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Conservation in the first internal transcribed spacer (ITS1) region of *Hematodinium perezi* (genotype III) from *Callinectes sapidus*

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ABSTRACT: *Hematodinium* spp. infections have been reported from blue crabs *Callinectes sapidus* in high-salinity waters of the USA from New Jersey to Texas. Recently, *H. perezi* (genotype III) has been proposed as the parasite species and genotype infecting blue crabs from Virginia; however, it is unknown whether this same genotype is present in blue crabs from other locations. To address this question, we collected 317 blue crabs from Massachusetts, Virginia, Georgia, Florida, Louisiana, and Texas to test for the presence of *H. perezi* (III) using a specific PCR assay targeting the first internal transcribed spacer (ITS1) region of the ribosomal RNA gene complex. To examine the genetic variation within *H. perezi* (III), ITS1 region sequences from the parasite in blue crabs from multiple locations were compared to each other and to those of *H. perezi* (III) found in alternate hosts from Virginia. In total, 34 distinct ITS1 sequence variants of the parasite were identified from blue crabs alone, and 38 distinct variants were identified when alternate hosts were included. However, a single ITS1 sequence variant appeared in all geographic regions and hosts, and also in blue crabs sampled from a previous study. The high similarity among all the ITS1 region sequences examined (>98%) and the observation of a single variant found throughout a large geographic range, strongly suggests that a single species and genotype of *Hematodinium*, specifically *H. perezi* (III), infects blue crabs from Virginia to Texas and multiple alternate host species in Virginia.

KEY WORDS: Dinoflagellate · Parasite · Blue crab · Crustacean · ITS1 region · Alternate hosts · Genetic variation

INTRODUCTION

Economically important fisheries for the blue crab *Callinectes sapidus* occur in 11 states in the USA from Delaware south to Texas (Milliken & Williams 1984). From 1989 to 1993, average annual blue crab landings from the Atlantic and Gulf coasts exceeded 96.6 kilotons (kt) with a dockside value of US$137 million in 1994 (Johnson et al. 1998). In the last decade, however, there have been major declines in harvest yields of many blue crab fisheries in the USA. In 2007, Chesapeake Bay had a total harvest of 19.7 kt, which was the lowest on record since 1945 (CBSAC 2010). Along coastal Georgia, blue crab landings dropped to 0.9 kt, well below the 45 yr average of 1.8 kt (Lee & Frischer 2004). While data indicate that the blue crab stock in Chesapeake Bay has begun to rebound over the last 2 yr, managers have

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decided to continue stringent conservation measures until the impact of these efforts can be fully assessed (CBSAC 2010, 2012).

While many factors are believed to affect blue crab populations and fisheries (Kennedy et al. 2007), 1 potential explanation for the decline of *Callinectes sapidus* populations may be a parasitic dinoflagellate, *Hematodinium perezi*. Recent studies have shown that this species is composed of 3 distinct genotypes, with the third genotype, herein referred to as *H. perezi* (III), infecting *C. sapidus* in Virginia waters (Small et al. 2012). To date, infections presumably of the same genotype of *H. perezi* have been reported in adult blue crabs from New Jersey south to Texas (Messick & Shields 2000), with prevalence ranging from 9 to 98% in some blue crab populations (Newman & Johnson 1975, Messick 1994, Messick & Shields 2000, Lee 2000, Gruebl et al. 2002). However, many of the reports documenting *Hematodinium* sp. infections in blue crabs identified the parasite based only on histological examination of tissues or hemolymph smears and did not incorporate molecular data to confirm the species or genotype of *Hematodinium* present. As morphological characters cannot confidently distinguish between different parasite species or genotypes within this genus (Stentiford & Shields 2005), molecular data are necessary to confirm that the same species and genotype of *Hematodinium* occurs throughout this range; this has previously been suggested as a research priority by Small & Pagenkopp (2011).

While the current molecular evidence suggests that there is 1 species of *Hematodinium* that appears to infect several different crustacean species from European waters (Small et al. 2007a, Hamilton et al. 2009, 2010, Eigemann et al. 2010), the North Pacific (Jensen et al. 2010), and Greenland waters (Eigemann et al. 2010), only 2 studies have used molecular techniques to examine the potential number of *Hematodinium* species within the blue crab range. One study demonstrated that *H. perezi* (III) appears to be present throughout the Delmarva Peninsula, Virginia, because of the high similarity in pathology, parasite morphology, and first internal transcribed spacer region (ITS1) region sequences from a variety of infected hosts, including blue crabs (Pagenkopp Lohan et al. 2012a). Additionally, the findings of Small et al. (2007b) suggest that *H. perezi* (III) likely occurs in Virginia and Georgia waters based on the results of a restriction fragment length polymorphism (RFLP) assay showing that the *Hematodinium* sp. obtained from blue crabs from Virginia and Georgia had identical profiles on a gel. However, sequence data are needed to confirm this hypothesis.

