensitivity and specificity, and the capability of producing a rapid and cost-effective diagnosis. However, before such benefits can be realized, it is important to conduct thorough field validations against traditional standard diagnostic techniques (Hiney & Smith 1998). Field validation trials for the pathogens listed above have been limited. Studies by Stokes et al. (1996) and Carnegie et al. (2000) demonstrated molecular diagnostics for *H. nelsoni* and *B. ostreae*, respectively, to be more sensitive than diagnosis by histological examination.

In contrast, the QPX PCR primers developed here were not more sensitive than routine histological analysis. This result most likely relates to the fact that QPX infections are often focal or localized in clam tissues. Unlike some oyster parasites, it does not appear that QPX is dispersed through its host via circulatory activities. QPX cells are surrounded by viscous mucus that appears to retain clusters of parasitic cells focally. Hence, diffuse or systemic infections are less likely to occur. Sampling error is likely to be greater in the case of focal infections than systemic infections, as the chance of encountering infective cells in any particular subsample of tissue is reduced. False negative PCR assays were diagnosed as focal infections by histology; likewise, samples that were positive by PCR and negative by histology may have been focal infections located in the tissue used for DNA extraction. Given the focal nature of QPX infections, the error associated with tissue sampling should be evaluated. It may be that duplicate or triplicate tissue samples must be taken to improve the precision of PCR.

The sensitivity of the PCR assay using QPX-F and QPX-R2 primers could potentially be improved by altering the tissue sampling and DNA isolation protocols. The latter was tested during the last month of the field validation study by comparison of DNA isolation using GTC and the QIA. The PCR assay using QIA DNA seemed to be more sensitive for QPX detection than PCR with GTC DNA, even though a smaller tissue sample is used. PCR of the QIA DNA also had better agreement with QPX-positive histology samples than GTC did, especially with one round of amplification; however, the number of clams used for this comparison was too small to definitively determine the effect of DNA isolation method on PCR diagnosis. The deviation in PCR results for GTC and QIA may also be related to tissue sampling; further comparison should be made using an experimental design that incorporates an estimate of such error. While the application of PCR-based diagnosis of QPX may have little advantage over histological analysis for routine monitoring of QPX disease in wild and cultured clam populations, it may prove very useful for research purposes.

QPX has been documented in hard clams from Virginia, New Jersey, and Massachusetts, USA, and Prince Edward Island, Nova Scotia, and New Brunswick, Canada; however, differences in the presentation of the disease, in terms of both QPX morphology and host response, has raised speculation that different species or strains of the organism may exist (Ragone Calvo et al. 1998, Smolowitz et al. 1998). The 1745 bp QPX SSU rDNA sequence presented here showed 99% similarity to the sequence reported by Maas et al. (1999), both of which were derived from *in vitro* cultures of QPX cells from diseased clams collected in Massachusetts, and to the sequence reported by Ragan et al. (2000), which was derived from an *in vitro* QPX culture from infected hard clams collected in New Brunswick, Canada. The QPX SSU rDNA sequence reported here differed from the previously published sequences by only 2 bases and 1 base, respectively. The close similarity of the sequences of QPX originating from Massachusetts and New Brunswick suggests that the 2 organisms are very similar, likely within the same genus and possibly the same species. Additionally, the QPX probe cocktail consistently hybridized with QPX cells in tissue sections of QPX-infected clams from all 3 USA sites as well as from Canada, providing supporting evidence that the organisms observed in these geographically distinct areas are genetically very closely related.

To corroborate this observation, QPX SSU rDNA sequences were determined for selected paraffin-embedded infected hard clam samples from Virginia, New Jersey, and Massachusetts. Amplification of the Canadian material was unsuccessful, probably because of the glutaraldehyde fixation, which damages DNA. The sequences of the 435 bp target region were identical for VA and MA samples and nearly identical for the NJ sample (single base change from VA/MA sequence). If any other labyrinthulomycete had been present in these samples, it also would have been amplified by the LABY-A and LABY-Y primers and subsequently sequenced. However, each of the 3 samples generated only 1 PCR product. Multiple DNA clones of the amplification product had identical sequences, suggesting that only 1 labyrinthulomycete, QPX, was present in each sample. The sequence similarity of the putative QPX organisms infecting hard clams from Virginia, New Jersey and Massachusetts supports the ISH results, which suggests the close relationship of these organisms.

