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Molecular genetic variation within and among isolates of QPX (Thraustochytridae), a parasite of the hard clam *Mercenaria mercenaria*

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ABSTRACT: The thraustochytrid known as QPX (Quahog Parasite Unknown) has sporadically caused disease in the hard clam *Mercenaria mercenaria* along the east coast of North America since the 1960s. We hypothesized that genetically distinct QPX strains might be responsible for outbreaks of QPX disease in different areas and tested this hypothesis by comparing several QPX isolates recovered from the recent outbreak in Raritan Bay, New York with QPX strains isolated from 2 outbreaks in Massachusetts, USA. There was no variation in small subunit rDNA (SSU rDNA), 5.8S rDNA, or 4 mitochondrial gene sequences. In contrast, both of the ribosomal ribonucleic acid (rRNA) operon intergenic spacers, internal transcribed spacers 1 and 2 (ITS1 and ITS2), revealed substantial sequence variation. However, strain-specific sequences were not detected because the ITS sequence variation within QPX isolates was comparable to the variation between isolates. ITS1 sequences recovered from an infected clam by amplification with a QPX ITS2-specific primer were identical to those recovered from the QPX isolates.

KEY WORDS: Internal transcribed spacer 1 · Internal transcribed spacer 2 · SSU rRNA gene · 5.8S rRNA gene · Mitochondrial gene · Labyrinthulomycota

INTRODUCTION

The disease of hard clams *Mercenaria mercenaria* (also known as quahogs) caused by the protist called Quahog Parasite Unknown (QPX) was first reported in wild clams from New Brunswick, Canada during the 1960s. Since then, QPX has caused high mortalities in hatchery-reared clams from Prince Edward Island, Canada and in commercially harvested clam populations from Virginia, Massachusetts, and New Jersey, USA (Whyte et al. 1994, Ragone Calvo et al. 1998, Smolowitz et al. 1998, MacCallum & McGladdery 2000). In the summer of 2002, QPX infections suddenly appeared in the Raritan Bay (off the coast of Staten Island, New York) *M. mercenaria* population, causing significant clam mortality (Dove et al. 2004).

The development of QPX disease management strategies is hampered by an incomplete understanding of factors controlling the occurrence and severity of QPX infections. Environmental factors such as salinity and temperature appear to be important (Ragone Calvo et al. 1998). High clam population density and the planting of seed from non-local sources might also increase the susceptibility of clams to QPX (Ford et al. 2002). Genetic variability of the QPX parasite itself is also likely to be important in disease development, as it is in other shellfish pathogens (e.g. *Perkinsus marinus*; Reece et al. 1997, 2001). Because early observations suggested that some aspects of QPX disease presentation in Raritan Bay were different than those reported elsewhere (Dove et al. 2004), we set out to determine whether the QPX organism causing the Raritan Bay outbreak was genetically distinguishable from previously characterized QPX isolates.

Genes commonly used for phylogenetic and biodiversity studies include nuclear ribosomal ribonucleic acid (rRNA)-encoding genes (rDNA) and protein-coding genes in the mitochondrial genome (mtDNA).

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Small subunit (SSU or 18S) rDNA sequences are particularly well-suited to molecular genetic identification of novel organisms (Honda et al. 1999). SSU rDNA sequences from Canadian (AF261664; Ragan et al. 2000) and Massachusetts (AF155209, Maas et al. 1999; and AY052644, Stokes et al. 2002) QPX isolates have been deposited in GenBank. These sequences, along with morphology, suggest that QPX belongs to the family Thraustochytridae within the subphylum Labyrinthulomycota in the phylum Heterokonta (Cavalier-Smith et al. 1994, Whyte et al. 1994, Kleinschuster et al. 1998, Leander & Porter 2001), although QPX has not yet been assigned to a genus. The rate of evolutionary change in rRNA coding genes is relatively slow, and it is possible for organisms with identical SSU rRNA gene sequences to have genetic and physiological differences that reflect more rapid evolutionary change in other genes (e.g. Mo et al. 2002). Protein coding genes, including those in the mtDNA, generally evolve more rapidly than rRNA coding genes, particularly because the third codon position is not strongly constrained.

Intergenic regions, including those separating rRNA genes, also tend to be relatively weakly constrained and rapidly evolving. rRNA genes in eukaryotes are typically arranged in 10s to 100s of tandemly repeated units that include the coding regions for the SSU, 5.8S and large subunit (LSU or 28S) rRNAs. Intergenic regions known as internal transcribed spacers, ITS1 and ITS2, separate the SSU and 5.8S genes and 5.8S and LSU genes, respectively (Fig. 1). The ITS1, 5.8S, and ITS2 sequences are collectively known as the ITS region. Because they evolve faster than the rRNA coding genes, ITS1 and ITS2 sequences are often useful in distinguishing closely related genera, species, and strains (Alvarez & Wendel 2003, Coleman 2003).

To investigate the genetic variability among QPX isolates, we sequenced 4 mtDNA genes and portions of the rRNA operon from several new cultures of QPX isolated during 2 yr of the Raritan Bay outbreak, from the original Massachusetts QPX isolate, and from new Massachusetts QPX isolates.