The purpose of the current study was to investigate whether *Hematodinium perezi* (III) is the only genotype and species of this parasite present in blue crab populations over a wide geographic range. Sequence data from a portion of the ITS1 region of the ribosomal RNA (rRNA) gene complex were used to determine the number of genotypes of *H. perezi* present in blue crab populations from Massachusetts, Virginia, Georgia, and the Gulf of Mexico. The ITS region was chosen as it is a commonly used molecular marker for distinguishing between species and strains of free-living dinoflagellates (LaJeunesse 2001, Litaker et al. 2007, Small et al. 2009), closely related shellfish parasites (Dungan et al. 2002, Casas et al. 2004, Dungan & Reece 2006, Moss et al. 2008, Small 2012), and it was previously used to investigate the species and strain composition of other *Hematodinium* sp. isolates (Small et al. 2007a, 2012, Hamilton et al. 2009, 2010, Eigemann et al. 2010). The sequences obtained in this study from a portion of the ITS1 region of *H. perezi* were compared to ITS1 region sequences of *H. perezi* obtained from alternate hosts and to previously published ITS1 region sequences of *H. perezi* from blue crabs to examine the geographic range and temporal stability of the parasite.

MATERIALS AND METHODS

Sample collection

Blue crabs were sampled from 19 different locations in the USA (Fig. 1, Table 1). Blue crabs from the Delmarva Peninsula, Virginia, were collected via crab pots from 3 primary seaside sites (Chincoteague Bay, Wachapreague Creek, and Fisherman Island) in 2008 (May to December) and 2009 (April to December) using commercial crab pots. An additional 3 sites (Cherrystone Creek, Oyster Creek, and Metompkin Bay) were sampled only in 2008. Alternate hosts were collected as described by Pagenkopp Lohan et al. (2012a).

Blue crabs were purchased from local fisherman from 5 sites in Massachusetts (Oyster Pond, Cociut Bay, Bass River, Agawam River, and Westport River), 4 sites in Florida (Goodland, Port Charlotte, Tampa, and Panama City), 1 site in Louisiana (New Iberia), and 2 sites in Texas (Texas City and Corpus Christi Bay).

Extracted DNA samples of blue crabs infected with a species of *Hematodinium* from coastal Savannah,

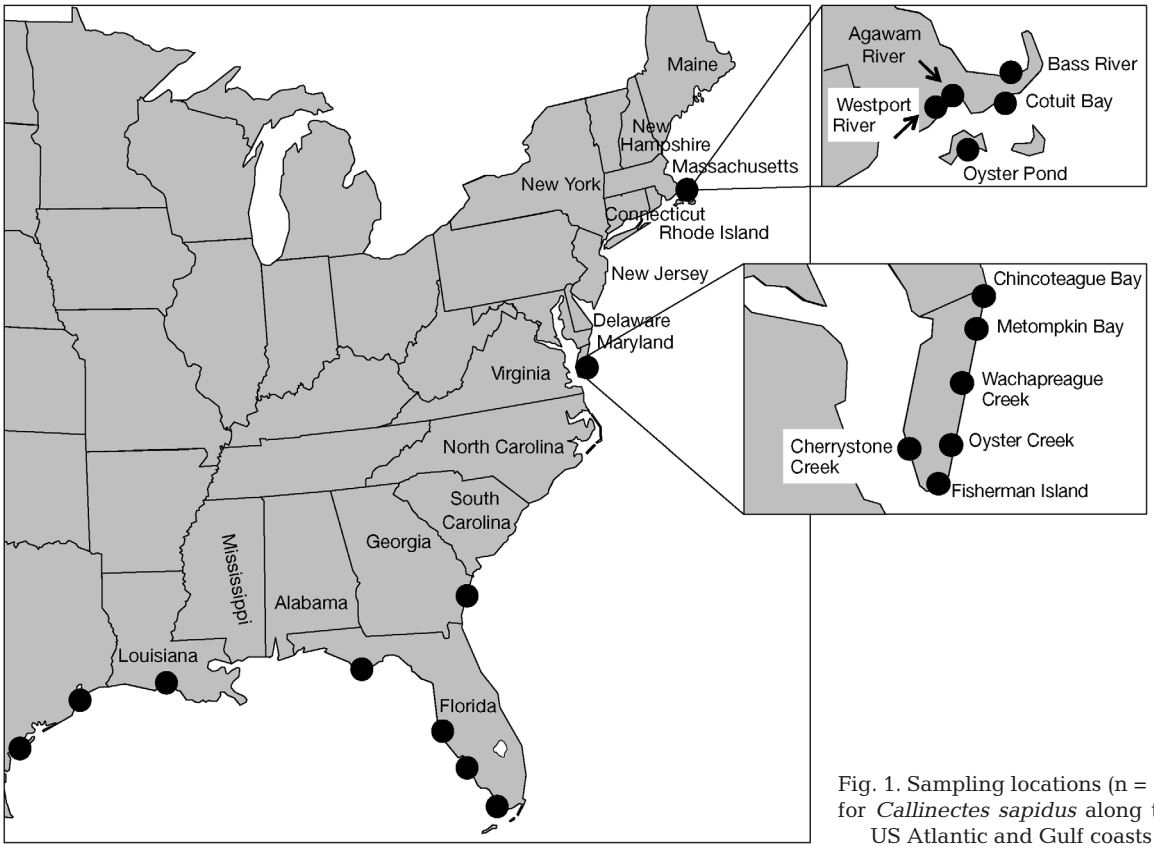

Fig. 1. Sampling locations (n = 19) for *Callinectes sapidus* along the US Atlantic and Gulf coasts

Georgia (n = 8), collected between 2000 and 2004 were obtained from M. Frischer and R. Lee (Skidaway Institute of Oceanography). Hemolymph samples stored in 95% ethanol from blue crabs infected with a species of *Hematodinium* from Corpus Christi Bay, Texas (n = 7), were obtained from J. Gain (Texas A&M University).

