INVESTIGATIONS OF INTERNAL INTERACTIONS BETWEEN THE PARASITIC BARNACLE *LOXOTHYLACUS TEXANUS* (RHIZOCEPHALA: SACCULINIDAE) AND ITS HOST *CALLINECTES SAPIDUS* (BRACHYURA: PORTUNIDAE) USING PCR TECHNIQUES

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ABSTRACT

We describe techniques that enable the preservation of tissues from the rhizocephalan barnacle, *Loxothylacus texanus*, inside the body cavities of blue crab hosts, *Callinectes sapidus*, in a manner that minimizes the degradative activities of hepatopancreatic enzymes. These procedures allow the extraction and amplification of both parasite and host 18S rDNA within the same sample and enable one to distinguish between parasitized and unparasitized crab tissue in as little as two weeks after infection, well before any external manifestations of the parasites. Two PCR-based approaches were taken to identify the presence of *L. texanus*. In the first approach, a set of primers specific for *L. texanus* was used to specifically amplify 18S sequence in a background of *C. sapidus* DNA or the DNA of other barnacle species. In the second approach, a set of general primers was used to amplify 18S sequence from *C. sapidus* and a variety of barnacle species. The products of this PCR were then digested with an enzyme that recognizes a restriction site present only in the *L. texanus* PCR product to yield a unique pattern of fragments. With these techniques, we could detect as few as five parasitic cypris larvae in water samples, as well as *L. texanus* in the tissue of a small crab collected from the field and in the four anterior periopods of a crab bearing the external stage of the parasite. In experiments with potential hosts of varying sizes and molt stages, we confirmed that the parasite was significantly more effective in infecting crabs less than 30 mm carapace width than larger individuals. These techniques will facilitate future investigations of ecological and physiological interactions between these important crustacean parasites and their hosts and will help to determine the economic impact of this parasite on blue crab fisheries.

KEY WORDS: bar coding identification, *Callinectes sapidus*, *Loxothylacus texanus*, PCR, Rhizocephala

INTRODUCTION

Interactions between the rhizocephalan barnacle, *Loxothylacus texanus* Boschma, 1933, and its blue crab host, *Callinectes sapidus* Rathbun, 1896, are of potential economic importance in the Gulf of Mexico (Guillory et al., 2001; Høeg et al., 2005). A good deal of information has been elucidated about the biology of this parasite and its interactions with hosts under laboratory conditions, but little in known about the ecology of *L. texanus* in the field or distribution of the parasite within the tissues of the host crab between the time of infection and the appearance of the parasitic externa.

Laboratory studies have shown that *L. texanus* female cypris larvae use carbohydrate chemical cues during initial interactions with a susceptible host (Boone et al., 2003, 2004). Once attached to a vulnerable crab, the cypris molts into a kentrogon that penetrates the host's exoskeleton with a stylet. The crab is actually infected a few days later when the worm-like vermigon larva of the rhizocephalan enters the host through the stylet (Glenner et al., 2000; Glenner, 2001; Lawrence, 2001). The vermigon migrates through the hemocoel and eventually settles posterior to the cardiac stomach of the host. Here it develops into the interna stage and is thought to grow along the intestine and send rootlets out into the hepatopancreas and other organs (Høeg, 1995; Glenner, 2001). After five to nine molts in residence, a virgin externa extrudes from the abdominal cavity of the

host (O'Brien, 1999). Following the settlement of a male cypris and penetration of the virgin externa by a specialized male trichogon larva, the external portion of the adult rhizocephalan (now the externa) expands and eventually occupies the area of the host that would contain the fertilized eggs of an unparasitized adult female host crab (Høeg, 1987; Høeg et al., 2005).