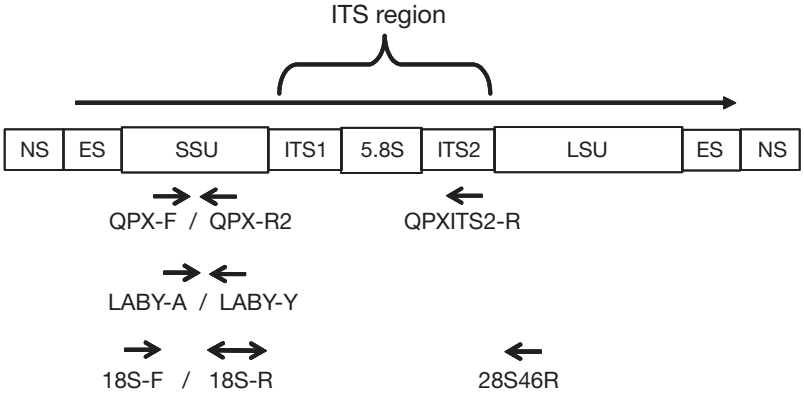

Fig. 1. Schematic of an rRNA operon, showing primer locations. Top 3 primers are Quahog Parasite Unknown (QPX)-specific, middle 2 are designed to recognize all Labyrinthulomycota, and bottom 3 are universal for eukaryotes. NS: nontranscribed spacer; ES: external transcribed spacer; SSU: small subunit or 18S rRNA gene; ITS1: internal transcribed spacer 1; 5.8S: 5.8S rRNA gene; ITS2: internal transcribed spacer 2; LSU: large subunit or 28S rRNA gene. - Arrow at top = direction and extent of pre-rRNA transcription. Arrows above primer names = approximate location and direction in which they prime DNA synthesis

MATERIALS AND METHODS

QPX isolation and cultivation. New QPX isolates were cultured using the method of Kleinschuster et al. (1998) from 2 presumptively QPX-infected hard clams *Mercenaria mercenaria* collected from Raritan Bay, New York, in October 2003 and from 4 clams collected in August 2004. New QPX isolates were also cultured from 3 clams collected in Wellfleet Harbor, Massachusetts in July 2005. QPX isolates are identified by clam collection location (NY or MA), year of collection (03, 04, or 05), clam sample group (3 digits that follow the year), and clam number within the sample group (01 to 30). Thus, QPX isolates NY0313808B and NY0313808C were from nodule biopsies B and C from Clam 08 of sample group 138 from Raritan Bay (Area 8) in 2003, and NY0314220A was from biopsy A from Clam 20 of sample group 142 collected in Raritan Bay (Area 16) in 2003. Isolates NY0400826, NY0400921, MA0505116, MA0505311, and MA0505325 were established similarly (only 1 biopsy was taken from each clam). Microscopic evaluation of morphological characteristics, such as size, cell wall thickness, and evidence of endogenous sporogony, was used to tentatively identify the cultures as QPX. QPX cultures were grown under ambient atmosphere at 23°C in 25 cm^2 canted-neck flasks (Falcon 3013) using the MEM formulated by Kleinschuster et al. (1998). Once a week, 10% of each QPX culture (0.5 ml of cells and medium) was passed under sterile conditions to a new flask with 5 ml fresh MEM. This regimen maintained cultures in log-phase growth and good appearance (the cells remained highly refractive under phase contrast microscopy) for over 2 yr. Cryopreserved QPX originally isolated from Massachusetts hard clams in 1997 (Kleinschuster et al. 1998) was purchased from the American Type Culture Collection (ATCC; no. 50749) in March 2004, thawed and maintained in culture in the same way as the new isolates.

To establish clonal QPX isolates, 1-wk-old cultures were diluted 5 to 10-fold with fresh MEM to dissociate cell aggregates and produce a sample dominated by separated QPX thalli. Cells were then picked individually with a sterile, disposable micropipette and transferred from the source flask to a new flask. Clonal isolates are identified by the original isolate names described above followed by C1 to Cn, where n is the number of clonal cultures established. Two clonal isolates (MA97C1 and MA97C2) were established from ATCC 50749. A nonclonal replicate isolate from 2003 NY clam 13808 biopsy B (NY0313808BR3) was also examined. All isolates were successfully cryopreserved in MEM containing 8% dimethylsulfoxide (Miyake et al. 2004).

Molecular genetic analyses. QPX cells were harvested from aliquots of 7 d old cultures in exponential growth phase. The mucoid material enveloping the cells was removed by adding phosphate-buffered saline or sterile artificial seawater and slowly aspirating and ejecting the suspension 10 to 15 times through a 20-gauge needle (BD PrecisionGlide). Washed QPX cells were recovered by centrifugation at 5500 *g* for 5 min. Genomic DNA was extracted using the Nucleospin Tissue Kit (BD Biosciences) following the manufacturer's protocol. The Nucleospin Tissue Kit was also used to purify DNA from tissue of 3 clams collected in Raritan Bay in June 2004: Clams 07 and 30 (CL0400907 and CL0400930, from Area 21) and Clam 11 (CL0401111, from Area 16).