Infection status

Infection status was assessed for blue crabs and alternate hosts from Virginia as described by Pagenkopp Lohan et al. (2012a). Briefly, for blue crabs and other large crustaceans, infection status was first assessed microscopically. Hemolymph smears were stained 1:1 with 0.3% Neutral red in crab saline and examined with a light microscope at 100× and 400× magnification for uptake of the dye (see Stentiford & Shields 2005). Mud crabs <10 mm carapace width (CW) were dissected, and small pieces of gill were stained and examined as above. If *Hematodinium* sp. cells were observed microscopically, then hemolymph samples were saved for molecular analysis. Hemolymph samples were preserved in 95% ethanol (500 µl hemolymph into 500 µl of 95% ethanol) and stored at −20°C for later molecular analysis. Whole crustaceans were first frozen, then transferred to 95% ethanol and stored for later molecular analysis. The infection status of the blue crab samples obtained from Massachusetts, Georgia, and the Gulf of Mexico was assessed solely via PCR (see next sub-section), as only DNA was obtained for those samples.

PCR amplification and sequencing

DNA extractions, ITS1 amplifications, and sequencing protocols for samples collected in Virginia were conducted as described by Pagenkopp Lohan et al. (2012a). Briefly, for blue crab samples, 200 µl of hemolymph in 95% ethanol were extracted with the Qiagen Tissue and Blood kit following the manufacturer's protocol for animal tissue. All DNA samples were eluted in 100 µl of buffer that was passed over the column twice, and incubated on the column for 5 min each time before centrifugation to increase DNA yield (Audemard et al. 2004). All extractions completed within the same day included a blank col-

Table 1. *Callinectes sapidus*. Locations in the USA where blue crabs were collected or obtained. The number of *C. sapidus* positive for *Hematodinium perezi* (III) via PCR analysis from which ITS1 sequences were generated, the number of amplicons that were either directly sequenced (D) or cloned (C) prior to sequencing, and the GenBank accession numbers for the consensus or unique sequences are given

Location	No. screened	No. positive	D or C	Accession no.
Massachusetts				
Martha's Vineyard, Oyster Pond	52	0	–	
Cociut Bay Estuary	15	0	–	
Bass River	15	0	–	
Agawam River	15	0	–	
Westport River	15	0	–	
Virginia				
Cherrystone Creek	6	6	D	JN380145–JN380147, JN380157, JN380169, JN380091
Oyster Creek	5	5	D	JN380148–JN380149, JN380162–JN380163, JN380166
Metompkin Bay	5	5	D	JN380150, JN380158–JN380160, JN380165
Chincoteague Bay	8	8	D=4, C=4	JN380144, JN380152–JN380155, JN380173–JN380176
Wachapreague Creek	6	6	D=3, C=3	JN380151, JN380161, JN380164, JN380170–JN380172, KC192761–KC192763
Fisherman's Island	6	6	D=4, C=4	JN380156, JN380167–JN380168, JN380177–JN380179, KC192749–KC192753
Georgia				
Savannah	8	8	C	JN380092–JN380113
Florida				
Goodland	16	2	C	JN380114–JN380116
Port Charlotte	3	0	–	
Tampa	17	1	C	JN380124, KC192754
Panama City	21	5	C	JN380117–JN380123, KC192755–KC192760
Louisiana				
New Iberia	31	0	–	
Texas				
Texas City	36	0	–	
Corpus Christi	38	7	C	JN380125–JN380143
Total	318	59	186	

umn extraction, which, when subjected to PCR analysis alongside samples, served as a control for extraction contamination. After extraction, the DNA was aliquoted (~20 µl) to avoid contamination of the entire stock of extracted DNA. The remaining stock DNA (~80 µl) was stored at –20°C and the 20 µl aliquot was stored at 4°C. Extracted DNA was quantified using a NanoDrop 2000 (Thermo Scientific).

For blue crabs collected from the Gulf of Mexico, genomic DNA isolation involved solubilization in a 10% cetylmethylammoniumbromide (CTAB) solution, followed by a phenol:chloroform extraction (Grabowski & Stuck 1999). For blue crabs collected from Massachusetts, genomic DNA was extracted from approximately 100 mg of muscle from a walking leg using the Qiagen Tissue and Blood kit according to the manufacturer's instructions. DNA was eluted off the column twice with 50 µl of water and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Genomic DNA extracted from blue crabs from the Gulf of Mexico and Massachusetts was diluted to a stock concentration of 10 ng μl^{-1} prior to PCR amplification.

