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Detection and Discovery of Crustacean Parasites in Blue Crabs (*Callinectes sapidus*) by Using 18S rRNA Gene-Targeted Denaturing High-Performance Liquid Chromatography▽†

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Recently, we described a novel denaturing high-performance liquid chromatography (DHPLC) approach useful for initial detection and identification of crustacean parasites. Because this approach utilizes general primers targeted to conserved regions of the 18S rRNA gene, a priori genetic sequence information on eukaryotic parasites is not required. This distinction provides a significant advantage over specifically targeted PCR assays that do not allow for the detection of unknown or unsuspected parasites. However, initial field evaluations of the DHPLC assay suggested that because of PCR-biased amplification of dominant host genes it was not possible to detect relatively rare parasite genes in infected crab tissue. Here, we describe the use of a peptide nucleic acid (PNA) PCR hybridization blocking probe in association with DHPLC (PNA-PCR DHPLC) to overcome inherent PCR bias associated with amplification of rare target genes by use of generic primers. This approach was utilized to detect infection of blue crabs (*Callinectes sapidus*) by the parasitic dinoflagellate *Hematodinium* sp. Evaluation of 76 crabs caught in Wassaw Sound, GA, indicated a 97% correspondence between detection of the parasite by use of a specific PCR diagnostic assay and that by use of PNA-PCR DHPLC. During these studies, we discovered one crab with an association with a previously undescribed protist symbiont. Phylogenetic analysis of the amplified symbiont 18S rRNA gene indicated that it is most closely related to the free-living kinetoplastid parasite *Procryptobia sorokini*. To our knowledge, this is the first report of this parasite group in a decapod crab and of this organism exhibiting a presumably parasitic life history.

Parasites, parasitoids, and infectious diseases are recognized as significant biotic factors affecting individuals, populations, communities, and ecosystems and have been implicated in global-scale declines of a wide range of marine and terrestrial species (11, 17, 22, 25, 27). For example, the world's coral reefs are currently declining at an alarming rate and although the role of disease is clearly prominent, many of the responsible disease agents remain unknown (12, 32, 36). Historically, discovery and investigation of disease processes have relied exclusively on labor-intensive histological and culture-based techniques. Methodological limitations have therefore been recognized as a major impediment to the study of emerging diseases in marine species. The lack of knowledge about the existence and biology of many parasites and parasitoids limits our ability to recognize the emergence of new diseases and to identify complex life histories and vectors involved in disease etiology. These difficulties are particularly acute for those trying to understand linkages between environmental stressors and disease since such studies demand the analysis of large numbers and diversities of samples (21).

During the past decade, new molecular diagnostic techniques have enabled detection and quantification of a large range of parasites, parasitoids, and pathogens in marine (35) and terrestrial (13) species. These approaches, used in concert with more classical methodologies, are fostering a renaissance in the investigation of disease and parasitism in many environments and systems. For example, a PCR-based molecular assay for the blue crab (*Callinectes sapidus*) parasite *Hematodinium* sp. was recently developed for routine high-throughput analysis of large numbers of crabs (9, 14). Application of this technique has led to the recognition of a link between an epidemic of the parasitic dinoflagellate *Hematodinium* sp. and drought conditions in the southeastern United States (24). However, while this PCR-based approach and other similar ones offer useful tools for investigation of known parasites, they do not enable discovery of new parasites and parasitoids, because these assays require a priori parasite-specific genetic sequence information. The assay described here offers the distinct advantage of providing the means for routinely detecting unknown or unsuspected parasites and allows the development of specific PCR primers in the event that new parasites are discovered.

To overcome the inherent limitations of specific PCR-based and antibody-based diagnostic techniques, we recently described a denaturing high-performance liquid chromatography (DHPLC) technique that allows the detection of eukaryotic parasites without the requirement of previous sequence knowledge (40). This assay involves two main processes; first, ribosomal gene fragments associated with each host/parasite spec-

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imen are PCR amplified using universal eukaryote-targeted rRNA gene primers; second, the amplicons are separated and resolved by DHPLC. Following separation, chromatographically distinct gene fragments can be collected and identified by sequencing and phylogenetic analysis. Using this approach, 498-bp fragments of cloned 18S rRNA genes derived from the blue crab (*C. sapidus*) and the blue crab parasite *Hematodinium* sp. were reliably distinguished and identified after the optimization procedure (40). However, preliminary results from field-caught blue crabs indicated that the sensitivity of the assay was too low for parasite detection in wild crab populations. In this study, we present a significant improvement to the DHPLC approach by the inclusion of a peptide nucleic acid (PNA) probe and demonstrate the successful use of this assay for detection and discovery of known and unknown parasites in blue crabs. This approach enabled us to detect and identify a previously unidentified kinetoplastid symbiont in blue crabs.