The nearly full-length QPX SSU rRNA coding region was amplified from purified QPX DNA using the universal primers for eukaryotic organisms of Medlin et al. (1988) modified by removing the restriction site extensions, yielding primers 18S-F and 18S-R (Table 1), producing an approximately 1800 bp amplicon. Smaller fragments of SSU rDNA were amplified from clam (CL0400907 and CL0401111) DNA using the QPX- or labyrinthulomycete-specific primers (QPX-F with QPX-R2 and LABY-A with LABY-Y, respectively; Table 1) designed by Stokes et al. (2002). rRNA operon fragments including the ITS region were amplified from QPX DNA using the reverse complement of primer 18S-R, 18S-RR, and primer 28S46R (Table 1; Van der Auwera et al. 1994). The ITS region was amplified from clam (CL0400930) DNA with primers 18S-RR and QPXITS2-R (Table 1), designed based on the new sequences reported here. Primer locations are indicated in Fig. 1. All synthetic oligonucleotide primers were purchased from Integrated DNA Technologies. Each PCR reaction (total volume 25 µl) contained 10 µl of 2.5X PCR mix and 0.4 µl of 25 mM Mg acetate stock solution (Eppendorf), 4.1 µl of distilled deionized water (ddH_2O), 5 µl of 1 µM stock of each primer, and 0.5 µl template DNA. PCR reaction conditions consisted of denaturation at 94°C for 30 s, annealing at 50°C (SSU rDNA) or 46.8°C (ITS region) for 1 min, and extension at 72°C for 2 min for 35 cycles, followed by a final extension step at 72°C for 10 min.

To amplify portions of mtDNA encoding cytochrome oxidase I (*coxI*), cytochrome *b* (*cob*), and NADH dehydrogenase subunits 1 and 7 (*nad1* and *nad7*), the homologues from the *Thraustochytrium aureum* mitochondrial genome (GenBank accession number AF288091) were each aligned with at least 11 of the most similar sequences in GenBank (as identified by the basic local alignment search tool BLAST; Altschul et al. 1990), and degenerate primers were designed (Rose et al. 1998) to conserved motifs as near as possible to the N- and C-termini (Table 1). The amplified fragments were approximately 1290, 670, 545, and 970 bp for *coxI*, *cob*, *nad1*, and *nad7*, respectively. PCR reaction conditions were as described for rRNA operon primers, except that the annealing temperature was 38°C, and 15 additional cycles were sometimes required to produce sufficient product for cloning.

An aliquot of each PCR reaction was checked by agarose gel electrophoresis (1.5% in 1X Tris-Acetate-EDTA [TAE] buffer) for amplicons of the expected size before the PCR product was analyzed further. PCR products were purified from agarose gels using a QIAEX II kit (Qiagen) following the manufacturer's instructions, ligated

Table 1. PCR primer sequences

Primer name	Sequence (5′ to 3′)
18S-F	AACCTGGTTGATCCTGCCAGT
18S-R	TGATCCTTCTGCAGGTTCACCTAC
18S-RR	GTAGGTGAACCTGCAGAAGGATCA
28S46R	ATATGCTTAARTTCAGCGGGT
LABY-A	GGGATCGAAGATGATTAG
LABY-Y	CWCRAACTTCCTTCCGGT
QPX-F	ATCCTCGGCCTGCTTTTAGTAG
QPX-R2	GAAGTCTCTACCTTTCTTGCGA
QPXITS2-R	GCCACCTATTCCCAAAAGAGGA
coxIN	TGGCTGTTCTCCACTAACCACAAGGAYATHGG
coxIC	AAGACCCAAGAAGTGGATNGGRAARAANGT
cobN	CAGATTATAACTGGAGTCTTCTTAGCNATGCAYTAYAC
cobC	AGTGACCATTGGATTCGCTGGDATRTARTTRTC
nad1N	TTCGGAGTAACTCAAGCACTAGCNGAYGG
nad1C	TCGGAAACCCATACCAGAATARAGNACRTTRTANCC
nad7N	CCGCATGTAGGTCTATTGCATAGAGGNACNGARAA
nad7C	CTGTAGGTGACTGAATCCAGGAGCYTTDATYTTRCA

into the pGEM-TEasy vector and transformed into *E. coli* JM109 following the manufacturer's instructions (Promega). Plasmid DNA was purified from white colonies using the Wizard Plus SV Minipreps plasmid DNA purification system (Promega) according to the kit instructions. The presence of an insert of the expected size was confirmed by *Eco*RI restriction digestion and gel electrophoresis.

Cloned amplicons were sequenced completely on both strands using SP6 and T7 primers and the ABI Big Dye Terminator v3.1 sequencing mix and instructions (PE Biosystems), and electrophoresis was performed at the Stony Brook University New York DNA sequencing facility. For the 1800 bp, nearly full-length SSU rDNA fragments, QPX-F and LABY-Y (Table 1, Fig. 1; Stokes et al. 2002) were also used as internal sequencing primers. To check for heterogeneity of amplicons and to produce a consensus sequence free of *Taq* errors, several clones from each PCR reaction were sequenced. Chromatograms were edited in Chromas 2.3 (Technelysium), and sequences were submitted to GenBank BLAST for a preliminary assessment of their identity. The new sequences reported here have been deposited in GenBank under accession numbers DQ641141 to DQ641208.

Sequences were aligned with homologues downloaded from GenBank using the CLUSTAL W function of BioEdit Sequence Alignment Editor 7.0.5.2 (Hall 1999) followed by manual optimization. Phylogenetic trees were constructed with MEGA 3.1 (Kumar et al. 2004). Neighbor-joining SSU rDNA trees were constructed with distances estimated by the Jukes-Cantor model with different rates among sites (gamma = 1) and pairwise deletion of sites with gaps, and neighbor-joining mtDNA trees were constructed from inferred amino acid sequences with a Percent Accepted Mutation (PAM) matrix. Five hundred bootstrap replicates were performed. The secondary structures of QPX ITS1 and ITS2 were predicted with the Mfold webserver using default settings (Zuker 2003).