To confirm the presence of high molecular weight DNA in extractions, a general metazoan primer set, nSSU A and nSSU B (modified from Medlin et al. 1988, see Moss et al. 2007) was used to amplify DNA extracted from samples. A second PCR assay using the primers HITS1F and HITS1R, which amplify a 302 base pair (bp) portion of the ITS1 region of *Hematodinium perezi* (Small et al. 2007b), was used to confirm infection status. For some of the samples from the Mexican Gulf of Mexico coast of Florida, 2 rounds of the ITS1 PCR were performed to increase the number of amplicons available for cloning. These PCRs were conducted as described above with 5 µl of DNA in the first amplification, and 0.5 µl from the first amplification was used as the template for a second amplification. A third PCR assay using primers Hemat-F-1487 and Hemat-R-1654, a modified version of the Gruebl et al. (2002) assay, which amplifies a 187 bp portion of the SSU rRNA gene of all known

members of the genus *Hematodinium*, was applied to blue crab samples from Massachusetts and the Gulf of Mexico due to the possibility of encountering other species of *Hematodinium* in these locations. A negative control, consisting of no DNA, was included in all of the diagnostic PCR assays, and a positive control, consisting of either DNA extracted from a culture of *H. perezi* (III) or a sample that had reliably amplified in the past, was also included in all diagnostic PCR assays.

The ITS1 region PCR products were cloned and sequenced as described by Moss et al. (2008) or sequenced directly. Prior to cloning, those amplicons from samples from the Gulf Coast of Florida that were produced through 2 rounds of PCR were first excised from the agarose gel. The excised PCR products were purified using a Qiagen gel extraction kit (Qiagen) and eluted in 30 µl of buffer. Amplicons of a portion of the ITS1 region were cloned with a TOPO TA cloning kit (Invitrogen) using half of the total volume of cells recommended by the manufacturer's instructions. Plasmid DNA was extracted from individual colonies following a boil-prep method, and colonies were screened using the M13 F/R vector primers supplied in the cloning kit. An aliquot of the PCR products (5 µl) was visualized under UV light on a 2% (w/v) agarose gel after ethidium bromide staining. PCR products of the expected size based on the insert (302 bp) and flanking vector region amplified with the M13 primers (~500 bp) were purified with exonuclease I (EXO) and shrimp alkaline phosphatase (SAP). The purified PCR product was used as the template for sequencing with M13 F/R primers and the Big Dye Terminator kit (Applied Biosystems) using 1/8 of the reaction size recommended by the manufacturer. All samples were bidirectionally sequenced. The sequence reactions were precipitated using an ethanol/sodium acetate protocol (ABI User Bulletin 2002). The precipitated sequencing products were resuspended in Hi-Di formamide (Applied Biosystems) and electrophoresed on a 3130xl Genetic Analyzer (Applied Biosystems).

For the samples from Georgia, 3 to 4 clones per sample were sequenced. For the samples from the Gulf Coast of Florida, 1 to 5 clones per sample were sequenced. For the additional 7 samples from Texas, 7 to 8 clones per sample were sequenced. For the 6 samples from Virginia whose amplicons were cloned, 1 to 4 clones per sample were sequenced. Due to the low variability observed previously, and to reduce the cost per sample, the majority of samples from Virginia were directly sequenced.

Sequence analysis

Sequences were edited in CodonCode Aligner and were aligned and compared using CodonCode Aligner and MacClade 4.07 OSX (Madison & Madison 2005) to determine similarity. When clones from the same sample showed identical ITS1 region sequences, the consensus sequence for the clones was used in the analyses. The sequences from this study were then compared to *Hematodinium perezi* (III) sequences from infected blue crabs collected in 2005, which were previously deposited in GenBank (Small et al. 2007b; accession numbers DQ925227–DQ925236), to determine whether there were changes in the ITS1 sequences of the parasites over time in Virginia coastal waters. An alignment with all of the ITS1 region consensus sequences of *Hematodinium* sp. from blue crabs was run through TCS 1.21 (http://darwin.uvigo.es/software/tcs.html; Templeton et al. 1992), with gaps as a fifth state, to determine whether any geographic groupings occurred. ITS1 sequence variants were defined as consensus sequences that differed by at least 1 bp or indel.

A second TCS 1.21 analysis was conducted as described above, which combined the *Hematodinium perezi* (III) ITS1 region sequences obtained from the above blue crab sequences and those obtained from alternate hosts (Pagenkopp Lohan et al. 2012a; accession numbers JN368149–JN368194), to determine whether any host groupings occurred.