MATERIALS AND METHODS

Collection and genomic DNA extraction of hemolymph from C. sapidus. Blue crabs, *C. sapidus*, were collected in the Wassaw Sound estuary (Georgia; 31.9°N, 80.9°W) by trawling or using standard commercial traps baited with Atlantic menhaden (*Brevoortia tyrannus*). Crabs were collected over a 6-year period from 2000 to 2006 as part of a routine sampling program (24). Crabs were bled at the hemal sinus with a 1-ml syringe, and the hemolymph was transferred to sterile 1.5-ml microcentrifuge tubes. To prevent coagulation, 50 μl of 0.5 M EDTA was added to the hemolymph samples. Alternatively, samples were kept cool and processed immediately. In these cases, anticoagulant was not required. There was no apparent negative impact on DNA extraction or PCR efficiency due to EDTA addition in these studies, but since EDTA has been reported to be an inhibitor of PCR (23), we chose to exclude it when possible. Genomic DNA was extracted and purified from hemolymph samples by using a DNeasy blood and tissue kit (Qiagen) as previously described (9), and the presence of the *Hematodinium* sp. was determined using a *Hematodinium* sp.-specific PCR assay (14).

***Hematodinium* sp.-specific PCR.** The specific detection of *Hematodinium* sp. in crab hemolymph samples was routinely accomplished by means of a PCR assay using the *Hematodinium*-specific 18S rRNA gene-targeted primers Hemat-F-1487 (5′-CCT GGC TCG ATA GAG TTG) and Hemat-R-1654 (5′-GGC TGC CGT CCG AAT TAT TCA C) as previously described (14). These primers produce a diagnostic 186-bp gene fragment that can be identified by agarose gel electrophoresis in 1.2% gels stained with GelStar nucleic acid stain (Cambrex). The presence of the correct-sized amplicon was routinely taken as evidence of *Hematodinium* infection.

PNA-PCR DHPLC assay. PCR amplification was performed using the high-fidelity, thermal-stable Optimase DNA polymerase (Transgenomics, Omaha, NE). A 498-bp 18S rRNA gene fragment was amplified from each sample by using the degenerate general eukaryotic primers Univ 18S-1131F (5′-AAA CTY AAA GRA ATT GAC GG-3′) and Univ 18S-1629R (5′-GAC GGG CGG TGT GTR C-3′) as previously described (40), except that a *C. sapidus*-specific PNA blocking probe was incorporated into the reaction as described below.

To overcome an inherent PCR bias associated with amplification of rare target genes, the *C. sapidus*-specific PNA probe BC 18S-1361S (H-GGT GTC CAG TTC GCA G-NH_2) was designed using the software package Primer Premier version 5.00 (Premier Biosoft International) and synthesized by Applied Biosystems. The PNA probe was freeze dried and stored at −20°C until use. Before use, the PNA probe was suspended in 10 mM Tris-HCl, pH 7.5, and heated for 10 min at 50°C. A final PNA concentration of 0.5 μM was used in all PNA-PCRs. However, initial investigation of the PNA concentrations required to inhibit amplification of blue crab 18S rRNA genes were conducted and concentrations ranging from 0.25 μM to 4 μM of PNA yielded equivalent results (data not shown). The PNA hybridization probe was incorporated into a standard PCR cycle by including a PNA annealing step (65°C) following denaturation at 95°C. In addition, the elongation reaction typically conducted at 72°C was reduced to 70°C to reduce the possibility of PNA-DNA denaturation during the extension step. The specific PNA-PCR conditions were 25 cycles of 95°C (30 s), 65°C (30 s), 50°C (30 s), and 70°C (30 s). After 25 amplification cycles, an additional elongation cycle (70°C for 5 min) was completed. For analysis of the crab hemolymph samples collected in November 2006, artifactual heteroduplex formation was reduced by reconditioning PCR essentially as recommended by Thompson et al. (39). A fresh 20-μl PCR mixture containing 2 μl of the amplicon produced during the 25 PNA-PCR cycles was used for six cycles under identical conditions.

To investigate sensitivity for detection of rare genes against a background of dominant genes by PNA-PCR, an 8-by-8 factorial design concentration matrix study was conducted (see Table S1 in the supplemental material). Mixtures of purified, nearly complete 18S rRNA genes from the *Hematodinium* sp. and *C. sapidus* cloned into the bacterial plasmid vector pCR4-TOPO (Invitrogen Corp.) were created such that the relative concentrations of both genes ranged from 1:1 to $1:10^7$ (*Hematodinium* sp./*C. sapidus*) copies. The absolute concentrations ranged from 10^2 to 10^9 gene copies. All PNA-PCRs utilized the 1131F and 1629R primers, producing a 498-bp amplicon as described above.