RESULTS

QPX isolates

The 3 vegetative life stages previously reported for QPX (Smolowitz et al. 1998) were observed in the new New York and Massachusetts QPX cultures. Thalli grew and matured into sporangia containing numerous endospores. The endospores were released by rupture of the sporangia and, in turn, matured to form thalli. The QPX cultures produced a great deal of mucoid material. All QPX isolates were morphologically similar and were maintained in culture for several months without any alteration in growth characteristics.

QPX mtDNA sequences

Degenerate primers (Table 1) were used to amplify fragments of the *coxI*, *cob*, *nad1* and *nad7* genes from 4 QPX isolates (NY0313808BC8, NY0314220AC6, MA0505311C5 and MA97C1), and 3 cloned amplicons from each isolate were sequenced. For all 4 genes, the cloned amplicons from each isolate, as well as all cloned amplicons considered together, differed from one another by less than 0.5%, which is similar to our observed *Taq* polymerase error rate during amplification of a homogeneous template, and suggests that these QPX isolates shared identical mtDNA sequences encoding these 4 genes. Further evidence that the observed variations were due to *Taq* error is as follows: (1) most sequence variants occurred in only 1 cloned amplicon; (2) most changes were T to C or A to G, transitions we commonly observe as *Taq* error; and (3) most were nonsynonymous and resulted in nonconservative inferred amino acid changes. The consensus sequences for the 4 QPX mtDNA genes have been submitted to GenBank under accession numbers DQ641205 to DQ641208. Phylogenetic analysis of concatenated inferred amino acid sequences (Fig. 2) showed that, as expected, the QPX mtDNA sequences were most closely related to the only other available Labyrinthulomycota mtDNA sequences (from *Thraustochytrium aureum*) with strong (100%) bootstrap support, and also group with the other Heterokonts (46% bootstrap support).

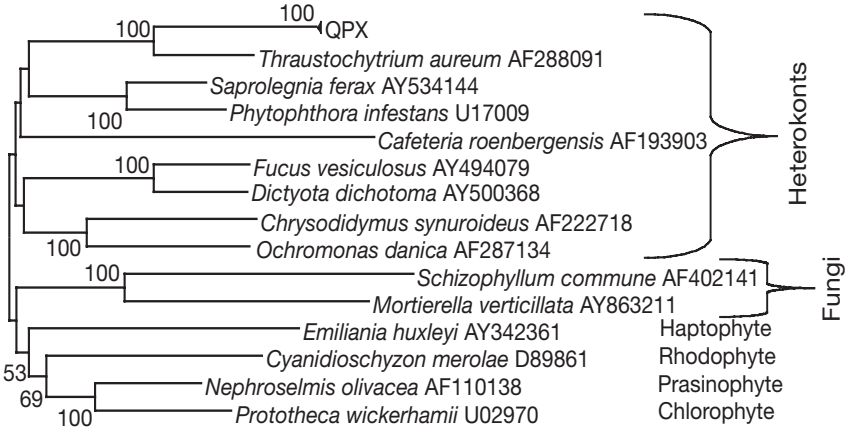

Fig. 2. Concatenated mtDNA-deduced amino acid sequence neighbor-joining phylogenetic tree. Bootstrap values (as % of 500 replications in which the branch was supported) indicated for nodes with support >50%

QPX SSU rDNA sequences

Universal primers 18S-F and 18S-R (Table 1, Fig. 1) were used to amplify nearly full-length SSU rDNA sequences from 6 clonal QPX isolates (NY0314220AC1, NY0314220AC5, NY0314220AC6, NY0313808BC8, NY0313808CC1 and MA97C1). The consensus of all cloned amplicons sequenced from each isolate (GenBank accession number DQ641204) was identical to the consensus of the 3 QPX SSU rDNA sequences previously reported (excluding the primer sites, the consensus was identical to GenBank accession number AF261664; AF155209 had 1 C for T substitution and AY025644 had 1 single base deletion). The consensus SSU rDNA sequence of the nonclonal QPX isolate NY0313808BR3 was also identical. Shorter fragments of SSU rDNA amplified from clonal isolates MA97C1, NY0313808BC4, NY0313808BC8 and NY0314220AC1 with various combinations of primers LABY-A, LABY-Y, QPX-F, and QPX-R2 (Table 1, Fig. 1; Stokes et al. 2002) also yielded sequences identical to the QPX consensus. Sequence variation among cloned amplicons (usually 6 or more) from a single QPX isolate and among all cloned amplicons (43 full-length plus 32 shorter) was less than 0.5%, which was consistent with our observed *Taq* error rate. The 13 (of 147) variable positions that showed the same change in 2 or more cloned amplicons might reflect real sequence variation among copies of the SSU rRNA gene, but in no case were these differences specific to a QPX isolate (restricted to 1 isolate or found in all cloned amplicons from a single isolate).

