The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Differential Gene Expression in Five Isolates of the Clam Pathogen, Quahog Parasite Unknown (QPX)

Ewelina Rubin[a,1], Arnaud Tanguy[b,c], Emmanuelle Pales Espinosa[a] & Bassem Allam[a]

a School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, 11794-5000 New York, USA
b UPMC Université Paris 6, UMR 7144, Equipe Génétique et Adaptation en Milieu Extrême, Station Biologique de Roscoff, Roscoff 29682, France
c UPMC Université Paris 6, UMR 7138, Systématique, Adaptation et Evolution, Paris 75005, France

Keywords
oligoarrays; virulence.

Correspondence
B. Allam, School of Marine and Atmospheric Science, Stony Brook University, Stony Brook, NY 11754-5000, USA
Telephone number: +1-631-632-8745;
FAX number: +1-631-632-8915;
e-mail: Bassem.Allam@stonybrook.edu

Received: 16 June 2016; revised 30 January 2017; accepted January 30, 2017.
Early View publication March 10, 2017

doi:10.1111/jeu.12400

ABSTRACT

Quahog parasite unknown (QPX) is a thraustochytrid protist that infects the hard clam, *Mercenaria mercenaria*, causing significant economic losses along the northeastern coast of North America. Previous investigations noted differences in growth dynamics and virulence in QPX cells from different geographic locations. In order to probe the molecular determinants for these variations, we investigated the transcriptomic profiles of five geographically distinct QPX isolates using custom 15k 60-mer oligonucleotide arrays. A total of 1,263 transcripts were differentially expressed (DE) among the five QPX isolates. The hierarchical clustering of gene expression profiles showed that the QPX isolates from Raritan Bay (RB, NY) and from Provincetown Harbor (MA) were more similar to each other and diverged from QPX isolates from Peconic Bay (PB, NY) and Old Plantation Creek (VA), which had more similar gene expression profiles. The most prominent difference was based on 78 transcripts coding for heat shock proteins DE between the five QPX isolates. The study generated contrasting transcriptomic profiles for QPX isolated from northern (MA) and deeper (RB, NY) locations as compared to southern (VA) and shallower (PB, NY) areas, suggesting the adaptation of the parasite to local environmental, in particular temperature, conditions.

QUAHOG parasite unknown (QPX) is an undescribed thraustochytrid (Labyrinthulomycetes, Tsui et al. 2009) that infects hard clam (a.k.a. northern quahog, *Mercenaria mercenaria*) populations in different geographic locations along the northeastern coasts of the United States and maritime Canada. The first published report on the QPX organism infecting clams described a disease outbreak in a shellfish hatchery on Prince Edward Island (Canada) in 1989 (Whyte et al. 1994). In the summer of 1995, QPX disease outbreaks were reported on the coast of Massachusetts near Provincetown (Smolowitz et al. 1998). Between 1996 and 1997, QPX was reported in clams from the coast of Virginia, and in the summer of 2002 severe mortality events were documented from Raritan Bay, New York (Dove et al. 2004). Since the clam mortalities occurred in distant geographic locations, at first it was unclear if they were caused by the same organism. It was noted that QPX cells on histology slides of clam tissues from Massachusetts (MA) and Virginia (VA) differ in morphological characteristics, including retention of Groccott's methanamine silver and Alcian blue stains by the extracellular material around the QPX cell wall (Ragone Calvo et al. 1998). In the search for strain differences, Stokes et al. (2002) compared partial variable region 9 of the small subunit ribosomal gene (SSU) of QPX cells preserved in the histology samples from four geographical regions (New Brunswick, MA, VA and New Jersey) suggesting that all QPX organisms investigated were most likely the same species. Previous studies, however, have shown that QPX cultures derived from NY and MA exhibit differences in growth rate in response to different temperatures with the NY isolates having optimal growth at 23 °C and the MA isolate at 20 °C (Perrigault et al. 2010). In addition, clam challenge experiments also showed that three different QPX isolates (two from NY and one from MA) caused different clam mortality rates thus suggesting variability in their virulence characteristics (Dahl et al. 2008). These findings suggested the possible presence of

multiple QPX strains or species through the parasite geographic range. This study aimed at finding molecular differences between QPX isolated from multiple geographic locations. Transcription profiling using cDNA microarrays has been applied to identify the molecular basis of phenotypic differences between isolates of the same species for protozoan pathogens (Baptista et al. 2006; Bozdech et al. 2008; Davis et al. 2007; Llinas et al. 2006). These studies show that gene expression patterns differ between highly related isolates and strains and reveal genes responsible for their different phenotypes. The most differentially expressed transcripts between isolates are usually investigated for their genetic polymorphisms within the gene coding sequence (e.g. Baptista et al. 2006), or within the 5′-UTR (untranslated region) or 3′-UTR which determine the binding of transcription factors and gene expression regulation (e.g., Rodrigues et al. 2010). For example, transcription profiles of six *Trypanosoma cruzi* isolates revealed 30-fold transcription regulation of the gene coding for NADH dehydrogenase subunit 7 (Baptista et al. 2006), which then revealed a large deletion within the coding sequence in some of the isolates.