Although vermigon larvae can be seen under thin areas of the host cuticle for a brief period immediately after infection (Glenner et al., 2000; Lawrence, 2001), for practical purposes, the vermigon and interna stages are undetectable by external examination. The stages from cypris settlement through successful development of the interna are of ecological interest, for their success or failure determine whether a crab becomes infected. Additionally, it cannot be assumed that the habitat in which crabs bearing externae of sacculinids are found is necessarily where they were infected. In one series of laboratory infections of *C. sapidus* with *L. texanus*, the time from exposure to cypris larvae to emergence of virgin externae ranged from 94 to 216 days (O'Brien, 1999). Such duration would provide ample time for host crabs to migrate out of the habitat in which they were infected (see Rasmussen, 1959). There is, at present, little direct evidence available about what occurs in the field, especially regarding the parameters that influence infection success following larval settlement. Therefore, a method to ascertain infection status during these cryptic stages of the parasite's

lifecycle, as well as a way to measure the distribution of larvae in the field, would be of great utility in studying the ecology and interactions of rhizocephalans with blue crabs.

Thus, our goal was to develop a relatively inexpensive molecular approach to detect the presence of *L. texanus* in *C. sapidus* tissues in the absence of other visual signs of infection and to characterize the temporal and spatial abundance of *Loxothylacus* larvae in the plankton. The cryptic nature of endoparasites is an obstacle to distinguishing between parasitized and unparasitized hosts, especially when hosts have been parasitized only recently. Traditional detection techniques such as visual examination of host tissues by dissection or fecal examination (typically only effective with intestinal parasites) have the potential to produce unacceptable numbers of false negatives, even with experienced investigators. Neither approach is particularly effective in detecting a small parasite in a relatively large host, something that is critically important to researchers studying factors that influence parasite transmission and prevalence. In the mid to late 1980s, the development of the polymerase chain reaction (PCR) enabled the detection of minute amounts of DNA through an enzymatic amplification process (Saiki, 1985; Saiki et al., 1988). Parasitologists used this process to address important questions that previously had been impractical to investigate (Barker, 1994) and successfully modified the technique for their own systems. For example, Rognlie et al. (1994) used reverse transcriptase-PCR (RT-PCR) to detect *Fasciola hepatica* in infected intermediate hosts, while Hanelt et al. (1997) employed standard PCR to detect 100 pg of trematode DNA in infected gastropods and, using nested PCR, were able to extend the detection limit to as little as 10 fg (equivalent to one infective larva). More recently, Jannotti-Passos et al. (2006) reported a multiplex PCR technique that enabled the simultaneous detection of the presence/absence of *Schistosoma mansoni* in three species of *Biomphalaria* that serve as intermediate hosts in Brazil.

Here we describe two PCR-based approaches to investigate the biology of *L. texanus*. The first is a standard PCR approach in which *L. texanus*-specific primers were used to detect the presence of the organism in a background of host DNA. The second is a PCR-RFLP analysis that uses restriction enzymes to distinguish between *L. texanus* and all other taxa present in the DNA extract. For both approaches we utilized 18S rDNA. This region is well conserved among phylogenetic lineages and has been used for taxonomic classification in a number of barnacle groups (e.g., Abele et al., 1992; Arrotin and Demoulin, 1993; Nakayama et al., 1996) including rhizocephalans (e.g., Murphy and Goggin, 2000; Glenner and Hebsgaard, 2006).

The simple techniques presented here enable the preservation of host and parasite tissues in the field, allowing for storage and transportation without need for refrigeration. The preserved tissues are suitable for DNA extraction and, subsequently, PCR assays that selectively recognize parasite DNA in a background of host tissue extracts, making it possible to distinguish between parasitized and non-parasitized crabs collected before external manifestations of the parasite are apparent. Additionally, the assays allow for specific detection of *Loxothylacus* DNA, without cross-recognition of other common barnacle species, and without the need for DNA sequence data, thus facilitating their use for screening planktonic samples.