Phylogenetic analysis of the more than 150 Labyrinthulomycota SSU rDNA sequences available in GenBank as of May 2007 strongly supported placement of QPX in the Thraustochytrid phylogenetic group of Honda et al. (1999). QPX was most closely related (with over 90% bootstrap support) to a Thraustochytrid C9G, isolated from hard clam gills (AF474172; Anderson et al. 2003), and *Thraustochytrium pachydermum* (AB022113). These organisms may also be related to Thraustochytriidae strains BS1 and BS2 (AF257314 and AF257315), which were isolated from a contaminated tunicate *Botryllus schlosseri* cell culture (Mo et al. 2002), although this relationship was unstable.

QPX ITS region sequences

The ITS region was amplified from 12 clonal QPX isolates (NY0313808BC1, NY0313808BC8, NY0313808CC1, NY0314220AC1, NY0314220AC5, NY0314220AC6, NY0400826C5, NY0400921C6, MA0505311C5, MA0505116C4, MA0505325C1, and MA97C1) plus the nonclonal NY0313808BR3 with primers 18S-RR and 28S46R (Table 1, Fig. 1). A product of approximately 420 bp was produced, and 3 to 5 cloned amplicons were sequenced for each QPX isolate, generating 57 ITS region sequences (submitted to GenBank under accession numbers DQ641141 to DQ641197). The locations of boundaries between coding and spacer regions were estimated by comparison to the sequences reported from *Perkinsus marinus* and other organisms, and by the complementarity between the 3′ end of the 5.8S rRNA and 5′ end of the LSU rRNA (see final paragraph of this section, below).

The 5.8S rRNA gene was 156 bp in length and the consensus sequence from each of the 13 QPX isolates was identical. Variability among cloned amplicons from a single QPX isolate and among all cloned amplicons (0.3%) was comparable to expected *Taq* error, and most differences were found only in 1 cloned amplicon, except for 1 position where 4 cloned amplicons (2 from NY0314220AC1 and 1 each from NY0313808BC1 and NY0313808BC8) shared a T to C change. The QPX 5.8S rDNA consensus sequence is represented by the 5.8S rDNA sequence submitted to GenBank under accession number DQ641157 (and others) and appears to be the first Labyrinthulomycota 5.8S rDNA sequence in GenBank. The most similar sequence returned by BLAST was from the heterokont *Hyphochytrium catenoides* (GenBank accession number X80346).

ITS1 and ITS2 were much more variable than the mtDNA or rRNA genes. Overall, the 57 cloned amplicons differed on average by 3.6% in ITS1 (3.8% including the large insertion in 2 cloned amplicons) and by 2.4% in ITS2. Much of the variation was found within individual QPX isolates: the cloned amplicons from each QPX isolate differed from each other by an average of 3.1% for ITS1 (4.1% including the large insertion) and 2.3% for ITS2. These differences are greater than the expected *Taq* error rate. The ITS1 and ITS2 region sequences are summarized in Fig. 3, in which the 57 sequences for each of ITS1 and ITS2 have been combined into 21 and 26 (respectively) types of identical sequences. While some of the sequence types represented by only 1 cloned amplicon may be the result of *Taq* error, evidence that most of the variation in ITS1 and ITS2 was real includes the fact that the variation in these regions was much greater than in SSU rDNA, 5.8S rDNA, or mtDNA, and that many of the variants were recovered from multiple independent PCR reactions using different template DNA.

ITS1 averaged 76.7 bp in length, except for 2 cloned amplicons (1 each from NY0314220AC5 and NY0313808BR3) containing a 24 bp insertion that was nearly identical to the first 24 bp of the 5.8S rRNA gene (with T instead of A in the last position). ITS1 had very

A QPX ITS1 Sequences

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                  ....|....|....|....|....|....| ....|....|....|....|....|....| ....|....|....|....|....|....| ....|....|....|
                            10        20        30        40        50        60        70        80        90        100
1   (16,10)       CCACATCTTT-ACTTTTTTTTGTTGTTCCT ATTTTATTTTTT-ATCATTTTCAAATTTTT TCAAAAATTT-AAACATTTTT--------- ----------
2   (7,6)         ..........-.................... ...........--..............- -.........-.......... --------- ----------
3   (4,3)         ..........-..-................. ...........--.................. .........-.......... --------- ----------
4   (4,3)         ..........-..-................. ...........-.................. .........-.......... --------- ----------
5   (4,3)         ..........-..-................. ...........--..............- -.........-.......... --------- ----------
6   (3,3)         ..........-.................... ............-...........T..... .........-.......... --------- ----------
7   (2,2)         ..........-.................... ....-.......-.................. .........-.......... --------- ----------
8   (2,2)         ..........-..-................. ...........--.................. .........-.......... --------- ----------
9   (2,2)         ..........-.................... ...........-..............- -.........T.......... --------- ----------
10  (2,1)         ..........-..-................. ...........-..............- -.........T.......... --------- ----------
11  (1,1)         ..........-..-....C........... ...........-..............- -.........T.......... --------- ----------
12  (1,1)         ..........-..-................. ...........-.................. .........-.......... --------- ----------
13  (1,1)         ..........-..-................. ............T.........G....... .........-.......... --------- ----------
14  (1,1)         ..........-..A................. ...........-........TC....... .........-.......... --------- ----------
15  (1,1)         ..........-.........C......... ...........-........TC....... .........-.......... --------- ----------
16  (1,1)         ..........-.................... ...........-.................. .........-.......... --------- ----------
17  (1,1)         ..........-..-...........C.... ...........-.................. .........-.......... --------- ----------
18  (1,1)         ..........-..C................. ...........-..............C. .........-.......... --------- ----------
19  (1,1)         ..........T.................... ...........-.................. .........-.......... --------- ----------
20  (1,1)         ..........-.................... ...........-.................. .........-..........ATGACTTTT AGCGATGGATGTCTT
21  (1,1)         ..........-.......C........... ...........-.................. .........-..........ATGACTTTT AGCGATGGATGTCTT
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B QPX ITS2 Sequences