DHPLC. All DHPLC analyses were performed using a WAVE DHPLC system equipped with a DNASep HT cartridge (Transgenomic, Omaha, NE; catalog no. DNA-99-3710) essentially as previously described (40). The PCR products resulting from the PNA-PCR amplification reactions were loaded directly onto the DHPLC instrument without additional purification. The DHPLC conditions were a column temperature of 59.5°C and a linear acetonitrile elution gradient from 15 to 17.5% acetonitrile (60 to 70% buffer B). Elution gradients were formed using WAVE optimized buffer A, consisting of 0.1 M triethylammonium acetate, and WAVE optimized buffer B, consisting of 0.1 M triethylammonium acetate in 25% (vol/vol) acetonitrile. All elution gradients were run over a 9-min period with a solvent flow rate of 0.35 ml min^{-1}, yielding a total DHPLC sample run time of 13.2 min, including cleaning and equilibration. All elution reagents were purchased from Transgenomic (Omaha, NE). Analysis of chromatographic data was facilitated using Navigator software version 1.6.2 (build 12) (Transgenomic, Omaha, NE).

Fraction collection, cloning, and sequencing. For identification of DHPLC chromatographic peaks by sequencing, peaks were collected using a FCW-200 fraction collector (Transgenomic, Omaha, NE) integrated with the WAVE DHPLC system. Fragments were subsequently reamplified using a HotMaster *Taq* DNA polymerase kit (Eppendorf, North America, Inc.), universal 18S rRNA gene-targeted primers 1131F and 1629R (ca. 0.2 μM [1 ng/μl] of each primer), 2 mM Mg^{2+}, and 0.2 mM of each deoxynucleoside triphosphate. The PCR amplification conditions included an initial denaturation step at 95°C (5 min), followed by 25 cycles of 95°C (30 s), 50°C (30 s), and 72°C (30 s), and finally a 5-min extended elongation step at 72°C in the last cycle. PCR was performed using a GenAMP 9700 thermal cycler (PerkinElmer, Inc., Wellesley, MA). Amplified gene fragments were visualized and sized by agarose gel electrophoresis in 1% gels stained with GelStar nucleic acid stain (Cambrex), extracted and purified using Quantum Prep Freeze ′n Squeeze DNA gel extraction spin columns (Bio-Rad Laboratories, Inc.), and dissolved in 30 μl MilliQ water. The concentration of each fragment was quantified using a Turner Design TD-700 fluorometer (Turner Designs, Sunnyvale, CA) after staining with PicoGreen (Molecular Probes, Eugene, OR). Amplified gene fragments were ligated into a pCR4-TOPO vector and transformed into One Shot chemically competent cells by using a TOPO TA cloning kit (Invitrogen Corp.) according to the manufacturer's instructions. For sequencing, plasmids were purified using a High Pure plasmid isolation kit (Roche) and sequenced by capillary electrophoresis using a CEQ DTCS quick-start sequencing kit with a CEQ 8000 eight-channel capillary sequencer (Beckmann Coulter, Inc.). At least three clones produced from each DHPLC peak were sequenced, and in all cases, the sequences recovered were identical (data not shown). The identities of fragments were initially determined by comparison to sequences in GenBank, using the BLASTn utility to search the nr/nt nucleotide collection (http://www.ncbi.nlm.nih.gov/). In some cases, it was possible to sequence directly from reamplified PCR products without subcloning (data not shown), but in all cases in this study, fragments were cloned prior to sequencing.

Following the initial identification of a kinetoplastid symbiont from crab 47, nearly the full 18S rRNA gene was amplified from purified genomic DNA from this crab, cloned, and sequenced. Initial attempts to amplify the complete gene as a single fragment repeatedly failed, so the gene was amplified in two parts. The 5′ end was amplified using the Universal eukaryotic primer Univ F-15 (5′ CTG CCA GTA GTC ATA TGC 3′) and paired with a symbiont-specific primer, Bodo R-1381 (5′ ACA ATC CTG GTT CCT ACT 3′). The 3′ end of the gene was amplified using the symbiont specific primer Bodo F-1393 (5′ GAA GCT ACC TCC AGT T 3′) and the general eukaryotic primer Univ R-1765S (5′ ACC TTG TTA CGA CTT 3′). Each of these amplicons was cloned and sequenced as described above such that nearly the complete gene (1,991 bp) was sequenced and assembled in both the forward and the reverse directions.

Phylogenetic analysis. The phylogenetic identity of the unknown symbiont in crab 47 detected by the PNA-PCR DHPLC assay was determined by phyloge-

TABLE 1. Detection of *Hematodinium* sp. 18S rRNA genes in multitemplate DNA mixtures by specific PCR, PCR-DHPLC, and PNA-PCR DHPLC[a]

Ratio of *Hematodinium* sp. to blue crab (*C. sapidus*) cloned inserts	n^b	% Detection for:		
		Hematodinium sp.-specific PCR	PCR-DHPLC	PNA-PCR DHPLC
1:1	8	100	100	87.5
1:10	7	100	100	100
1:100	6	100	0	100
1:1,000	5	100	0	100
1:10,000	4	100	0	75
1:100,000	3	100	0	67
1:1,000,000	2	50	0	0
1:10,000,000	1	0	0	0

[a] PCR and PCR-DHPLC were performed as described in references 14 and 40, respectively, and PNA-PCR DHPLC was performed as described in this study.