Materials and Methods

Sources of Parasite, Barnacle, and Crab Material

Juvenile *C. sapidus* (not bearing externae) were collected by hand from Fowl River (30°26′N, 88°06′W) and Dog River (30°34′N, 88°5′W) on the western shore of Mobile Bay, AL; Airport Marsh (30°15′N, 88°07′W) on Dauphin Island, AL; and Pointe aux Pins (30°22′N, 88°18′W), Grand Bay, AL. Adult parasitized crabs (bearing externae) were collected by otter trawl from inshore waters near Dauphin Island, AL; Mississippi Sound, MS; and Dickerson Bay of Apalachee Bay (Wakulla County), FL. The latter were purchased from Gulf Specimens Laboratory, Panacea, FL. All crabs infected with *L. texanus* externae were separated from the uninfected specimens (those bearing no externae) and maintained in separate 680 L tanks with independent water supplies and filtration systems. These crabs were used as a source of *L. texanus* larvae. *Balanus eburneus* were collected from substrata in Mobile Bay, AL, Fowl River, AL, and from pier pilings in Pensacola, FL.

Tissue Preservation, DNA Extraction, and PCR Amplification

The oral cavity of each crab was injected by syringe with 3-6 cc of 95% ethanol and stored in 95% ethanol until DNA extraction was performed. For each specimen, total genomic DNA was extracted from approximately 25 mg of stomach region tissues using the DNeasy Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Larval stages of *L. texanus* also were preserved in 95% ethanol and extracted using the DNeasy Tissue Kit.

To amplify a 0.5 kb portion of the 18S rDNA region, we used universal crustacean primers HI (5′-GTG CAT GGC CGT TCT TAG TTG-3′) and 329 (5′-TAA TGA TCC TTC CGC AGG TTC ACC TAC G-3′) from Spears et al. (1994). To detect the presence of *L. texanus* against a background of *C. sapidus*, however, it was necessary to design a primer internal to those previously published. All publicly available 18S sequences for *C. sapidus*, *L. texanus* and its relatives were downloaded from GenBank and used to design a new primer specifically for *Loxothylacus*. From these sequences, we created a new reverse primer, Loxo3 (5′-ACG TTT GAT TGC GCG CGC ACT GTC TGC-3′), to be used with the previously published HI primer of Spears et al. (1994). While largely specific to *Loxothylacus*, variation within the priming site of Loxo3 potentially results in amplification of *Balanus eburneus* Gould, 1841, an unrelated, non-parasitic barnacle species. Therefore, we optimized the stringency, i.e., annealing temperature and magnesium concentration, of our PCR reaction to prevent amplification of *B. eburneus* when the Loxo3 primer was used. For each DNA extract, two assays were performed. One assay contained the universal crustacean primers HI and 329, while the second contained HI and Loxo3 primers, intended to amplify only *Loxothylacus*. Amplifications under the latter were expected to succeed only if *L. texanus* was present in the DNA extract.

PCR reactions included 20 mM Tris HCl pH 8.0, 50 mM KCl, 0.2 mM dNTPs, 1.75 mM MgCl$_2$, 0.4 μM HI primer, 0.4 μM 329 or Loxo3 primer, 0.0125 units of *Taq* polymerase and 0.2 mg/mL of unacetylated BSA. The PCR profile was as follows: 95°C for 5 min; 30 cycles of 95°C for 40 s, 66.8°C for 25 s, and 72.0°C for 3 min; with a final 10 min extension of 72.0°C. The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV transilluminator.

Sensitivity of PCR to Presence of Parasite

The sensitivity of the standard PCR to the amount of parasite DNA present was determined by serially diluting purified parasite DNA and subjecting the samples to PCR as described above. For potential use in analysis of plankton samples collected from the field, it is necessary to know the minimum number of dispersal naupliar larval stages and/or the infective cypris larvae that must be present in order to get a DNA signal with PCR analysis. To this end, we isolated: 1, 5, 10, 30, 50, 100, and 300 cypris larvae that had metamorphosed from naupliar larvae three days after release from infected crabs in the laboratory and extracted DNA from each group. Each group was analyzed using the PCR process described above.