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                  ....|....|....|....|....|....| ....|....|....|....|....|....| ....|....|....|....|....|....| ....|....|....|....|....|....| ....|....|.
                            10        20        30        40        50        60        70        80        90        100       110       120       130
1   (8,5)         TTTTTCTCACTTGCTTTTTT-ATAGAGCAG TTTGTGGGCCTGTTTTCTTGTTTCCTCTTT T-GGGAATAGGTGGCCTTGAGAATAGTATT TCAACAAATAATCCTAGCAATTATTTAACC TTTTACAACAT
2   (6,5)         ....................-......... ..............................- -.............................. ..............................-.T ...........
3   (3,3)         ..................A..T......... ..............................C -.............................. ..............................-.T ...........
4   (3,3)         ....................-A.A....... ..............................- -.............................. ..............................-.T ...........
5   (3,3)         ....................-......... ..............................- -.............................. ..............................T ...........
6   (3,3)         ....................--........ ..............................- -.............................. ..............................  ...........
7   (3,2)         ..................A..T......... ............C.................C -.............................. ..............................  ...........
8   (3,2)         ....................-......... ..............................C -.............................. ..............................  ...........
9   (3,2)         ....................--A....... ..............................C -.............................. ..............................  ...........
10  (3,2)         ....................--........ ...........A.C................C -.............................. ..............................-.T ...........
11  (2,2)         ....................--........ ..............................- -.............................. ..............................-.T ...........
12  (2,2)         ....................--........ ............C................. --.............................. ..............................  ...........
13  (2,2)         ....................-......... ..............................- -.............................. ..............................-...T ...........
14  (1,1)         ....................-......... ..............................C -.............................. .....C........................  ...........
15  (1,1)         ..................A..T......... ..............................C -.............................. ....................C.....T ...........
16  (1,1)         ....................-......... ..............................- -.............................. ..............................T -.........
17  (1,1)         ....................-......... ..............................C -.............................. ..............................  ...........
18  (1,1)         ....................-C........ ..............................- -.............................. ..............................  ...........
19  (1,1)         ....................-......... ......A.......................C -.............................. ..............................T ...........
20  (1,1)         ....................-......... ....................A........C -.............................. ..............................T ...........
21  (1,1)         ....................-......... ............C................. .T............................. ..............................  ...........
22  (1,1)         ..................A..T.......G. ............C................C -.................G............ ..............................  ...........
23  (1,1)         ....................-......... ..............................- .T............................. ..............................  ...........
24  (1,1)         ....................-......... ..............................- -.........-.................... ..............................-.T ...........
25  (1,1)         ..........C.........-......... ..............................- -.............................. ..............................-.T ...........
26  (1,1)         ....................--........ ..C........................... -.............................. ..............................T ...........
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Fig. 3. Sequence summaries for QPX: (A) ITS1 and (B) ITS2. Values in parentheses for each sequence type = number of cloned amplicons with that sequence and number of QPX isolates in which the sequence type was found (e.g. for ITS1 sequence Type 1, 16 cloned amplicons from 10 QPX isolates shared this sequence). (.) identical to top sequence; (–) alignment gap

low GC content (averaging 17.3%). ITS2 averaged 128.5 bp in length and had an average GC content of 33.5%. Much of the sequence variation in ITS1 was due to single base insertion/deletion events (indels) rather than the single nucleotide changes typical of *Taq* error observed in the rDNA and mtDNA coding regions. Single nucleotide indels also occurred in ITS2, but substitutions were more common in ITS2 than in ITS1. The ITS1 and ITS2 regions of individual cloned amplicons were not necessarily linked (Table 2); that is, cloned amplicons sharing an ITS1 sequence type may not also share an ITS2 sequence type (and vice versa).

ITS1 and ITS2 are thought to fold into secondary structures required for the processing of pre-rRNA into mature rRNA subunits (Coleman 2003, Gottschling & Plötner 2004). QPX ITS1 was predicted by Mfold to contain 1 stem-loop structure (from positions 41 to 74 in Fig. 3A, $\Delta G = -1.6$). QPX ITS2 was predicted by Mfold to have a more complex structure (Fig. 4). Including the region of complementarity between the 3′ end of the 5.8S rRNA and 5′ end of the LSU rRNA (positions 1 to 21 and 151 to 171 in Fig. 4, respectively), this structure has a predicted ΔG of −43; without it, the predicted ΔG of ITS2 is −26. Of the 22 variable positions in ITS2 (Fig. 3B), 13 were in bases predicted to be unpaired (Fig. 4), and 11 of these 13 were found in more than 1 cloned amplicon. In contrast, of the 9 variable ITS2 positions among bases predicted to be paired, only 1 occurred in more than 1 cloned amplicon (and none of the 4 variable positions in the 5.8S and LSU rRNA complementary regions were found in more than 1 cloned amplicon). The fact that most of the ITS2 variants found in only 1 cloned amplicon (8 of 10) were predicted to occur in paired bases, where they may disrupt the secondary structure, suggests that they were more likely due to *Taq* error than real variation. Conversely, the fact that most of the ITS2 variants found in multiple cloned amplicons (11 of 12) were predicted to occur in unpaired bases, where the change would be unlikely to disrupt the ITS2 secondary structure, suggests that they represent real sequence variants.