Lastly, ITS1 region sequences from *Hematodinium perezi* genotypes I and II (Small et al. 2012) were downloaded from GenBank (accession numbers EF065708–EF065716, EF153724–EF153727, EF173451–EF173454) and added to the alignment of parasite ITS1 region sequences from blue crabs from this study, the previously deposited ITS1 sequences of *H. perezi* (III) from blue crabs, and those from alternate hosts. This alignment was used to calculate the average genetic distances between the sequences in MEGA 5.05 (Tamura et al. 2011). For this calculation, the partial ITS1 region sequences were combined into 4 groups: ITS1 region sequences from *H. perezi* (I) from GenBank, ITS1 region sequences from *H. perezi* (II) from GenBank, ITS1 region sequences from *H. perezi* (III) from alternate hosts, and ITS1 region sequences from *H. perezi* (III) from *Callinectes sapidus*. Genetic distance was calculated with a partial deletion of gaps and missing data with site coverage cutoff at 95% and a p-distance model.

RESULTS

In total, 186 sequences and 97 consensus sequences of a portion of the ITS1 region of *Hematodinium* sp. were obtained from infected blue crabs from 10 of the 19 locations surveyed (Fig. 1, Table 1). *Hematodinium* sp. DNA was not amplified from any blue crabs from Massachusetts or Louisiana using either of the parasite PCR assays. *Hematodinium* sp. DNA was amplified with both parasite PCR assays from 8 blue crabs from Georgia, 7 blue crabs from Texas, and 11 blue crabs from the Gulf Coast of Florida (Table 1). *Hematodinium* sp. DNA was amplified from 1 sample from Panama City, Florida, with the parasite SSU PCR assay, but not with the parasite ITS1 PCR assay. Although weak amplicons were observed, ITS1 region sequences of *Hematodinium* could not be obtained from 2 additional samples from Florida (1 from Tampa and 1 from Panama City). As no ITS1 sequence data were obtained from these 3 blue crabs from Florida, they were excluded from the analysis.

Of those parasite sequences obtained from blue crab hosts, 34 distinct ITS1 sequence variants were observed; however, 1 variant was observed in all geographic regions (n = 43 consensus sequences; Fig. 2). Six of the 9 sequences from the 2005 study matched the most common ITS1 variant from this study, while the other 3 sequences from 2005 differed from the most common ITS1 variant by a single nucleotide substitution or indel. From 34 amplicons, 161 clones were sequenced from a portion of the ITS1 region of *Hematodinium* sp. (Table 2). Of these cloned sequences, 74% (n = 118) were the most common ITS1 variant observed, 17% (n = 28) matched another clone that was not the most common ITS1 variant, and 9% (n = 15) were only observed a single time. The sampling site with the most unique ITS1 consensus sequences was Georgia (n = 11), although most of these sequences differed by only a single nucleotide polymorphism or indel. Only the most common ITS1 variant was detected in the amplicons from blue crabs that were directly sequenced.

When ITS1 region sequences of the parasite from alternate hosts were combined with those from blue crabs, a total of 38 distinct ITS1 sequence variants were observed (Fig. 3). The most common ITS1 variant was observed in all hosts examined (n = 58 consensus sequences). Six additional ITS1 variants were observed either in multiple locations or in multiple host species. Of the 40 clones obtained from alternate hosts, 80% (n = 32) were the most common ITS1 variant observed, 10% (n = 4) matched another clone that was not the most common ITS1 variant, and 10% (n = 4) were only observed a single time (Table 3). With the exception of a single amplicon obtained