The aim of this study was to identify possible transcriptome-wide gene expression differences among QPX isolates, and to identify genes with the highest expression differences among QPX isolates. Five QPX isolates were investigated including three isolates with previously sequenced and nearly identical full length small subunit (SSU) RNA gene and two new QPX isolates for which SSU gene sequences were obtained during preliminary assays (Table 1, Data S1). The similarity of almost full length SSU gene sequences from these five isolates (pairwise identity: 99.32–99.94%) suggests that the isolates are most likely the same species considering that 3–5% difference in the SSU gene is typically used as a threshold to designate species within the Labyrinthulomycetes (Mo et al. 2002; Pan et al. 2016). The transcriptomes of these five isolates, herein considered to represent the same species, were then profiled with a specific focus on exploring the potential molecular basis for virulence differences among QPX isolates and for possible genetic adaptations to local environmental conditions.

MATERIAL AND METHODS

QPX cultures

Five QPX isolates were used in this study: three from New York, one from Massachusetts (MA-QPX), and one from Virginia (Fig. S1). The NY QPX included two isolates from Raritan Bay previously shown to display different virulence characteristics (NY0313808BC7 and NY0314220AC6, here designated NY-1 and NY-2 respectively Dahl et al. 2008) as well as an isolate cultured from a diseased clam collected from the Peconic Bay (NY070348D, or NY-3). The MA-QPX isolate was purchased from American Type Culture Collection (Number 50749), and the Virginia isolate (VA-QPX) was cultured from a diseased clam originating from Old Plantation Creek, VA. All isolates were continuously propagated (sub-cultured every 2 wk) in minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (Sigma-Aldrich) media in triplicate cultures (MEM-FBS, Kleinschuster et al. 1998) at 23 °C for about 12 mo before the initiation of the experiment. Each culture flask (25-ml sterile vented and canted Falcon® culture flasks, Corning, NY) contained 5 ml sterile media inoculated with 100 µl of an exponentially growing 4-d-old QPX culture (2×10^5 cells/ml). Cultures ($n = 3$ replicate cultures per strain except for MA-QPX where $n = 2$) were incubated at 23 °C for 6 d to reach the exponential growth phase (6×10^6 cells/ml). At the end of the incubation period, the cultures were diluted with equal volumes of filtered artificial seawater and passed several times through a syringe (3-ml volume syringe without a needle, Becton, Dickinson and Company, Franklin Lakes, NJ) to facilitate liquefaction of QPX mucus secretion. The mixtures were then transferred into 15-ml conical tubes (Falcon®, Corning, NY) and centrifuged at 1,000 *g* for 20 min at 4 °C. The supernatant

Table 1. List, geographic origin and SSU gene sequences of the five QPX isolates used in the study (see Data S1 for details on sequence generation)

	Location	Strain ID	Length (bp)	SSU accession number	References
QPX-1	Raritan Bay, NY	NY0314220AC6	1,746	DQ641204	Qian et al. (2007)
QPX-2	Raritan Bay, NY	NY0313808BC7	1,746	DQ641204	Qian et al. (2007)
QPX-3	Cape Cod Bay, MA	MA97C1	1,746	DQ641205	Qian et al. (2007)
			1,744	AF155209	Maas et al. (1999)
			1,745	AY052644	Stokes et al. (2002)
			1,794	AF261664	Ragan et al. (2000)
QPX-4	Peconic Bay, NY	NY070348D	1,769	KX965675	This study
			1,752	KX965676	This study
			1,764	KX965677	This study
			1,750	KX965678	This study
OPX-5	Old Plantation Creek, VA	VA0374A1	1,750	KX965681	This study
			1,750	KX965680	This study
			1,751	KX965679	This study

was discarded and cell pellets were collected and kept on ice for immediate RNA extraction. Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH) and the manufacturer's protocol were used to isolate RNA from all samples and RNA quality and quantity were estimated spectrophotometrically by Nandorop™ (Thermo Fisher Scientific, Wilmington, DE) and using an Agilent 2100 Bioanalyzer (Santa Clara, CA).

Oligoarray design and data analysis

The oligoarray platform and protocols used in this study were described earlier (Rubin et al. 2014). Briefly, previously generated transcriptomic data were used for the production of an 8 × 15k 60-mer oligonucleotide array using the Agilent eArray application (http://www.genomics.agilent.com/en/Custom-Design-Tools/eArray). These included 6,781 curated annotated sequences and 8,297 nonannotated sequences (Rubin et al. 2014). Probes (1 probe/sequence) were synthesized in situ along with positive and negative controls using 8 × 15k-feature Agilent format slides. Labeled (Cy3 or Cy5) complementary RNA (cRNA) was synthesized from 150 ng of RNA purified from cultures using the Two-Color Microarray-Based Gene Expression Analysis Protocol (Quick Amp Labeling) following manufacturer's protocol (Agilent, Marlborough, MA). Labeled cRNA was purified using Illustra CyScribe GFX Purification Kit (GE Healthcare Marlborough, MA) and used for array hybridization (300 ng of each Cy3- and Cy5-labeled cRNA). Array hybridization and scanning were conducted as previously described (Rubin et al. 2014). LIMMA package in R software was used to normalize the intensity data and to remove within-array (method: global lowess) and between-array (method: quantile) nonbiological variation (Smyth and Speed 2003). After normalization, the intensities in separate color channels were exported into an excel spreadsheet for further data quality control and trimming. The spot intensities which were less than two-fold of the background intensities were eliminated from further analysis. The relative mRNA expression levels for each transcript were calculated as the ratio of the fluorescence intensity in individual samples and the mean fluorescence intensity of that transcript in all strains ($n = 3$ replicates per strain except for MA $n = 2$). Hierarchical clustering of samples and genes (Pearson correlation) and the determination of statistically significant differentially expressed genes (ANOVA, $P < 0.01$) were completed in the TM4-suite using MeV program (Saeed et al. 2003, 2006). The final criteria for differential gene expression were significance by ANOVA analysis and a one and one-half fold increase (up-regulation) or one and one-half fold decrease (down-regulation) from the mean (Rubin et al. 2014).