QPX rRNA operon sequences from infected clams

rRNA operon sequences identical to those from cultured QPX isolates were also recovered directly from QPX-infected clams. Three cloned fragments of SSU rDNA amplified from clam CL0401111 DNA using primers LABY-A and QPX-R2 (Table 1, 374 bp amplicon) were identical to the QPX consensus sequence, as were 10 of the 12 cloned SSU rDNA fragments amplified from clam CL0400907 DNA using primers LABY-A and LABY-Y (Table 1, 417 bp amplicon for QPX). A primer was designed to match the ITS2 sequence of QPX (QPXITS2-R; Table 1, Fig. 1) and used in combination with primer QPX-F or 18S-RR to amplify most of the QPX ITS region from clam CL0400930 DNA. Six cloned amplicons were sequenced and have been submitted to GenBank under accession numbers DQ641198 to DQ641203. The consensus sequences of the 5.8S rDNA and amplified part of SSU rDNA matched the consensus sequences from cultured QPX isolates. All 6 of the QPX ITS1 sequences recovered from this clam also matched ITS1 sequences recovered from the QPX isolates: 2 were identical to Type 8, and 1 was identical to each of Types 2, 3, 4, and 10 (Fig. 3). The fragment of ITS2 amplified was too short for complete comparison, but matched ITS2 sequences from QPX isolates in the shared region.

Table 2. ITS1/ITS2 sequence type as in Fig. 3 for each ITS region clone from the 13 QPX isolates examined. NC: no clone (only 3 cloned amplicons sequenced for these isolates)

Isolate	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
NY0313808BC1	13/18	2/1	11/2	1/5	5/4
NY0313808BC8	2/1	14/19	7/11	6/3	5/8
NY0313808BR3	2/2	4/4	21/25	1/9	1/9
NY0313808CC1	6/3	16/21	1/12	1/10	1/10
NY0314220AC1	6/3	7/11	1/15	5/8	5/8
NY0314220AC5	10/2	12/5	4/4	20/24	10/2
NY0314220AC6	1/10	2/1	1/16	15/20	2/2
NY0400826C5	1/9	9/6	1/13	NC	NC
NY0400921C6	8/1	9/17	3/4	NC	NC
MA97C1	2/1	1/6	17/22	8/2	3/1
MA0505311C5	2/1	18/23	3/1	1/13	3/5
MA0505116C4	4/7	4/7	1/6	NC	NC
MA0505325C1	1/7	1/12	19/26	NC	NC

DISCUSSION

Differences in the presentation of QPX disease in hard clams, in terms of both QPX morphology and host response, have raised speculation that different strains of QPX may exist (Stokes et al. 2002). The establishment of numerous QPX cultures from several infected hard clams collected in New York and Massachusetts in 2003, 2004, and 2005 allows this hypothesis to be tested by a comparative analysis of morphological, molecular, and pathogenic characteristics among QPX isolates. The identity of SSU and 5.8S rDNA sequences as well as