Further assertions regarding the relatedness of geographically distinct QPX organisms requires phylogenetic analyses, including the determination of pairwise sequence distances. The phylogenetic analyses conducted for this study support earlier observations that QPX belongs within the Labyrinthulomycota (Whyte et al. 1994, Smolowitz et al. 1998, Maas et al. 1999, Ragan et al. 2000). Honda et al. (1999) examined the SSU rDNA of 6 genera of labyrinthulids and thraustochytrids and found signature sequences that provided separation into the 2 major phylogenetic groups, the TPG and LPG. The phylogenies presented here agree with those groupings, with QPX in the TPG. Analyses that included more recently deposited sequences for *Labyrinthula* species suggest that some members of that genus form a separate sister group to the major clade containing the TPG and LPG (Fig. 5).

Pairwise sequence distances for taxa within the Labyrinthulomycota were calculated to assess whether it was reasonable to claim, based on the SSU rDNA sequences, that the QPX samples from geographically distinct sites were likely to be the same species. Within some phyla, genera demonstrate great variability within portions of the SSU rDNA and sequence information aids in species determination. For example, the plasmodial life stages of *Haplosporidium costale* and *H. nelsoni* are difficult to differentiate by histological examination; however, with the use of SSU rDNA-based tools, these can be readily discriminated (Stokes & Burreson 2001). In other phyla, inter-specific SSU rDNA sequences are highly conserved within a genus, so that species differentiation is difficult or not possible using this gene. This is true, for example, of *Perkinsus* species (Kotob et al. 1999, Reece et al. 2001), *Marteilia* species infecting mussels and oysters in Europe (Berthe et al. 2000, Le Roux et al. 2001), and of rhizocephalans that parasitize green crabs (Murphy & Goggin 2000).

Pairwise distance comparisons and phylogenetic analyses conducted for this study, however, support as-

sertions by other researchers that there needs to be a major reevaluation of the taxonomy for the phylum Labyrinthulomycota (Honda et al. 1999, Maas et al. 1999, Ragan et al. 2000). Labyrinthulomycete species currently placed in the same genus based on morphological characters have often failed to group together in molecular phylogenetic studies based on SSU rDNA sequences (Honda et al. 1999, Ragan et al. 2000). In this study, species in the same genus sometimes showed greater uncorrected 'p' distances than was observed between species in different genera. In addition, it was found that in some cases, even sequences reportedly from the same species showed much greater pairwise distances than was observed among species currently placed in different genera. For example, in the region of SSU rDNA for which data were overlapping, 2 sequences for *Schizochytrium aggregatum* (AB022106 and AF265336) and those for *Thraustochytrium striatum* (AB022112 and AF265338) showed uncorrected 'p' distances of 0.27588 and 0.19909, respectively. By contrast, however, in this same region the sequences for *Japonochytrium* sp. (AB022104) and *Ulkenia profunda* (L34054) had an uncorrected 'p' distance of only 0.06762. The species identifications and/or sequence data need to be carefully reevaluated in these instances where sequences from the same species differ so significantly. This confusion with species and genus designations rendered it nearly impossible to make assertions about the levels of sequence divergence expected among species within a genus for this phylum. Although only 2 polymorphic sites were found among the QPX A-Y fragment sequences, a single nucleotide difference was also observed between the *U. profunda*2 and *U. radiata* sequences and between the *Labyrinthula yorkensis* and *Labyrinthuloides minuta*2 sequences in this same region of the gene. Phylogenetic analyses based on DNA sequence data for other genes may help resolve the inconsistencies between taxonomies suggested by the current SSU rDNA and morphological data. In addition, in order to determine if different presentations of so-called QPX disease are due to different strains or species of the pathogen, it will be necessary to obtain more sequence data for different geographic isolates and to examine other more variable regions of the QPX genome. The conserved nature of SSU rDNA renders it inappropriate for identification of strain differences; however, sequences not under structural constraint, such as the internal transcribed spacer (ITS) regions, can be very useful for such discrimination. Molecular diagnostics targeting the ITS regions recently have been developed to distinguish among species and/or strains of several disease agents, such as *Perkinsus* (Reece et al. 2001) and *Piscirickettsia salmonis* (Heath et al. 2000) and these regions also may be useful for QPX discrimination.

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