[b] Number of independent assays in the 8-by-8 factorial design experiment (see Table S1 in the supplemental material).

netic comparison with 27 representative *Bodonidae* sequences and 11 representative *Trypanosomatidae* sequences. Phylogenetic analyses were rooted against the euglenoid *Euglena gracilis* (GenBank accession number AF283308). After all sequences were trimmed to the length of the smallest sequence in this database (1,948 bp), the sequences were aligned using the ClustalW version 1.8 multiple sequence alignment algorithm (38). Alignments were viewed and refined based on secondary structure considerations (31), using BioEdit version 7.0.5.3 (16). Neighbor-joining phylogenetic trees were inferred and drawn using the TREECON software package (version 1.3b) (41, 42), with the Kimura two-parameter model for inferring evolutionary distances (18). Because of the high level of conservation between 18S rRNA genes and the fact that only near-full-length gene sequences were utilized, insertions and deletions were not taken into account and all alignment positions were considered. Bootstrap estimates (1,000 replicates) of confidence intervals were also made using the algorithms in TreeconW.

Nucleotide sequence accession number. The nucleotide sequence determined for this study was submitted to GenBank (accession number EU045326).

RESULTS

DHPLC detection of the *Hematodinium* sp. Although previous studies demonstrated that the 498-bp 18S rRNA gene amplicons from the *Hematodinium* sp. and *C. sapidus* could be resolved by DHPLC analysis (40), in preliminary studies we were unable to detect the presence of the *Hematodinium* sp. in blue crabs known to be infected (data not shown). Thus, here we investigated the detection sensitivity of *Hematodinium* sp. genes in the presence of the *C. sapidus* 18S rRNA gene with and without the inclusion of a *C. sapidus*-specific PNA hybridization blocking probe. Utilization of a standard PCR assay with universal primers resulted in the amplification and DHPLC detection of the *Hematodinium* sp. gene only when it was at an abundance such that the ratio of the *Hematodinium* sp. to *C. sapidus* genes was ≤1:10 gene copies (Table 1). Inclusion of the *C. sapidus*-specific PNA 1361S-BC hybridization blocking probe in the PCR increased the detection sensitivity of the *Hematodinium* sp. by 4 orders of magnitude (Table 1). The *Hematodinium* sp. could be detected after PNA-PCR by DHPLC when it was present at a ratio of 1:100,000 with respect to *C. sapidus* genes. The presence of the PNA in the PCR did not affect the ability of DHPLC to resolve 18S rRNA gene amplicons from the *Hematodinium* sp. and *C. sapidus*, although the presence of the PNA appeared to increase chromatographic baselines and sensitivity of parasite detection (Fig. 1). Background was further reduced by including a six-cycle reconditioning PCR prior to DHPLC analysis. This yielded an increased sensitivity of 37.5%, enabling us to detect many of the minor peaks from the chromatogram (Fig. 2).

To determine whether this increased level of sensitivity would be sufficient to detect the presence of parasites in wild crabs, 76 blue crabs were analyzed with both the *Hematodinium* sp.-specific PCR diagnostic assay (14) and the PNA-PCR DHPLC method developed in this study. Of these, 20 were identified as being

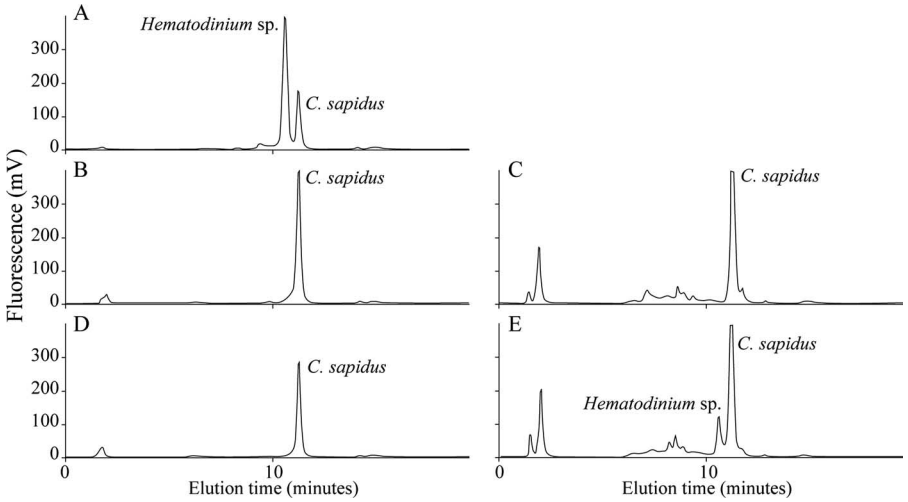

FIG. 1. Detection of *Hematodinium* sp. infection in blue crabs (*C. sapidus*) by DHPLC with and without inclusion of the *C. sapidus*-specific PNA PCR blocking probe BC 18S-1361S. (A) Detection of *Hematodinium* sp. and *C. sapidus* 18S rRNA gene amplicons produced from plasmid-cloned genes. These products were used throughout the study as chromatographic references. (B) DHPLC chromatogram of a *Hematodinium* sp.-free blue crab in the absence of the blue crab-specific PNA. (C) PNA-PCR DHPLC chromatogram of a *Hematodinium* sp.-free blue crab in the presence of the blue crab-specific PNA. (D) DHPLC chromatogram of a *Hematodinium* sp.-infected blue crab in the absence of the blue crab-specific PNA. (E) PNA-PCR DHPLC chromatogram of a *Hematodinium* sp.-infected crab in the presence of the blue crab-specific PNA.