PCR-RFLP Detection of *Loxothylacus*

Due to the strict stringency control required in the PCR assay described above to distinguish between *L. texanus* and other barnacle species (see
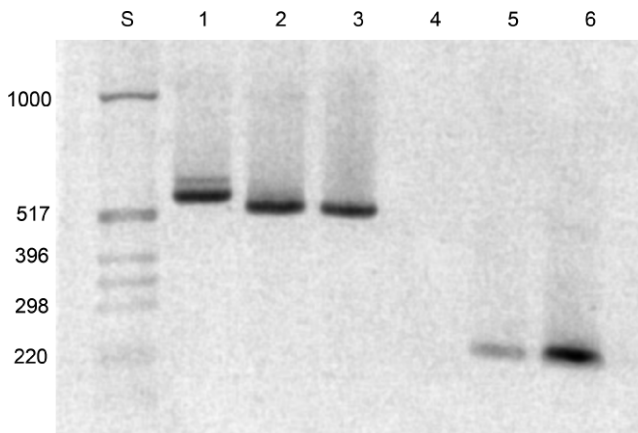
Fig. 1. Loxo3 primer specificity. PCR was conducted on *L. texanus*-infected *C. sapidus*, *L. texanus* externa, and the sessile barnacle *Balanus eburneus*. PCR was conducted in the presence of the general primer pair (HI and 329) (lanes 1-3) or the *L. texanus* species-specific primer pair (lanes 4-6). [lanes 1 & 4 - *B. eburneus* DNA, lanes 2 & 5 - DNA from the cheliped of an infected crab, and lanes 3 & 6 - the externa of a parasitized crab (parasite tissue).]

results below), we developed a second approach to determine the presence of *L. texanus* DNA that would help to avoid this problem. We used all 18S sequences previously downloaded from GenBank (see above) to screen for restriction sites specific to *L. texanus*. We did this using CLC Combined Workbench 2 (CLC Bio, Cambridge, MA, USA), which screens the REBASE database for all the restriction enzymes with cut sites in the provided sequences. We edited the GenBank sequences to include only the fragments that would be generated by the universal 18S crustacean primers HI and 329 of Spears et al. (1994). The enzyme screen identified *Taa*I (Fermentas International Inc., Glen Burnie, MD, USA) as one of several enzymes that had a RFLP pattern specific to *L. texanus*. The enzyme was used according to the manufacturer's recommendations and digestion products were separated using 1.5% agarose gel electrophoresis. Fragment patterns were compared among samples.

Effect of Crab Size on Infection Success

Juvenile *C. sapidus* (carapace width 10.1-56.3 mm) were collected at Airport Marsh, Dauphin Island, AL. Carapace width measurements were recorded for parasitized crabs obtained over a period of five years from 1999 to 2004. Crabs with externae that were about to release larvae could be recognized by their dark brown mantle cavity and were isolated in separate, aerated 19 L buckets of filtered seawater (25 ppt). The larvae are non-feeding and were maintained in aerated buckets until the cypris stage was reached (O'Brien, 1999).

Crabs were exposed to parasites by placing them in 10 L of seawater containing an undetermined number of cypris larvae in aerated 75 L aquaria for three days. The cuticle of each crab was then examined for signs of larval settlement. Only individuals with at least one visible kentrogon were used in the remainder of the experiment. Following exposure, crabs were maintained in a recirculating seawater system (300 L) as described in Boone et al. (2003). Equal numbers of crabs were fixed with 95% ethanol at two weeks, one month, and two months post exposure. DNA was extracted from each individual and analyzed using standard PCR (as described above) with HI and Loxo3 primers.

Detection of Rhizocephalan Roots in Host Periopods

All ten periopods were removed from a parasitized crab bearing an externa after the animal had been fixed with ethanol. Muscle tissue was then removed from each leg and analyzed for the presence of the parasite using standard PCR (as described above) with HI and Loxo3 primers.