RESULTS

A total of 1,263 transcripts were found to be differentially expressed (DE) among the five QPX isolates (ANOVA, $P < 0.01$ in conjunction with 1.5-fold change, Fig. 1). Of these, 547 sequences gave a positive BLASTx hit in the NCBI non redundant (nr) data base and 474 transcripts were annotated with a putative function based on gene ontology (GO) terminologies (Table S1). The 475 transcripts were grouped into 17 functional categories representing their biological or molecular function (Fig. 2). This classification resulted in 78 DE transcripts representing response to temperature, 70 DE transcripts categorized into metabolic processes, 60 DE transcripts related to transmembrane transport, and 50 DE belonging to cellular biosynthetic processes (Fig. 2). Multiple sequence alignments of the 78 transcripts coding for heat and cold shock proteins revealed that 55 sequences represented multiple isoforms of one heat shock protein (93% pairwise identity) with a predicted molecular weight of 15 kDa. Another 12 transcripts encode for a different small heat shock protein of around 18 kDa, and the remaining 11 sequences encode two 16 kDa, one 40 kDa, three 70 kDa, one 90 kDa, and one 96 kDa heat shock proteins. Contrasting trends where noticeable among the different strains for various heat and cold shock proteins (Fig. 3). For example, relatively high expression of heat shock proteins 15 kDa (NCBI accession no: GALJ01014247), 16 kDa (GALJ01005668), and 18 kDa (GALJ01008689) was detected in NY-1 as compared to NY-3 or VA-QPX. Concomitantly, NY-1 displayed the lowest expression for the cold shock proteins 70 kDa (GALJ01013231), 90 kDa (GALJ01004566), and 100 kDa (GALJ01004707).

In addition, the differentially expressed transcripts were sorted by the highest-fold change, which revealed that the expression of 131 transcripts changed by five or more folds. Nineteen of these sequences had predicted functions (Table 2). These included the two heat shock proteins (15 and 18 kDa), two serine peptidases (one from the S8 and one from the S10 family), a nonribosomal peptide synthase, a catalase, a g-protein-coupled receptor and three transmembrane transporters (Table 2).

DISCUSSION

Oligoarray methodology was applied to generate transcription profiles of five QPX isolates originating from locations along the geographic range of QPX disease distribution in the United States. The study identified 1,263 transcripts to be differentially expressed (DE) among the five isolates (Fig. 1), with the highest number of DE and annotated transcripts belonging to heat shock response and metabolic processes. It should be noted that more than half of the DE transcripts (62%) were not annotated limiting the data interpretation, which is consistent with the lack of genetic functional investigations of some protozoan lineages (Rubin et al. 2014). Some of the transcriptomic responses can be linked to QPX response to the temperature of the experimental cultures in comparison to the natural temperature regimes at the original source of each isolate. Since the different QPX isolates were cultivated for an extended period of time prior to the experiment (giving the isolates sufficient time to acclimate to the

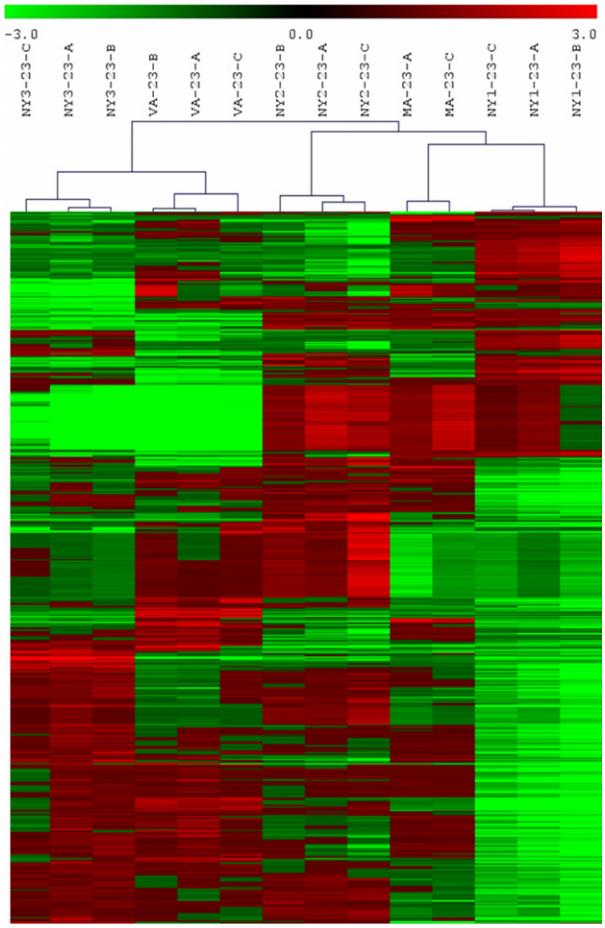

Figure 1 Hierarchical clustering of 1,263 QPX transcripts (Pearson's correlation centered) identified to be differentially expressed among five QPX isolates (NY-1 and NY-2—from Raritan Bay, NY; NY-3—from Peconic Bay, NY; MA—from Provincetown Harbor, MA; VA—from Old Plantation Creek, VA).