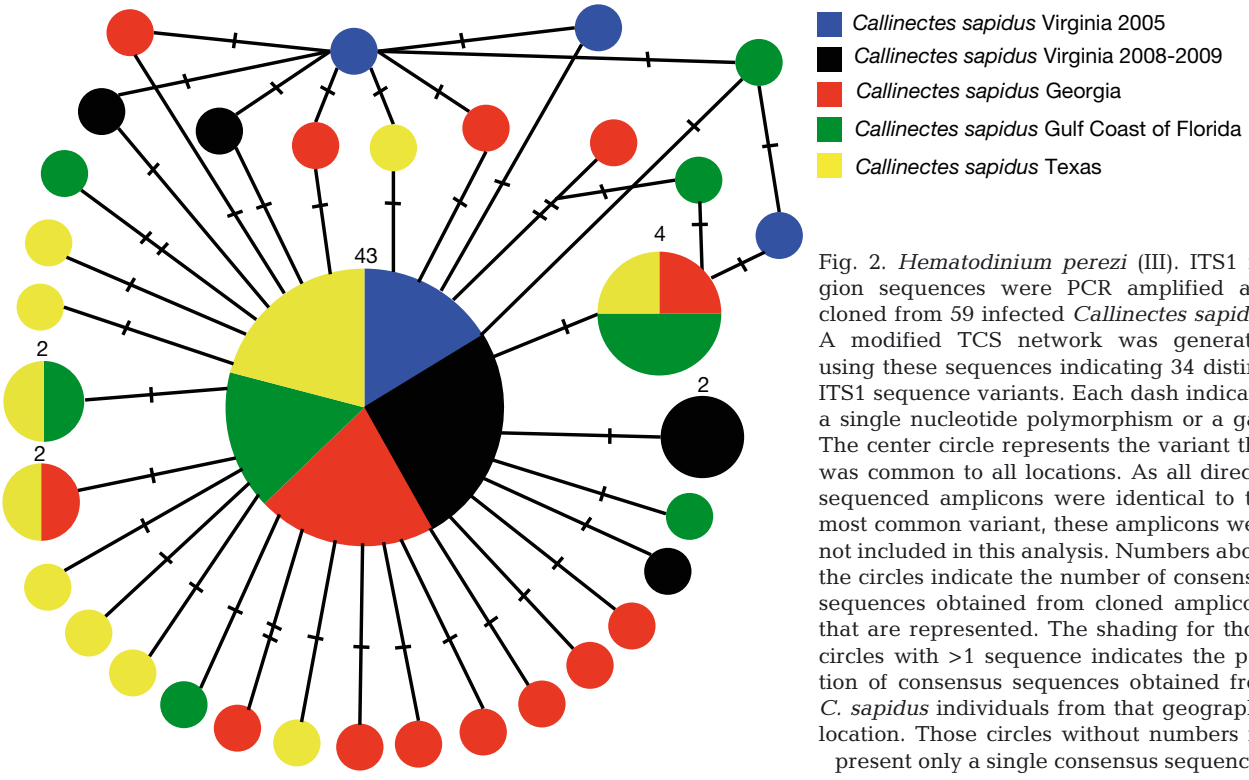

Fig. 2. *Hematodinium perezi* (III). ITS1 region sequences were PCR amplified and cloned from 59 infected *Callinectes sapidus*. A modified TCS network was generated using these sequences indicating 34 distinct ITS1 sequence variants. Each dash indicates a single nucleotide polymorphism or a gap. The center circle represents the variant that was common to all locations. As all directly sequenced amplicons were identical to the most common variant, these amplicons were not included in this analysis. Numbers above the circles indicate the number of consensus sequences obtained from cloned amplicons that are represented. The shading for those circles with >1 sequence indicates the portion of consensus sequences obtained from *C. sapidus* individuals from that geographic location. Those circles without numbers represent only a single consensus sequence

Table 2. *Hematodinium perezi* (III). ITS1 region amplicons (obtained from infected *Callinectes sapidus*) that were cloned and sequenced. The number of sequences obtained from each location is indicated, as well as the number of clones that matched the most common ITS1 variant (most common seq.), the number that did not match any other sequences (unique seqs.), and the number that matched at least 1 other sequence in this study (shared seq.)

Location	No. amplicons cloned	Number of clones			
		Sequenced	Most common seq.	Unique seqs.	Shared seq.
Chincoteague Bay, VA	4	12	9	2	1
Wachapreague Creek, VA	3	12	11	1	0
Fisherman's Island, VA	4	16	14	0	2
Savannah, GA	8	30	15	12	3
Goodland, FL	2	6	4	0	2
Tampa, FL	1	6	5	1	0
Panama City, FL	5	25	17	4	4
Corpus Christi, TX	7	54	43	8	3
Total	34	161	118	28	15

from an infected <10 mm mud crab, the remaining directly sequenced amplicons obtained from infected alternate hosts were all identical to the most common ITS1 variant.

The average genetic distance between the partial ITS1 region sequences of *Hematodinium perezi* (III) obtained from *Callinectes sapidus* and the alternate hosts was only 0.002 (99.8% similarity) with the genetic distance ranging between 0 and 0.016 (100 to 98.4% similarity). The average genetic distances between the ITS1 region sequences from *H. perezi* (I) infecting *Liocarcinus depurator* from the English Channel, the ITS1 region sequences from *H. perezi* (III) obtained from *C.*