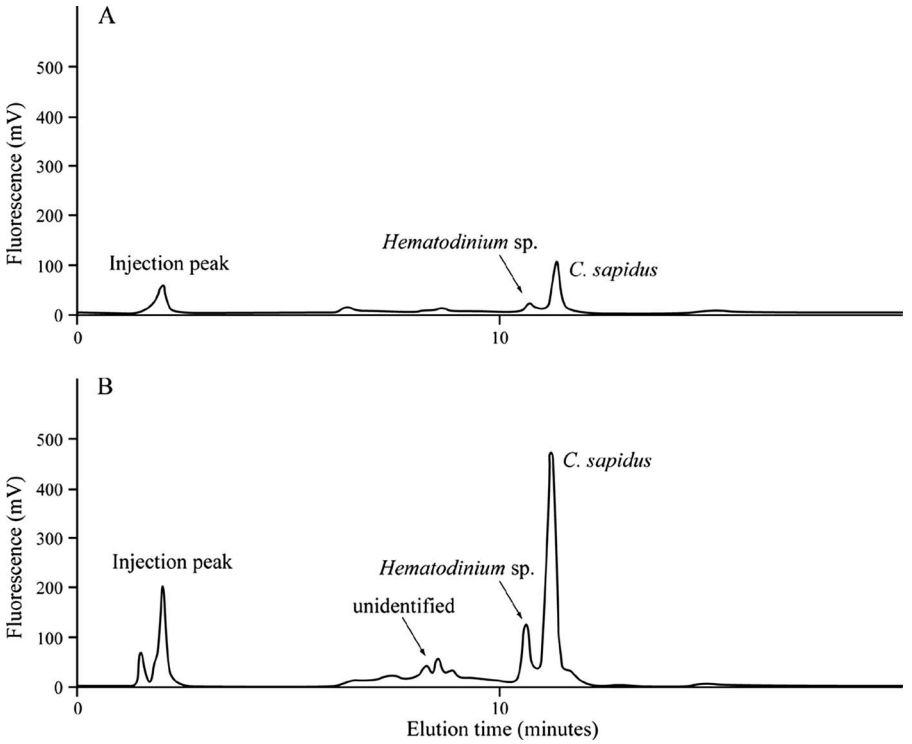

FIG. 2. DHPLC profiling of a *Hematodinium* sp.-infected blue crab before (A) and after (B) six-cycle PCR reconditioning. PCR products are in similar proportions in original and reconditioned reactions, while the sensitivity of detection is increased. Although not clearly apparent in this example because chromatographs are shown at the same fluorescence scale, as described by Thompson et al. (39), chromatographic baselines were also reduced after reconditioning PCR.

infected by the *Hematodinium* sp. and 56 were *Hematodinium* free (see Table S2 in the supplemental material). Following PNA-PCR DHPLC, the *Hematodinium* sp. peak was observed in infected crabs, demonstrating that this approach could be used to detect parasitized animals in the wild (Fig. 1). The identities of both the *Hematodinium* sp. and the *C. sapidus* chromatographic peaks were confirmed by sequencing representative samples. Of the 20 crabs shown to be parasitized by the *Hematodinium* sp. with the *Hematodinium* sp.-specific assay, the parasite was detected in all of these crabs by the PNA-PCR DHPLC assay (see Table S2 in the supplemental material). However, in two crabs (135 and WS7) a small *Hematodinium* signal was detected by PNA-PCR DHPLC in crabs believed to be *Hematodinium* free based on the results of the *Hematodinium*-specific PCR assay. These DHPLC peaks were also collected, sequenced, and confirmed to be the *Hematodinium* sp. Overall, there was a 97% correspondence between the specific PCR and general PNA-PCR DHPLC assays.