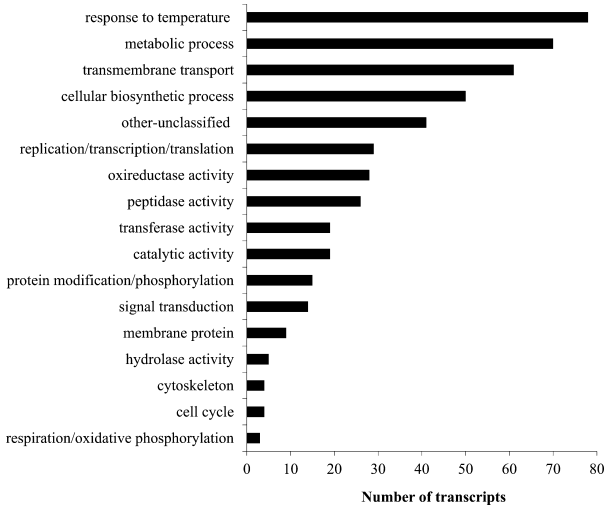

Figure 2 Number of differentially expressed transcripts among five Quahog parasite unknown isolates, grouped into biological and molecular function categories based on gene ontology terminology.

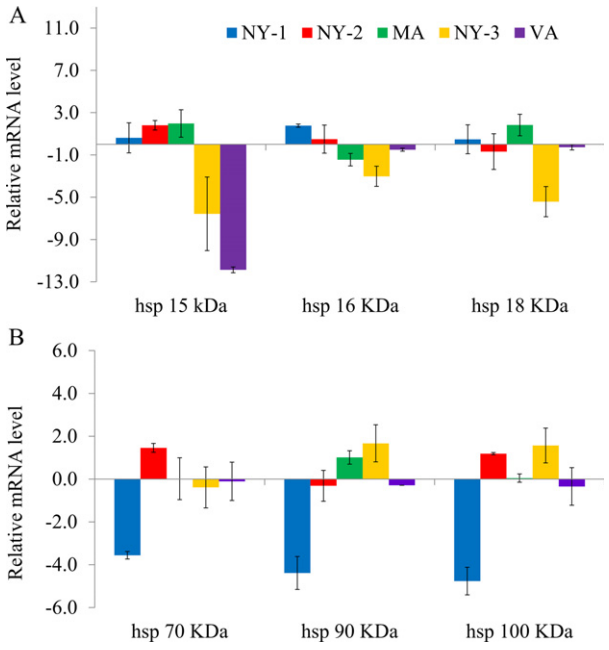

Figure 3 Differentially expressed heat and cold shock proteins among five Quahog parasite unknown isolates.

culture conditions), the divergent transcriptional signatures are most likely the result of genetic or epigenetic differences. In the study, all experimental cultures were incubated at 23 °C, which was thermally stressful for the MA-QPX isolate in comparison to VA-QPX and NY-3 isolates as highlighted by the expression profiles of small heat shock proteins in the 20 kDa family (specifically 15 and 18 kDa, Fig. 3). The low molecular weight heat shock proteins (15, 16, and 18 kDa) have been previously shown to be up-regulated in the New York isolate NY-1 grown at its suboptimal growth temperature of 27 °C (Rubin et al. 2014). On the other hand, the high molecular weight heat shock proteins (70, 90, and 100 kDa) were previously documented to be up-regulated in QPX (NY-1) cells grown at cold (13 °C) temperatures (and were consequently defined for QPX as cold shock proteins, Rubin et al. 2014). The present experiment confirms these results as the high molecular cold shock proteins were strongly downregulated in NY-1 QPX in comparison to the mean expression of all isolates (Fig. 3). These results are in agreement with the observations of Perrigault et al. (2010) who demonstrated maximal growth for the Raritan Bay isolates of QPX (NY-1 and NY-2) at 23 °C while the MA isolate (MA-QPX) displayed a 20% reduction in growth when the temperature increased from 20 to 23 °C. In addition, the VA-QPX, which is the most southern isolate of the parasite, showed strong down-regulation (12-fold decrease) of the 15 kDa heat shock protein, suggesting

Table 2. Annotated transcripts differentially expressed by five or more folds among the five QPX isolates (ANOVA, $P < 0.01$)