RESULTS

The oligonucleotide primers HI and 329 were conserved across numerous taxa and were not appropriate for direct and sole amplification of *L. texanus* in the presence of other tissues. They did however, allow us to assess the quality of
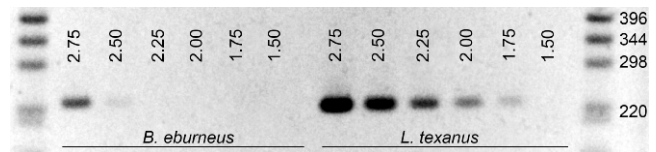

Fig 2. Effect of Mg^{2+} concentration on specificity of the PCR assay. Mg^{2+} concentration in the standard PCR assay was varied incrementally. Primers HI and Loxo3 were used.

our DNA preparations, and served as positive controls for standard PCR. For specific recognition of *L. texanus* 18S rDNA, it was necessary to develop an additional primer. Our newly constructed primer (Loxo3), when used with HI, produced a PCR product of 237 bp. PCR assays using these primers reliably produced a band of this expected size for pure *L. texanus* tissue and *C. sapidus* tissue known to be infected with *L. texanus*, but not for those of known uninfected *C. sapidus* tissues (Fig. 1).

Although this technique distinguished between *C. sapidus* and *L. texanus*, there remained a problem distinguishing between the parasite and other barnacle species due to sequence conservatism within the Loxo3 priming site. By decreasing the concentration of magnesium in the PCR, we were able to increase the stringency of the reaction, thereby eliminating amplification of all but *L. texanus* DNA (Fig. 2). DNA extracted from *Balanus eburneus*, a common non-parasitic barnacle in the Gulf of Mexico, was used to test this assumption. *Balanus eburneus*, which amplified under normal conditions, failed to amplify when $MgCl_2 \leq 2.50$ mM, whereas *L. texanus* could still be amplified with $MgCl_2$ concentrations as low as 1.75 mM. While we did not assay the DNA of other barnacles directly, sequence similarity assessment indicates that other barnacle species have sequence variation equal to or greater than that of *B. eburneus* within the Loxo3 priming site, suggesting that this method would work equally well in the presence of other taxa.

Because standard PCR requires very tight control of assay stringency to maintain specificity of the Loxo3 primer against other barnacle species, we also used PCR-RFLP analysis. In this assay the general primers were used to amplify an approximately 500 bp sequence common to most barnacles and the host crab. The resulting PCR products were digested with a restriction enzyme that yielded a pattern of digestion products specific to *L. texanus*. By screening potential restriction sites for the 18S sequences of all members of Cirripedia, which included sacculinid rhizocephalans, found in GenBank (Table 1), we can make some conclusions regarding the utility of the PCR-RFLP approach in this context. Of those available sequence data, 15 contain the restriction site for *Taa*I, but none of these will yield restriction fragments of the sizes found within *L. texanus*. The size differences used to differentiate between organisms are easily discernible by agarose gel electrophoresis (Fig. 3).

We have also examined the sensitivity of the PCR techniques concerning both DNA concentrations in the PCR mix and the number of cypris larvae that can be detected. The assay produces detectable PCR products from as little as 100 pg of total *L. texanus* DNA and with as few as five larvae in a single reaction (Fig. 4). Rheinsmith et al. (1974)

Table 1. Representatives of the Cirripedia, Rhizocephala, and Sacculinidae with 18S DNA sequence present in GenBank. Members listed in bold possess sequences that contain priming sites for 329 and HI universal primers of Spears et al. (1994).