Detection and discovery of parasites in the blue crab *C. sapidus*. During the examination of the 76 blue crab samples, 25 exhibited DHPLC peaks with retention times that did not correspond to the *Hematodinium* sp. or *C. sapidus* (see Table S2 in the supplemental material). Unidentified DHPLC peaks from two of these crabs originally collected from Wassaw Sound, GA, in the spring and summer of 2004 (23 April and 4 August) were identified by sequencing. The first crab (male, no. 47) had four major peaks in the DHPLC chromatogram (Fig. 3A). The second peak, at 8.5 min, corresponded to an unclassified proteobacterium (GenBank accession number

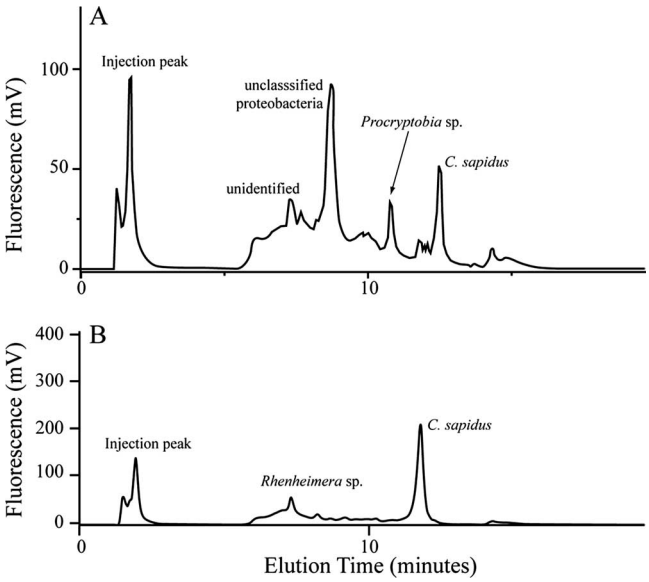

FIG. 3. Detection of unknown symbionts in wild-caught blue crabs by PNA-PCR DHPLC. (A) Detection of a kinetoplasid-like symbiont and a bacterial infection in a male blue crab (no. 47) collected on 23 April 2004 in Wassaw Sound, GA. The first peak (7.3 min) was not identified, since we were unable to obtain sequence from this fragment. (B) Detection of a bacterial associate in a male blue crab (no. 35) collected on 4 August 2004 in Wassaw Sound, GA. Neither crab was infected by the *Hematodinium* sp.