		Top blast match result			Fold expression change				
NCBI accession no.	Sequence description	Accession no.	E-value	Organism	NY-1	NY-2	NY-3	VA	MA
GALJ01001803	Ammonium transporter	XP_003062934	2.4E-21	*Micromonas pusilla*	0.3	1.9	0.4	−5.4	−2.1
GALJ01003935	abc Transporter	EJK46845	2.1E-87	*Thalassiosira oceanica*	−6.6	1.9	−0.5	0.4	0.0
GALJ01006762	abc Transporter	XP_001023596	3.9E-13	*Tetrahymena thermophila*	1.3	1.1	−1.2	−5.1	1.7
GALJ01004301	Calcium transporting ATPase	XP_002184696	0.0E+00	*Phaeodactylum tricornutum*	−2.0	2.0	0.5	−6.9	0.2
GALJ01003864	Non-ribosomal peptide synthetase	WP_004272016	4.8E-07	*Azospirillum amazonense*	−2.9	1.2	2.3	−1.3	−21.6
GALJ01004530	Zinc finger, CCHC-type	XP_003103852	1.9E-05	*Caenorhabditis remanei*	1.8	1.4	−6.8	−0.3	−2.2
GALJ01005083	von Willebrand factor domain	ETV79340	1.8E-79	*Aphanomyces astaci*	−5.2	−0.5	1.6	−0.1	1.1
GALJ01005112	Catalase	AEX91749	6.4E-34	*Acanthamoeba castellanii*	−5.4	1.2	−2.4	1.3	2.3
GALJ01006102	Bacitracin synthetase	AHG22620	7.7E-13	*Serratia fonticola RB-25*	−17.6	−6.1	−0.6	0.8	−5.3
GALJ01006786	Lipase	EFA75426	1.5E-09	*Polysphondylium pallidum*	−0.6	0.9	1.8	−7.5	−4.7
GALJ01007968	Long chain fatty acid synthetase	YP_006821641	2.9E-23	*Alcanivorax dieselolei*	2.5	1.4	−1.8	−9.0	−9.4
GALJ01011177	Vacuolar protein sorting-associated protein	EXX57160	2.0E-10	*Rhizophagus irregularis*	0.2	2.2	−7.9	−2.3	1.4
GALJ01014317	Zinc finger, ZZ-type	XP_007299136	1.8E-13	*Stereum hirsutum*	−8.2	1.2	0.4	1.9	−1.5
GALJ01003905	Tubulin alpha chain	XP_006433708	3.1E-88	*Citrus clementina*	−5.9	−0.6	1.3	1.3	1.5
GALJ01004863	Histone acetyltransferase	XP_001417549	9.4E-26	*Ostreococcus lucimarinus*	−6.2	0.5	−1.2	1.7	1.2
GALJ01014247	Heat shock protein 15 kDa	BAK07374	7.0E-21	*Hordeum vulgare*	0.6	1.8	−6.6	−11.9	2.0
GALJ01008689	Heat shock protein 18 kDa	WP_003690914	4.0E-20	*Lactobacillus mali*	0.5	−0.7	−5.4	−0.3	1.8
GALJ01005514	Carboxypeptidase y	XP_002550523	7.4E-10	*Candida tropicalis*	−1.8	0.4	−5.5	2.4	−1.4
GALJ01005606	Subtilisin kexin type peptidase	ABI79453	6.0E-56	*Quahog parasite X*	−4.8	−7.4	−7.3	1.2	3.4

divergence in the expression of that gene based on latitudinal origin of QPX (Fig. 3). Interestingly, the NY-3 isolate (isolated from Peconic Bay) also exhibited down-regulation of all three small heat shock proteins by three-, five-, and seven-fold for the 16, 18, and 15 kDa heat shock proteins, suggesting the adaptation of this isolate to warm temperatures (peaking at 27 °C in summer) prevailing in that shallow water embayment as compared to the NY isolates sourced from the deeper and cooler (summer temperature peaking at 23 °C) Raritan Bay site (NY-1 and NY-2).

The expression profiles of heat and cold shock proteins suggest that NY-3 QPX and VA-QPX are more related to each other than the other 2 NY isolates and the MA isolate. Differential expression of heat shock proteins has been documented for many aquatic organisms living in shallow, stagnant waters (Feder and Hofmann 1999). In addition, expression of heat shock proteins varies along the temperature gradient related to geographic distribution across degrees of latitude (Feder and Hofmann 1999). In fact, heat shock protein-related sequences have been used to study differences among strains within the same species. For example, the nucleotide sequence polymorphisms of the heat shock protein 16 gene allow for assignment of *Trypanosoma cruzi* strains into two main genetic groups (Perez-Morales et al. 2009). In addition, genetic polymorphism within the 5′ or 3′ untranslated regions (UTRs) of heat shock protein 70 is responsible for the regulation of gene expression among strains of *T. cruzi* (Rodrigues et al. 2010). The strong variation in the expression of the 15 kDa heat shock protein (GALJ01004863, Fig. 3 and Table S1) reported here across different QPX isolates suggests that the coding region or the genomic regions involved in the regulation of this transcript could represent a genetic marker for the study of intraspecific QPX variation.

This study also suggests that QPX isolates from different locations exhibit adaptation to the salinity of local waters. The VA-QPX isolate used in this study originated from a clam mortality outbreak which took place in 2009 at an aquaculture farm located at Old Plantation Creek (OPC), a small embayment connected to Chesapeake Bay, Virginia. The water salinity of OPC varies between 15 and 25 ppt which makes it an unusual location for QPX disease, because previous QPX disease outbreaks in VA were only reported for high salinity (30–35 ppt locations of the Atlantic coast (Ragone Calvo et al. 1998). In addition, NY-1, NY-2, and MA isolates where shown to have optimal growth salinity around 34 ppt (Perrigault et al. 2010; Ragone Calvo et al. 1998). The cultures of all five isolates in this study were prepared in minimal essential medium

supplemented with fetal bovine serum and adjusted to 34 ppt with salts (Kleinschuster et al. 1998). Interestingly, under these growth conditions, only VA QPX cells were found to have higher expression of an aquaporin (GALJ01012077, Table S1) and lower expression of two different sodium/hydrogen ion antiporters (GALJ01005367, GALJ01007636, Table S1). Aquaporins are transmembrane proteins that export and import water out of and into the cell and participate in osmoregulation of microbial, plant, and animal cells (Echevarria and Ilundain 1998; Fu and Lu 2007). The sodium/hydrogen ion antiporters belong to the major facilitator superfamily (MFS) and they exchange sodium and hydrogen ions across the cell membrane, allowing these ions to move along the concentration gradient (Pao et al. 1998). The down-regulation of the sodium transporter in VA-QPX in response to the high sodium concentration outside the cell suggests a phenotypic plasticity and acclimation ability of that isolate to the high salinity of the culture media.