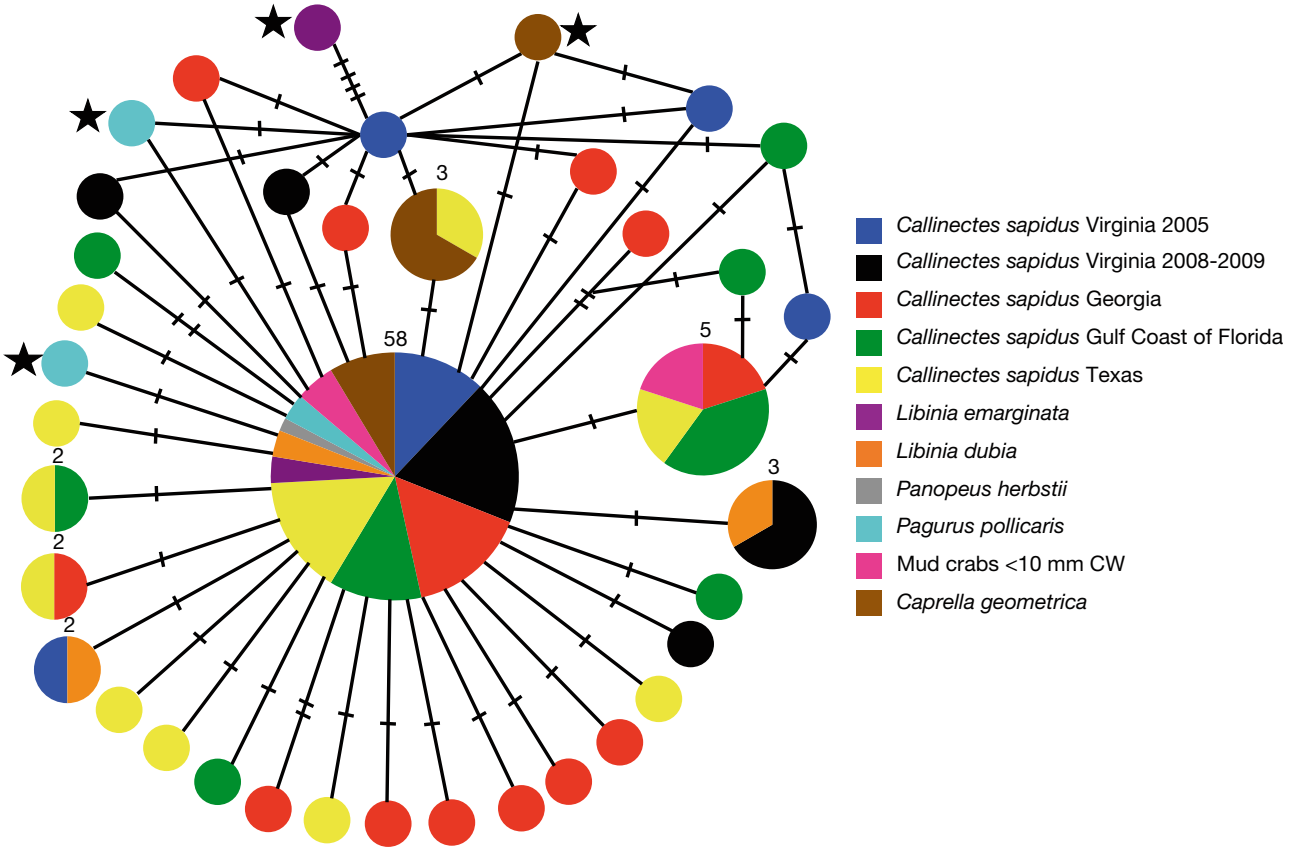

Fig. 3. *Hematodinium perezi* (III). ITS1 region sequences were PCR amplified and cloned from 59 infected *Callinectes sapidus* and 37 alternate host species. A modified TCS network was generated using these sequences indicating 38 distinct ITS1 sequence variants. The center circle represents a variant that was common to all locations and host species. As in Fig. 2, all directly sequenced amplicons that matched the most common variant were not included in this analysis, which includes all but 1 directly sequenced amplicon from an alternate host (mud crab <10 mm carapace width, CW), which was included. Each dash indicates a single nucleotide polymorphism or a gap. Stars indicate the 4 ITS1 variants observed only in alternate host species. Numbers above the circles indicate the number of consensus sequences represented. Those circles without numbers represent only a single consensus sequence

Table 3. *Hematodinium perezi* (III). ITS1 region sequences from alternate hosts that were included in the second TCS analysis. The numbers of samples that were PCR positive for *H. perezi* and the numbers of amplicons obtained via direct sequencing (D) or cloning (C) are listed, as well as the total number of clones obtained, the number of clones that matched the most common ITS1 variant, the number that did not match any other sequences (unique sequences), and the number that matched at least 1 other sequence in this study

Species	No. positive	D or C	Number of clones				GenBank accession no.
			Sequenced	With most common sequence	With unique sequences	With shared sequence	
Libinia emarginata	2	C	6	5	1	0	JN368149–51
Libinia dubia	3	D=1, C=2	6	4	0	2	JN368152–56
Pagurus pollicaris	2	C	8	6	2	0	JN368157–60
Eurypanopeus depressus	3	D	–	–	–	–	JN368161–63
Panopeus herbstii	3	D=2, C=1	3	3	0	0	JN368164–66
Caprella geometrica	3	C	8	5	1	2	JN368187–94
Mud crabs <10 mm	21	D=17, C=3	9	9	0	0	JN368167–86
Total	37	63	40	32	4	4	

sapidus, and those from the alternate hosts were 0.045 (95.5% similarity) and 0.044 (95.6% similarity), respectively, with the genetic distances ranging between 0.039 and 0.055 (96.1 to 94.5% similarity). The average genetic distances between the ITS1 region sequences from *H. perezi* (II) infecting *Scylla serrata* and *Portunus trituberculatus* from China, the ITS1 region sequences from *H. perezi* (III) obtained from *C. sapidus,* and those from the alternate hosts were 0.036 (96.4% similarity) and 0.036 (96.4% similarity), respectively, with the genetic distances ranging between 0.027 and 0.044 (97.3 to 95.6% similarity).

DISCUSSION

Based on the high similarity (>99%) among the partial sequences of the ITS1 region obtained in this study, we conclude that *Hematodinium perezi* (III) is the only species and genotype of *Hematodinium* infecting blue crabs from Virginia to Texas. In addition, this same species and genotype infects other crustaceans in the coastal waters of Virginia based on the >98% similarity observed among all *Hematodinium* sp. sequences from the region. The average genetic distance between the partial sequences of the ITS1 region of *H. perezi* (III) from the US and those from *H. perezi* (I) from Europe and *H. perezi* (II) from China is approximately 20 times greater than the average genetic distance among the sequences in crustacean hosts from the US Atlantic and Gulf coasts, providing additional evidence that *H. perezi* (III) is the only species and genotype of *Hematodinium* currently found in the region.