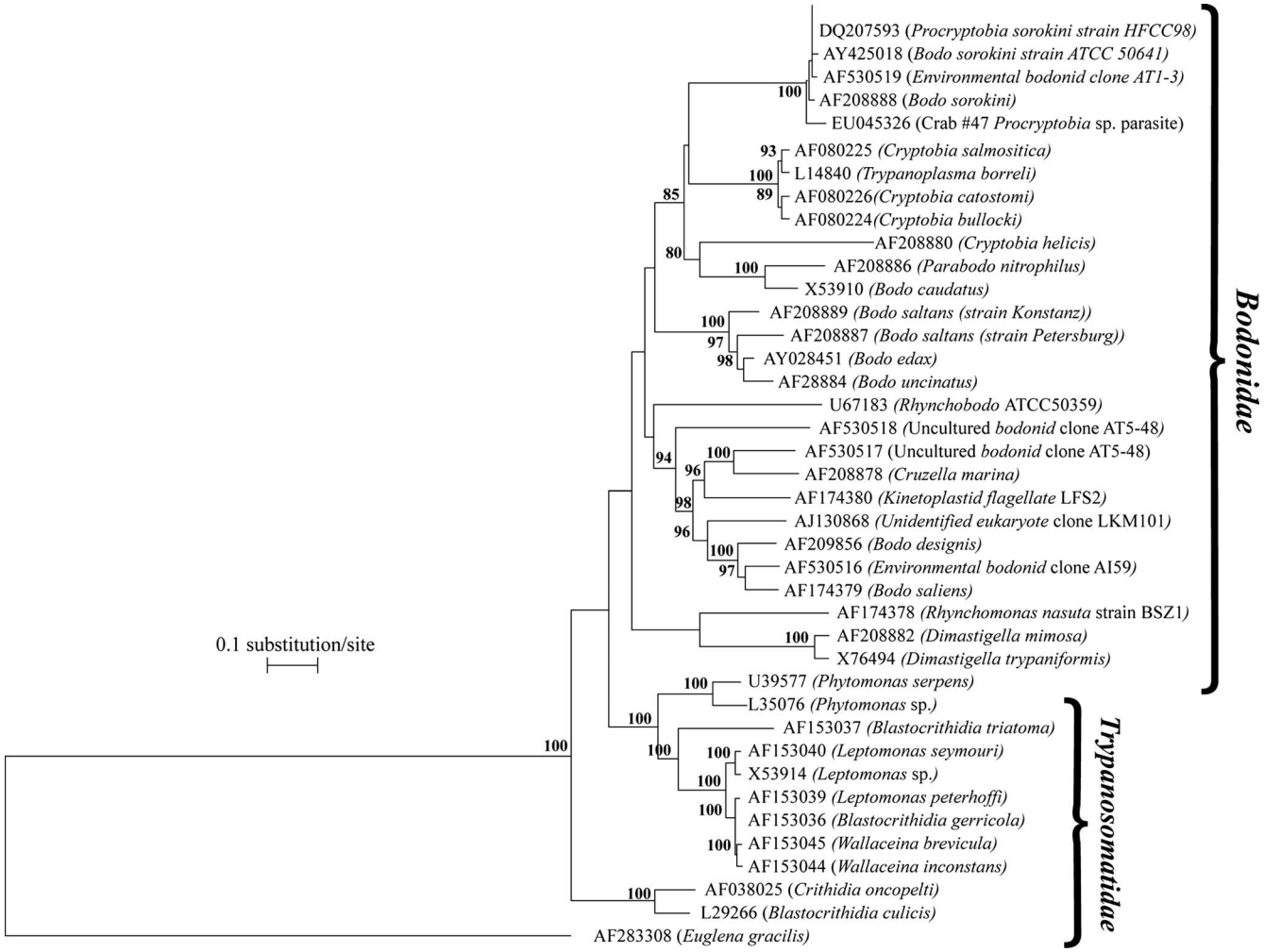

FIG. 4. Inferred taxonomic relationship between the unknown kinetoplastid symbiont detected by PNA-PCR DHPLC in blue crab 47, together with 38 *Bodonidae* and *Trypanosomatidae* species. The tree was rooted by the euglenoid *Euglena gracilis* (GenBank accession number AF283308). Phylogenetic analyses were based on comparison of nearly complete 18S rRNA gene sequences. All sequences in the alignment were trimmed to 1,948 bp, which was the shortest fragment in the alignment. GenBank accession numbers for each species are provided. Numbers for each node refer to the percentages of bootstrap iterations from 1,000, and the scale bar indicates 0.05 nucleotide substitutions per site.

AJ876947). The third peak (10.66 min) corresponded to the 18S rRNA gene from a *Protocrytobia* sp. (GenBank accession number DQ207593). The fourth peak was identified as *C. sapidus*. We were not able to obtain sequence information from the first peak (7.3 min). The second crab (male, no. 35), collected on 4 August 2004, had, in addition to a peak corresponding to *C. sapidus* (11.75 min), an additional peak that eluted at 7.31 min (Fig. 3B). Sequencing and an initial BLAST search suggested that this peak belonged to a *Gammaproteobacteria* species. The closest match in GenBank was with a *Rhenheimera* sp. in the family *Chromatiaceae* (GenBank accession number EF076758).

Because of the potential importance of discovering an undescribed eukaryotic symbiont of blue crabs (male, no. 47), we amplified, cloned, sequenced, and conducted a phylogenetic analysis based on nearly the complete 18S rRNA from this symbiont. These results indicated that the symbiont was a *Bodonidae* species clustering in the cryptobiid group sharing 99.9% nucleotide similarity with *Procryptobia sorokini* strain HFCC98 (DQ207593). Based on this analysis, this organism is almost certainly a member of the genus *Procryptobia* (42a) and likely *P. sorokini* (Fig. 4). This sequence was submitted to GenBank (accession number EU045326).

DISCUSSION

Globally, pathogens have been implicated in the decline of a wide range of marine and terrestrial systems, including species in the estuaries of the South Atlantic Bight (24). Although it is generally accepted that the incidences of disease and parasites may be influenced by accumulating environmental stressors, declining environmental quality, and ongoing climatic change (7), the major obstacle to exploring these hypotheses has been the difficulty of parasite detection, identification, and diagnosis (9, 14). To analyze the large number of samples required of ecological studies, approaches that are accurate, rapid, inexpensive, and not limited to known parasites and disease agents are needed.

Recently, we developed a DHPLC-based DNA-profiling assay that fulfils these criteria by allowing separation of mixed PCR amplicons, using a pair of universal eukaryote-targeted 18S rRNA gene primers (40). However, initial attempts to detect the presence of a specific dinoflagellate parasite *Hematodinium* sp. in wild-caught blue crabs by using this assay suggested that the sensitivity was too low. Indeed, it is well known that the predominance of a specific DNA template within a mixture of genes can bias molecular analysis by PCR (8, 20, 29). In this study, we attempted to overcome this bias by specifically inhibiting the PCR amplification of the blue crab 18S rRNA genes through incorporation of a PNA probe in the DHPLC assay.

PNAs are synthetic DNA analogs in which the deoxyribose phosphate backbone is replaced by an uncharged polyamid backbone, resulting in a high affinity and specificity to nucleic acids (28). Because PNAs lack a 5′ hydroxyl moiety, they are resistant to the 5′ nuclease activity of *Taq* polymerase and thus are not digested during DNA amplification (4). The presence of a PNA-DNA hybridization is therefore capable of blocking PCR amplification when it is bound within a targeted amplification region (2, 26, 37, 44). Incorporation of the *C. sapidus* 18S rRNA gene-specific PNA probe into the PCR increased the detection sensitivity of the *Hematodinium* sp. 18S rRNA gene in the presence of *C. sapidus* DNA by 4 orders of magnitude. This supports the hypothesis that PCR amplification bias toward dominant genes resulted in initial low assay sensitivity and further suggests that the PNA-PCR DHPLC assay would be capable of detecting parasites in wild-caught, infected crabs.