A main objective of this study was to gain insight into the molecular basis of virulence variation among the three QPX isolates that were previously shown to cause different mortality rates in clams during experimental challenge (Dahl et al. 2008). The DE transcripts were screened for sequences coding for proteins commonly associated with virulence in other pathogenic organisms. In the study by Dahl et al. (2008), the MA isolate caused highest percent mortality in clams from VA, NY-2 isolate caused the highest percent mortality in MA clams, the NY-1 isolate caused the highest percent mortality to NY clams, and finally both NY-1 and NY-2 isolates caused the highest percent mortality in clams from Florida. Overall the study concluded that both the genetic susceptibility of clams and the virulence of QPX isolates determine the disease outcome (Dahl et al. 2008). In this study, some QPX isolates were found to have higher expression of putative virulence-related factors than other isolates (Rubin et al. 2014). For instance, NY-1 QPX exhibited higher expression of three different peptidases (GALJ01002048, GALJ01005486, and GALJ01011145, Table S1) as compared to the other isolates. Among these three peptidases, only one (GALJ01002048) belongs to a family of peptidases associated with virulence (the papain-like peptidase family C1), while the other two peptidases (GALJ01005486 of the carboxypeptidase family S28, and GALJ01011145 of the oligopeptidase family S10) are less commonly involved in infection processes. On the other hand, NY-2 QPX exhibited higher expression of two virulence-related peptidases: a S8 or subtilisin-like peptidase (GALJ01010717, Table S1) and a C1 or papain-like peptidase (GALJ01009092, Table S1). Interestingly, contrasting expression of papain-like cysteine peptidases has been documented for two different strains of *Entamoeba histolytica* exhibiting different virulence phenotypes (Davis et al. 2007). The actual funtion of the specific QPX peptidase coded by that transcript has to be further investigated to confirm its role in QPX infectivity.

Furthermore, the MA-QPX cells were found to have more than a threefold higher expression of another subtilisin-like peptidase (GALJ01005606, Table S1). QPX possesses at least seven different sequences encoding subtilisin-like peptidases with amino acid similarities ranging from 36 to 79% (Rubin et al. 2015). It is important to note that QPX disease is endemic in MA and has been regularly causing severe mortality events in clams since the 1990s (Kraeuter et al. 2011; Smolowitz et al. 1998). In contrast, QPX epizootics in NY are rarer and the organism is often detected at background levels. Therefore, it can be speculated that the MA-QPX isolate is more virulent and the subtilisin peptidase gene (GALJ01005606, Table S1) could be responsible for that difference. On the other hand, a final outcome of QPX disease leading to clam mortality is not exclusively dependent on QPX virulence but also on genetic susceptibility of clam stocks (Dahl et al. 2008). In another study, when three different clam strains were kept in Barnstable Harbor, MA and in Dry Bay, NJ, all strains suffered the highest QPX prevalence and mortalities at the MA location (Kraeuter et al. 2011). Of course, environmental conditions, specifically stress factors that could favor disease development, have to be considered as well. In fact, QPX is widely considered as an opportunistic pathogen that is able to cause disease when the clams are stressed by environmental factors (Dahl et al. 2011; Perrigault et al. 2011). Therefore, the QPX disease outcome is the result of a combination of factors including environmental stressors (to clams or to the parasite), clam susceptibility, and QPX virulence, and all of these have to be accounted for in analysis of clam mortality events.

Subtilisin-like peptidases are common virulence factors in many pathogenic organisms including bacteria, fungi, and protozoa (Bonifait et al. 2010; McKerrow et al. 2006; Monod et al. 2002). However, further studies aimed at understanding the function and role of subtilisin peptidases in QPX virulence are required to determine which increase in expression contributes most to the variation in virulence among QPX isolates. The expression of these transcripts, however, was also found to be influenced by temperature changes (Rubin et al. 2014), and since the different isolates have different optimal temperatures, the expression of these peptidases might also be a result of adjustment of QPX metabolic rate in response to temperature.

Another noticeable difference between QPX isolates was the expression of several enzymes related to carbohydrate metabolism, some of which are likely associated with extracellular polysaccharide production. For example, dTDP-4-dehydrorhamnose reductase (GALJ01007497, Table S1) is an enzyme that catalyzes the production of L-rhamnose. The carbohydrate composition of QPX extracellular muco-polysaccharide secretion is unknown and rhamnose is not a major component of extracellular polysaccharides of two other thraustochytrids (Jain et al. 2005). However, this does not exclude the possibility of its presence in QPX mucus and variability in that sugar in the composition of mucus from different isolates. Similarly, the expression of UDP-glucose/GDP-mannose dehydrogenase (GALJ01002143, Table S1), an enzyme

most likely involved in polysaccharide biosynthesis in QPX (Rubin et al. 2014), was also differentially expressed between QPX isolates. Differences in the thickness and stain affinity of the mucus layer between MA-QPX and VA-QPX cells examined on histological sections of clam tissue has been previously noted (Ragone Calvo et al. 1998). In addition, the differences in mucus viscosity among QPX isolates are very noticeable during routine preparation of QPX subcultures (Rubin, personal observation). The carbohydrate and protein composition of mucus secretion of the studied QPX isolates needs to be completed to test whether the above transcripts are responsible for the reported differences in mucus characteristics.