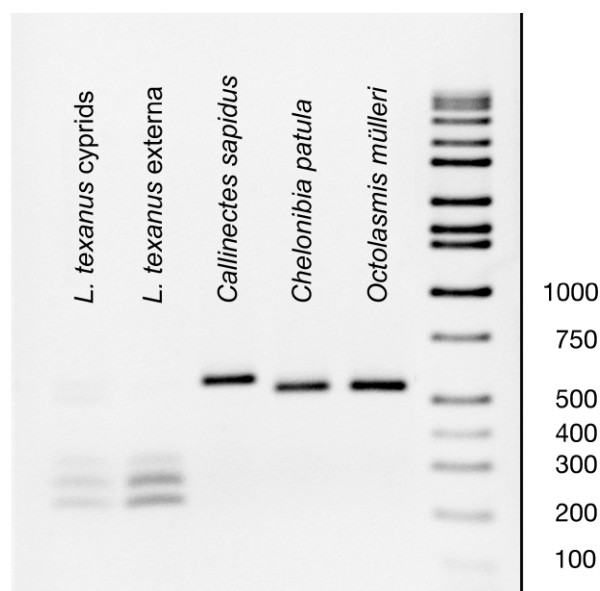| Species	GenBank acc. no.
Balanus balanus	AY520628
Balanus crenatus	AY520624
Balanus eburneus	BLNERRNA
Balanus glandula	AY520625
Balanus nubilus	AF201665
Balanus perforatus	AY520629
Bosmaella japonica	AY265369
Chthamalus bisinuatus	AY520644
Chthamalus challengeri	AY520643
Chthamalus fissus	AY789457
Chthamalus fragilis	CMHERRNAA
Chthamalus montagui	AY520642
Chthamalus stellatus	AY520641
Diplothylacus sinensis	DQ826568
Heterosaccus californicus	AY520657
Lernaeodiscus porcellanae	DQ826569
Loxothylacus panopaei	AY265364
Loxothylacus texanus	LOYERRNAA
Octolasmis lowei	OCLERRNAA
Peltogaster paguri	DQ826570
Peltogasterella sulcata	DQ826572
Polyascus gregaria	AY265363
Polyascus plana	AY265368
Polyascus polygenea	AY265362
Polysaccus japonicus	DQ826565
Pottsia serenei	DQ826567
Sacculina carcini	AY520656
Sacculina confragosa	AY265361
Sacculina leptodiae	AY265365
Sacculina oblonga	AY265367
Sacculina sinensis	AY265360
Sacculinidae sp.	AY859600
Septosaccus rodriguezii	DQ826571
Sylon hippolytes	DQ826564
Tetraclita japonica	AY520640
Tetraclita rubescens	AY789459
Tetraclita squamosa	AY520639
Tetraclita stalactifera	TEKERRNAA
Thompsonia littoralis	DQ826573

Fig. 3. Specificity of assay based on PCR followed by restriction digest (PCR/RFLP method). DNA was extracted from *L. texanus* cyprids, an externa of *L. texanus*, an uninfected specimen of *C. sapidus*, and two species of ectocommensal barnacles (*Octolasmis mülleri* and *Chelonibia patula*) that were taken from a specimen of *C. sapidus*.

studied genome size of crustaceans and described the genome size of "*Sacculina* sp." to be about 0.7 pg. If this value is typical of rhizocephalans, then the sensitivity of the assay would be approximately 3.5 pg.

Detection of Parasitized Crabs in the Field

A total of 65 juvenile crabs from Airport Marsh ($n = 14$), Pointe aux Pins ($n = 11$) and Fowl River ($n = 40$) were analyzed using PCR. While the majority of the crabs tested negative for the parasite, the presence of *L. texanus* DNA was detected in one of the crabs collected from the Fowl River site. Of approximately 100 adult *C. sapidus* collected at the Fowl River site during this period, one bore an externa of the rhizocephalan (JOB, unpublished).

Detection of Rhizocephalan Roots in Host Periopods

Rhizocephalan tissue was detected by PCR assays in both chelipeds and all of the second through fourth pairs of periopods (= walking legs) in the parasitized crab that was examined. No positive PCR signal was obtained from tissue removed from the fifth periopods (=←paddle) of that individual.

Crab Size at Exposure as a Factor Affecting Infection Success

Ninety two crabs of varying sizes were exposed to infective larvae. The largest crab to be infected was 43.0 mm CW. Parasitic infection could be detected in as little as two weeks post larval settlement. Detection rates from hepatopancreas tissue did not increase with time. Infection rates for intermolt crabs were relatively low with only 18% of the exposed individuals testing positive for parasite DNA. The parasite was significantly more successful in infecting crabs in the laboratory with carapace widths under 30 mm than larger individuals (Chi-Sq $= 6.78$; P-value $= 0.009$; $d.f. = 1$; See Table 2). Similarly although the carapace widths of field caught crabs bearing externae ($n = 50$) ranged from 41-104 mm, the average size at parasitic anecdysis (= terminal molt, sensu O'Brien & van Wyk, 1985) was 62.5 mm carapace width.