As a result of the increased sensitivity resulting from the addition of the PNA probe in the initial PCR, the PNA-PCR DHPLC assay was used to assay 76 blue crab hemolymph samples that had previously been collected and PCR assayed for the presence of the *Hematodinium* sp. as described by Gruebl et al. (14). In this sample set, 56 crabs were *Hematodinium* sp. free and 20 were infected. A 97% (74 of 76) correspondence between the two assays was observed, indicating that the sensitivity of the PNA-PCR DHPLC assay was sufficient to detect infections in wild crabs. In the two cases where there was not agreement between the two methods, the *Hematodinium* sp. was detected by the PNA-PCR DHPLC assay and not by the specific PCR (see Table S2 in the supplemental material) (crabs 135 and WS7). We cannot offer an explanation for these two cases where the *Hematodinium* sp. was detected by PNA-PCR DHPLC but not by the specific and more sensitive PCR method. Since stringent positive and negative controls were carried out, it seems unlikely that the PNA-PCR DHPLC assay is more prone to false positives than the specific PCR assay.

During the systematic assaying of the 76 blue crab samples, a relatively large fraction (34%) yielded unidentified DHPLC peaks with elution times not consistent with the amplicon from either *C. sapidus* or the *Hematodinium* sp. Each of these peaks in two crabs were collected and their identities determined by sequencing (Fig. 3). Of particular interest was a peak that eluted at 10.66 min, derived from a *Hematodinium* sp.-free male crab collected on 23 April 2004. Sequence similarity analysis of the collected fragment indicated that it was derived from a kinetoplastid-like organism most closely related to *Procryptobia sorokini* (Fig. 4). This group is composed of an important and diverse group of free-living and parasitic organisms (43). For example, in humans the causative agents of African sleeping sickness are the related euglenozoa parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense.* This group of parasites is also of great concern in marine systems. For example, the related kinetoplastid parasite *Cryptobia salmositica* is recognized as a lethal pathogen of salmonid fish in seminatural and intensive salmon culture facilities (45). Kinetoplastid parasites have also been reported to be in some crustacean species, including copepods (1). Although kinetoplastid infections are believed to be uncommon in blue crabs (33), a common parasitic infection in blue crabs is paramoebiasis disease, caused by the amoeba *Paramoeba pernicgosa* (19). Since a related group of parasites, *Neoparamoeba* spp., are known to contain kinetoplastid endosymbionts closely related to *Procryptobia sorokini* (6), detection of *P. sorokini* in blue crabs may be indicative of an amoeba infection rather than a kinetoplastid infection. However, in silico analyses of available *Paramoeba* and *Neoparamoeba* ribosomal gene sequences indicate that our universal primers would amplify these organisms, and therefore, had these crabs been infected by it, we would have detected its rRNA genes in subsequent DHPLC analyses. It is therefore highly unlikely that the infections of the blue crabs with kinetoplastids were due to the endosymbiont relationship between the crabs and *Neoparamoeba* spp. Interestingly, the particular kinetoplastid detected here, *Procryptobia sorokini*, is known only as a free-living organism found in oceanic and estuarine environments (5, 10). To our knowledge, this is the first report of *P. sorokini* being detected within a crustacean and therefore opens up the interesting possibility that this organism may have a parasitic life history.

An apparent bacterial infection was also detected in the hemolymph (Fig. 3). Although controversial during the 1970s (34), it is now generally recognized that bacterial infections in the hemolymph of blue crabs are often found (3). For example, there have been reports of bacteria isolated from 26% of blue crabs sampled from South Carolina estuarine tidal creeks (L. Burnett and K. Burnett, personal communication). The universal primers used in this study were designed to nonselectively amplify all 18S rRNA genes (40). Nevertheless, it was not possible to design absolute universal primer sets fulfilling this criterion. For example, highly divergent groups of eukaryotic organisms, including several fungal groups, would not be expected to be detected using the Univ 18S-1131F and -1629R primer sets (data not shown). Furthermore, because of the highly conserved nature of small-subunit ribosomal genes, these primers were expected to amplify a small number of bacterial 16S rRNA genes.

The discovery of a previously unrecognized blue crab symbiont and the detection of crab bacterial infections in this study demonstrate the capacity of the PNA-PCR DHPLC assay for both the detection and the discovery of parasites in wild populations of blue crabs. However, additional studies are required to determine whether this symbiont is a pathogen of *C. sapidus* and whether it is prevalent in wild crab populations. Survey studies of archived and newly caught blue crabs with *Protocryptobia* sp.-specific PCR primers are currently under

way to assess the prevalence of this symbiont in blue crab populations. Initial studies have so far detected this organism by using a *P. sorokini*-specific PCR assay in 12 additional crabs, but the full results of this study will be reported elsewhere. Because of the generality of the analytical approach, DHPLC technology should be widely applicable with only minor modification for detection of parasites in other metazoan species. Although the potential significance of parasitism and disease in nature, even at subepidemic levels, is broadly recognized (15, 25, 30), methodological limitations have hindered the exploration of the ecological significance of parasitism in most natural environments. The availability of novel and robust DHPLC technology should contribute to the understanding of parasitic interactions in nature.

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