In conclusion, this study shows that QPX isolates originating from distant geographic locations show different transcription profiles under the same culturing conditions, even after being subcultured under the same conditions for at least 1 year prior to the experiment. These findings suggest that the transcriptional profiles reflect an underlying genetic or epigenetic difference in QPX isolates that appear to be adapted to local environmental conditions such as temperature and salinity ranges. The quite high fold changes in the expression of some genes should be explored as genetic markers for the study of QPX genetic variation across its geographical range. However, the actual nucleotide polymorphism of these genes among QPX isolates would have to be determined to demonstrate their suitability for QPX genotyping or population level studies. This study also identifies potential virulence-related genes which are differentially expressed among different QPX isolates. These molecules are excellent subjects for further studies on molecular bases of virulence variation among QPX strains.

ACKNOWLEDGMENTS

We thank Drs. Eugene Burreson and Ryan Carnegie (Virginia Institute of Marine Science) for providing Virginia clams that served for the isolation of the VA strain of QPX. This research was supported by projects R/XG-19 and R/FBM-34, funded under award NA07OAR4170010 from the National Sea Grant College Program of NOAA to the Research Foundation of State University of New York on behalf of New York Sea Grant. The study was also partially supported by project 2012-38500-19656 funded by the Northeastern Regional Aquaculture Center and project IOS 1050596 supported by the National Science Foundation. The statements, findings, conclusions, views, and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations.

LITERATURE CITED

Baptista, C. S., Vencio, R. Z., Abdala, S., Carranza, J. C., Westenberger, S. J., Silva, M. N., Pereira, C. A., Galvao, L. M., Gontijo, E. D., Chiari, E., Sturm, N. R. & Zingales, B. 2006. Differential transcription profiles in *Trypanosoma cruzi* associated with clinical forms of Chagas disease: maxicircle NADH dehydrogenase subunit 7 gene truncation in asymptomatic patient isolates. *Mol. Biochem. Parasitol.*, 150:236–248.

Bonifait, L., de la Cruz Dominguez-Punaro, M., Vaillancourt, K., Bart, C., Slater, J., Frenette, M., Gottschalk, M. & Grenier, D. 2010. The cell envelope subtilisin-like proteinase is a virulence determinant for *Streptococcus suis*. *BMC Microbiol.*, 10:42.

Bozdech, Z., Mok, S., Hu, G., Imwong, M., Jaidee, A., Russell, B., Ginsburg, H., Nosten, F., Day, N. P., White, N. J., Carlton, J. M. & Preiser, P. R. 2008. The transcriptome of *Plasmodium vivax* reveals divergence and diversity of transcriptional regulation in malaria parasites. *Proc. Natl Acad. Sci. USA*, 105:16290–16295.

Dahl, S. F., Perrigault, M. & Allam, B. 2008. Laboratory transmission studies of QPX disease in the hard clam: interactions between different host strains and pathogen isolates. *Aquaculture*, 280:64–70.

Dahl, S. F., Perrigault, M., Liu, Q., Collier, J. L., Barnes, D. A. & Allam, B. 2011. Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog parasite unknown) disease development: I. Dynamics of QPX disease. *J. Invertebr. Pathol.*, 106:314–321.

Davis, P. H., Schulze, J. & Stanley Jr, S. L. 2007. Transcriptomic comparison of two *Entamoeba histolytica* strains with defined virulence phenotypes identifies new virulence factor candidates and key differences in the expression patterns of cysteine proteases, lectin light chains, and calmodulin. *Mol. Biochem. Parasitol.*, 151:118–128.

Dove, A. D. M., Bowser, P. R. & Cerrato, R. M. 2004. Histological analysis of an outbreak of QPX disease in wild hard clams *Mercenaria mercenaria* in New York. *J. Aquat. Anim. Health*, 16:246–250.

Echevarria, M. & Ilundain, A. A. 1998. Aquaporins. *J. Physiol. Biochem.*, 54:107–118.

Feder, M. E. & Hofmann, G. E. 1999. Heat-shock proteins, molecular chaperones, and the stres response: evolutionary and ecological physiology. *Annu. Rev. Physiol.*, 61:243–282.

Fu, D. & Lu, M. 2007. The structural basis of water permeation and proton exclusion in aquaporins. *Mol. Membr. Biol.*, 24:366–374.

Jain, R., Raghukumar, S., Tharanathan, R. & Bhosle, N. B. 2005. Extracellular polysaccharide production by thraustochytrid protists. *Mar. Biotechnol. (NY)*, 7:184–192.

Kleinschuster, S. J., Smolowitz, R. & Parent, J. 1998. In vitro life cycle and propagation of Quahog parasite unknown. *J. Shellfish Res.*, 17:75–78.

Kraeuter, J. N., Ford, S., Bushek, D., Scarpa, E., Walton, W., Murphy, D. C., Flimlin, G. & Mathis, G. 2011. Evaluation of three northern quahog (hard clam) *Mercenria mercenaria* (Linnaeus) strains grown in Massachusetts and New Jersey for QPX-resistance. *J. Shellfish Res.*, 30:805–812.

Llinas, M., Bozdech, Z., Wong, E. D., Adai, A. T. & DeRisi, J. L. 2006. Comparative whole genome transcriptome analysis of three *Plasmodium falciparum* strains. *Nucleic Acids Res.*, 34:1166–1173.

Maas, P. A. Y., Kleinschuster, S. J., Dykstra, M. J., Smolowitz, R. & Parent, J. 1999. Molecular characterization of QPX (Quahog parasite unknown), a pathogen of *Mercenaria mercenaria*. *J. Shellfish Res.*, 18:561–567.