DISCUSSION

As far as we know, this is the first report of successful extraction of rhizocephalan DNA from the body cavities of hosts. Previous investigations using PCR to study this group extracted DNA from either externae or planktonic larval stages of the parasites (Spears et al., 1994; Murphy and Goggin, 2000; Pérez-Losada et al., 2002; Glenner et al., 2003; Pérez-Losada et al., 2004; Glenner and Hebsgaard, 2006; Gurney et al., 2006; Tsuchida et al., 2006). A number of obstacles had to be overcome for the PCR techniques described here to be successful. First, the parasite stages of interest (vermigon and early interna) were most likely to be located near, or embedded within, the hepatopancreas, or
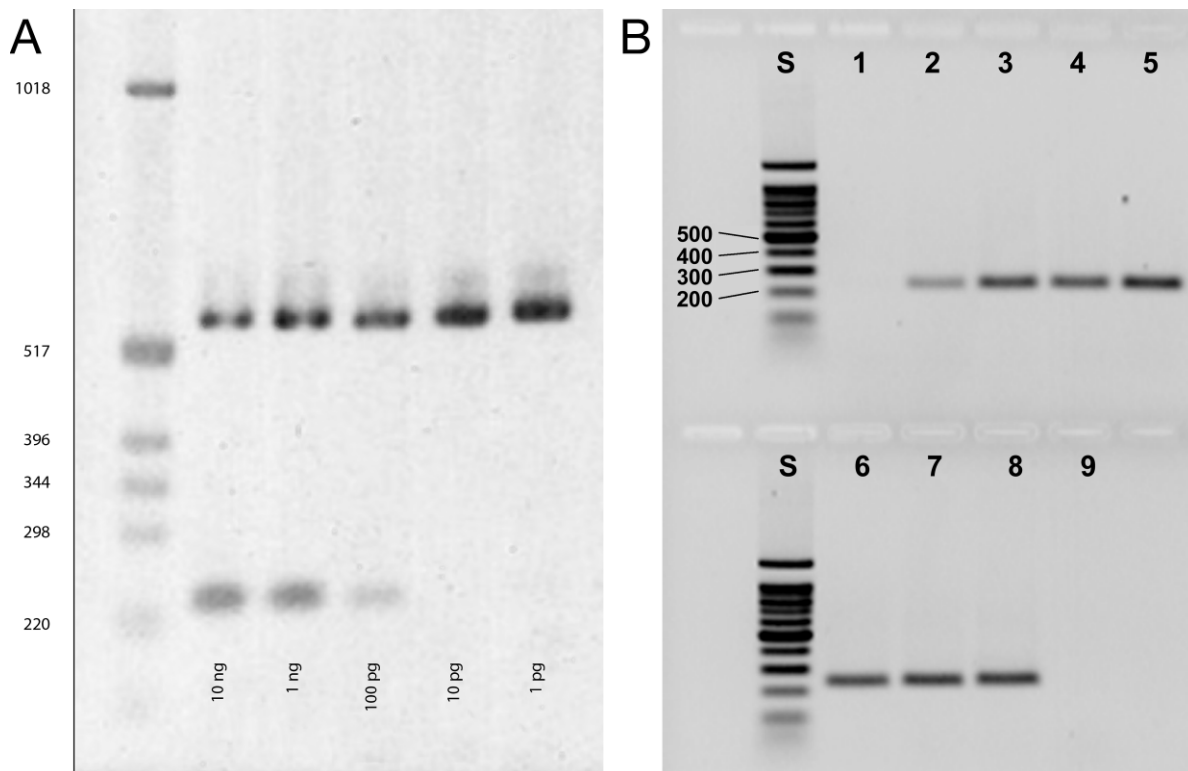
Fig. 4. Detection limits of the assay. A. Various amounts of *L. texanus* DNA were combined with 100 ng of *C. sapidus* DNA and subjected to PCR. Separate reactions run using either HI and 329 primers or HI and Loxo3 primers. After PCR, aliquots of both reactions were combined and run in a single lane of the gel. B. *L. texanus* cypris larvae were hand-counted and analyzed by PCR using Loxo3 and HI primers. [S = size standards, Lanes 1-7 represented 1, 5, 10, 30, 50, 100, and 300 larvae, respectively. Lane 8 - *L. texanus* externa (positive control) and lane 9 no DNA] (All samples showed a similar pattern when using the broad host range primers 329 and HI.)