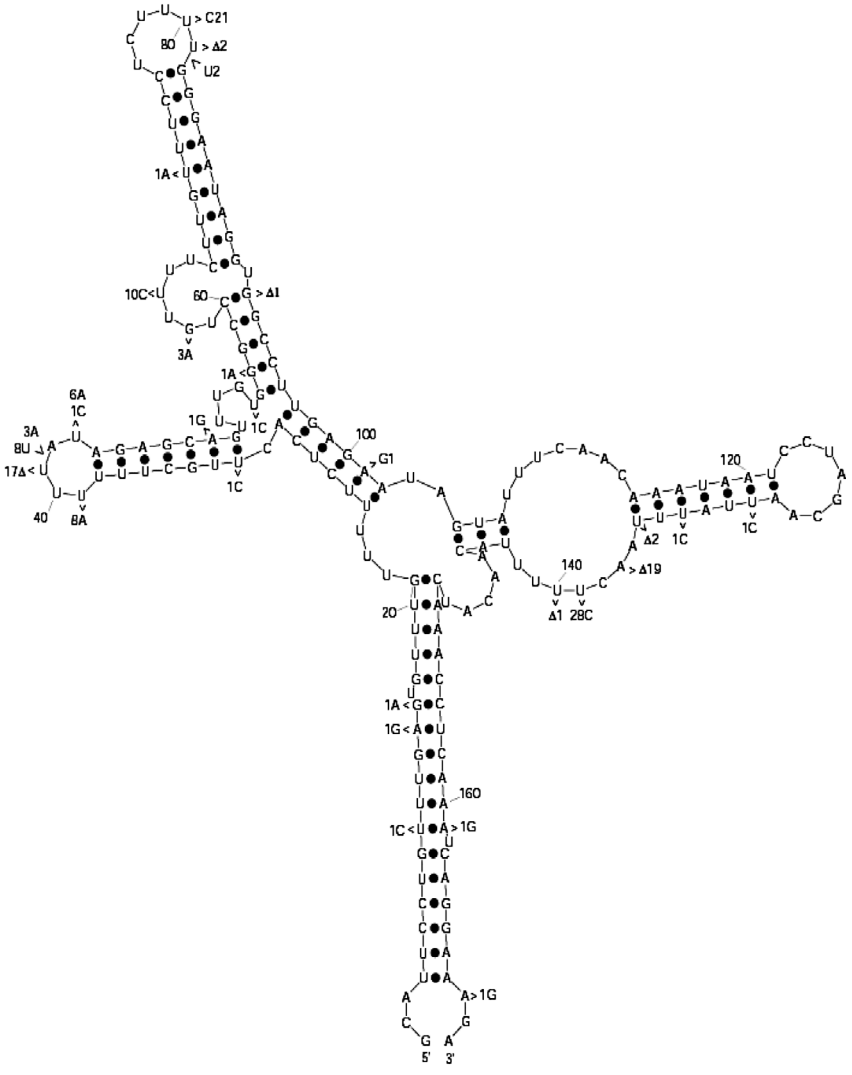

Fig. 4. QPX ITS2 predicted secondary structure from NY0313808BC1 clone 4 (ITS2 sequence type 5). Positions 1 to 21 are the 3′ end of the 5.8S rRNA, and positions 151 to 171 are the 5′ end of LSU rRNA. Variable positions indicated by (>); changes observed are indicated by the substituted or inserted nucleotide (A, C, G, or U) or by a deletion (Δ) together with the number of cloned amplicons in which that variant was observed

mtDNA protein coding sequences among the QPX isolates examined here is consistent with previous reports (Maas et al. 1999, Ragan et al. 2000), although Stokes et al. (2002) reported a 1-base substitution in samples from New Jersey amplified with primers LABY-A and LABY-Y. However, the ITS1 and ITS2 sequences reported here (Fig. 3) show that molecular genetic variation does exist in QPX.

It is generally thought that the many copies of the rRNA-encoding operon in a genome do not evolve independently; instead, they evolve in a concerted fashion that maintains a high degree of homogeneity among them. As a result, rRNA, ITS1 and ITS2 sequences are expected to be identical within and among individuals belonging to the same species, but to differ between species. However, it has become clear that the effectiveness of concerted evolution varies among organisms (Alvarez & Wendel 2003). If concerted evolution does not homogenize the rRNA operon copies faster than the processes that generate differences between copies operate, there will be differences in sequence among operon copies within the same genome, as we have observed for QPX. The importance of screening for intragenomic polymorphism of rRNA operon sequences, particularly in ITS1 and ITS2, before using them for phylogenetic analysis or for developing strain- or species-specific molecular diagnostics has been emphasized by researchers studying taxa as diverse as invasive algae (Fama et al. 2000), grasshoppers (Parkin & Butlin 2004), sponges (Worheide et al. 2004), and the oyster parasite *Perkinsus marinus* (Brown et al. 2004). As demonstrated by the success of the QPXITS2-R primer in amplifying QPX sequences from infected clam DNA, despite the sequence variation reported here, the ITS region should offer appropriate targets for the development of molecular genetic tools to detect and enumerate QPX.

Some of the processes responsible for concerted evolution are linked to meiosis, so concerted evolution may be less effective in organisms using primarily asexual rather than sexual reproduction (Fama et al. 2000, Gandolfi et al. 2001). Thus, the ITS sequence variation found within individual clonal isolates of QPX may suggest that QPX has a predominantly asexual mode of reproduction, but much more information about the ecophysiology and life history of QPX is required to resolve this question. The mixing and matching of different ITS1 and ITS2 sequence types in single rRNA operon amplicons (Table 2) may reflect real *in vivo* recombination among rRNA operon copies (Alvarez & Wendel 2003) or the formation of chimeras or other artifacts during PCR (Kanagawa 2003).

QPX ITS1 and ITS2 are somewhat unusual. They are both on the short side of reported ITS sequences and have low GC content (averaging 17.3% in ITS1 and 33.5% in ITS2 versus 45.5% in 5.8S rDNA and 44.5% in SSU rDNA). They also differ from the emerging pic-

ture of ITS secondary structure (e.g. Gottschling & Plötner 2004) in that ITS1 has only 1 predicted helix rather than several, and ITS2 has only 3 predicted helices rather than 4 (Fig. 4). The unusual predicted secondary structures for QPX ITS1 and ITS2 could reflect either the unique characteristics of this organism or the fact that structures predicted by programs like Mfold are not necessarily the real, *in vivo* structures assumed by RNA molecules. ITS region sequence data from related organisms would allow the structures predicted here to be tested by examining the conservation of homologous predicted secondary structures and the presence of complementary base changes (Coleman 2003, Gottschling & Plötner 2004).

The recovery of the same ITS1, SSU and 5.8S rDNA sequences from a QPX-infected clam as were found in QPX isolates provides evidence that the organism in culture is the one responsible for QPX disease in the field. However, our results do not preclude the possibility that molecular genetic and physiological variation exists among QPX isolates. In fact, our experimental pathology work (B. Allam, unpubl. data) suggests that there are differences in virulence among QPX isolates that did not show sequence differences in the genes investigated here. The loci we examined are likely to be selectively neutral with regard to the pathogenic phenotype of QPX, and variation may be more likely to exist at loci directly involved in, and potentially under selective pressure for, virulence.

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