McKerrow, J. H., Caffrey, C. R., Kelly, B. L., Loke, P. & Sajid, M. 2006. Proteases in parsitic diseases. *Annu. Rev. Pathol. Mech. Dis.*, 1:497–536.

Mo, C., Douek, B. & Rinkevich, B. 2002. Development of a PCR strategy for thraustochytrid identification based on 18S rDNA sequence. *Mar. Biol.*, 140:883–889.
Monod, M., Capoccia, S., Lechenne, B., Zaugg, C., Holdom, M. & Jousson, O. 2002. Secreted proteases from pathogenic fungi. *Int. J. Med. Microbiol.*, 292:405–419.
Pan, J., Del Campo, J. & Keeling, P. J. 2016. Reference tree and environmental sequence diversity of Labyrinthulomycetes. *J. Eukaryot. Microbiol.*, doi:10.1111/jeu.12342.
Pao, S. S., Paulsen, I. & Saier, M. H. 1998. Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.*, 62:1–34.
Perez-Morales, D., Ostoa-Saloma, P. & Espinoza, B. 2009. *Trypanosoma cruzi* SHSP16: characterization of an alpha-crystallin small heat shock protein. *Exp. Parasitol.*, 123:182–189.
Perrigault, M., Bugge, D. M. & Allam, B. 2010. Effect of environmental factors on survival and growth of Quahog parasite unknown (QPX) in vitro. *J. Invertebr. Pathol.*, 104:83–89.
Perrigault, M., Dahl, S. F., Espinosa, E. P., Gambino, L. & Allam, B. 2011. Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog parasite unknown) disease development: II. Defense parameters. *J. Invertebr. Pathol.*, 106:322–332.
Qian, H., Liu, Q., Allam, B. & Collier, J. L. 2007. Molecular genetic variation within and among isolates of QPX (Thraustochytridae), a parasite of the hard clam *Mercenaria mercenaria*. *Dis. Aquat. Organ.*, 77:159–168.
Ragan, M. A., MacCallum, G. S., Murphy, C. A., Cannone, J. J., Gutell, R. R. & McGladdery, S. E. 2000. Protistan parasite QPX of hard-shell clam *Mercenaria mercenaria* is a member of Labyrinthulomycota. *Dis. Aquat. Organ.*, 42:185–190.
Ragone Calvo, L. M., Walker, J. G. & Burreson, E. M. 1998. Prevalence and distribution of QPX, Quahog parasite unknown, in hard clams *Mercenaria mercenaria* in Virginia, USA. *Dis. Aquat. Organ.*, 33:209–219.
Rodrigues, D. C., Silva, R., Rondinelli, E. & Urmenyi, T. P. 2010. *Trypanosoma cruzi*: modulation of HSP70 mRNA stability by untranslated regions during heat shock. *Exp. Parasitol.*, 126:245–253.
Rubin, E., Pales Espinosa, E., Koller, A. & Allam, B. 2015. Characterisation of the secretome of the clam parasite, QPX. *Int. J. Parasitol.*, 45:187–196.
Rubin, E., Tanguy, A., Perrigault, M., Pales Espinosa, E. & Allam, B. 2014. Characterization of the transcriptome and temperature-induced differential gene expression in QPX, the thraustochytrid parasite of hard clams. *BMC Genom.*, 15:245.
Saeed, A. I., Bhagabati, N. K., Braisted, J. C., Liang, W., Sharov, V., Howe, E. A., Li, J., Thiagarajan, M., White, J. A. & Quackenbush, J. 2006. TM4 microarray software suite. *Methods Enzymol.*, 411:134–193.
Saeed, A. I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V. & Quackenbush, J. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques*, 34:374–378.
Smolowitz, R., Leavitt, D. & Perkins, F. 1998. Observations of a protistan disease similar to QPX in *Mercenaria mercenaria* (hard clams) from the coast of Massachusetts. *J. Invertebr. Pathol.*, 71:9–25.
Smyth, G. K. & Speed, T. 2003. Normalization of cDNA microarray data. *Methods*, 31:265–273.
Stokes, N. A., Ragone Calvo, L. M., Reece, K. S. & Burreson, E. M. 2002. Molecular diagnostics, field validation, and phylogenetic analysis of Quahog parasite unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria*. *Dis. Aquat. Organ.*, 52:232–247.
Tsui, C. K., Marshall, W., Yokoyama, R., Honda, D., Lippmeier, J. C., Craven, K. D., Peterson, P. D. & Berbee, M. L. 2009. Labyrinthulomycetes phylogeny and its implications for the evolutionary loss of chloroplasts and gain of ectoplasmic gliding. *Mol. Phylogenet. Evol.*, 50:129–140.
Whyte, S. K., Cawthorn, R. J. & McGladdery, S. E. 1994. QPX (Quahog Parasite X), a pathogen of northern quahaug *Mercenaria mercenaria* from the Gulf of St. Lawrence, Canada. *Dis. Aquat. Organ.*, 19:129–136.

[1]Present address: Graduate School of Oceanography, 215 S Ferry Rd, Narragansett, RI 02882, USA.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Map showing the geographical origins (stars) of the QPX isolates.
Table S1. Annotated transcripts that were differentially expressed among the five QPX isolates: (NY-1 and NY-2—from Raritan Bay, NY; NY-3—from Peconic Bay, NY; MA—from Provincetown Harbor, MA; VA—from Old Plantation Creek, VA).
Data S1. SSU rRNA gene sequences for two QPX isolates (NY070348D and VA0374A1).