Many studies on geographic variation, population structure, and identification of different species of phytoplankton have used variable regions of the genome, such as the ITS1 of the rRNA gene complex (Kooistra et al. 2001, Litaker et al. 2003, Ki & Han 2007, Zheng et al. 2008). Although the level of intraspecific variation in the ITS1 region for *Hematodinium perezi* (III) appears to be fairly low throughout this geographic region, it is consistent with what has been reported in other molecular studies on *Hematodinium* species (Hamilton et al. 2007, 2009, Small et al. 2007a,b, 2012, Eigemann et al. 2010); however, a direct comparison cannot be made between this study and those that used direct sequencing techniques (Hamilton et al. 2007, 2009, Eigemann et al. 2010) because some of the variation may not have been detected in those studies. In addition, the level of intracellular variation among the multiple copies of the ITS regions in the genome of this parasite has yet to be determined because clonal cultures have not been established. Therefore, we could not distinguish between genetic variations among the multiple ITS1 region sequences within a single parasite cell and multiple sequence variants from infections of multiple parasite strains within a single host individual (Pagenkopp Lohan et al. 2012b).

We found no genetic differentiation among *Hematodinium perezi* (III) populations from the US Atlantic coast and Gulf of Mexico based on the partial ITS1 region sequences. Previous studies have discovered population differentiation due to vicariance in several marine and estuarine species between the Atlantic coast and Gulf of Mexico using various types of genetic markers (Avise 1992, Pelc et al. 2009). Such studies have provided insight into the historical

expansion and contraction of the ranges of those species. However, population differentiation along the US Atlantic and Gulf coasts has only been examined for 1 marine parasite, *Perkinsus marinus* (Reece et al. 2001). In that study, different allele frequencies among 3 regions of the USA were found: Northeast Atlantic, Southeast Atlantic, and Gulf of Mexico. In the blue crab host for *H. perezi* (III), no genetic population structure has been detected along the US Atlantic seaboard and Gulf of Mexico using RFLP analysis of mtDNA (McMillen-Jackson & Bert 2004); however, there was a notable decline in genetic variation with increasing latitude along the eastern seaboard.

Although the ITS1 region is generally recognized as evolving more rapidly than other portions of the rRNA gene complex (Schlötterer et al. 1994), we found low variation in this region in *Hematodinium perezi* (III) from Virginia to Texas. Samples from Georgia had the largest number of unique ITS1 consensus sequences, but the majority of these sequences differed by a single nucleotide polymorphism or indel. Two scenarios may explain the low variation in the ITS1 region. First, the data are consistent with a relatively recent introduction event followed by rapid dispersal over a wide geographic region. Small et al. (2012) hypothesized that *H. perezi* may have spread from the waters of the UK to the east coast of the USA through various mechanisms including the unintentional introduction of infected *Carcinus maenas*, an invasive species, via ballast transfer. If *H. perezi* (III) was introduced to the east coast of the USA, it may have rapidly spread through the Atlantic and Gulf of Mexico coastal waters, either through dispersal of a potential free-living stage (Frischer et al. 2006, Li et al. 2010), through host movement and further introductions, or as a host generalist (Pagenkopp Lohan et al. 2012a) through infection of a broad number of hosts with consequent dispersal, leaving little time for the ITS1 region to accumulate mutations. While we do not know exactly how long *H. perezi* (III) has been present along the east coast of the USA, it was first discovered infecting blue crabs in 1968 by Newman & Johnson (1975). At the time of their sampling, the parasite was already present in South Carolina, Georgia, and Florida.

A second possible mechanism for the low variation in the ITS1 region of *Hematodinium perezi* (III) is that this locus is conserved due to a functional role. The spacer regions of the ribosomal gene complex are involved in the processing of primary RNA transcripts in yeast (Musters et al. 1990, Lalev et al. 2000), and conservation in the secondary structure of the ITS1 region has been observed in *Hematodinium* (Jensen et al. 2010) and other species of dinoflagellates (Gottschling & Plötner 2004). This latter finding may be attributable to a potential functional significance for this locus in dinoflagellates. However, the length of the spacer region that is needed to perform this function is unknown, and the majority of the ITS1 region appears to evolve at the same rate as neutral loci in multiple *Drosophila* species (Schlötterer et al. 1994).

In conclusion, partial ITS1 region sequences obtained from *Hematodinium*-infected blue crabs collected from various geographic locations and alternate hosts indicate that the same *H. perezi* genotype infects blue crabs from Virginia to Texas, and that it is capable of infecting a wide variety of other hosts. Although the ITS1 region sequences did not demonstrate any genetic population structure for *H. perezi* (III) in the geographic range examined, additional molecular markers with higher resolution, such as the microsatellite markers recently developed for this parasite (Pagenkopp Lohan et al. 2012b), may be able to elucidate genetic population structure or provide further strain-level partitioning over a similar geographic range.

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