digestive gland, of the host. This organ is a repository for degradative enzymes (Icely and Nott, 1992), which can lead to false negatives through degradation of parasite DNA during the extraction process. The treatment of specimens via injection of 95% ethanol directly into the hepatopancreas region and use of commercially available DNA extraction kits for use with small amounts of tissue solved these problems. Additionally, this preservation approach allows specimens to be preserved in the field, stored at room temperature, and still yield usable DNA at least three months later.

Furthermore, rhizocephalans are barnacles that typically parasitize decapods (Høeg, 1995); hence they are crustacean parasites of crustaceans. Their phylogenetic proximity to their hosts required a high degree of stringency of reaction condition used in the PCR. Additionally, other barnacles such as *Balanus amphitrite* Darwin, 1854, and *Chelonibia patula* (Ranzani, 1818) attach to the carapace (Williams, 1984) while *Octolasmis mülleri* (Coker, 1902) (Gannon, 1990) settles upon the gills of blue crabs. These circumstances increase the likelihood of species cross-contamination of crab tissues. Our data indicate that the methodology outlined here is sufficient for specific amplification of *Loxothylacus texanus*.

Consequently, important stages in the life cycle of the parasite occurring between successful penetration of host cuticle and the appearance of the externa, which have been impractical to investigate heretofore, are now accessible for

study. While successful settlement and metamorphosis into kentrogon larvae has been observed in the lab on crab carapaces regardless of size (Boone et al., 2003), host blue crabs in Mississippi Sound bearing *L. texanus* externae are usually smaller than their uninfected counterparts (Overstreet et al., 1983), suggesting that larger crabs are either not vulnerable to infection or do not survive the infection process. The data reported here indicate that size at exposure is an important factor in determining infection success. Significantly more small crabs than large crabs were infected in the laboratory by the parasite (Table 2). Considering that a blue crab will increase its size by approximately 25-30% with each molt and an infected crab will only molt 5-9 times before reaching a terminal molt (O'Brien, 1999), these data are consistent with size data obtained from parasitized field caught crabs whose average carapace width was only 65 mm (compared to a typical adult size of 120-170 mm CW).

Table 2. Infection success rate of *Loxothylacus texanus* exposed to potential hosts (*Callinectes sapidus*) of different sizes. *Carapace width.

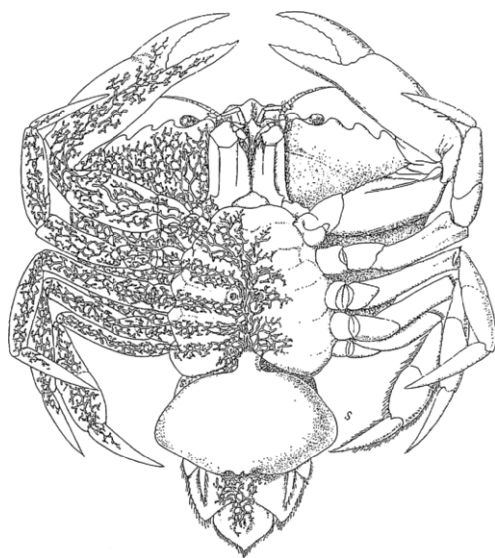| Crab size* at exposure (mm)	Number of crabs exposed	Number of crabs parasitized	Prevalence or success rate (%)
10-20	47	13	27.7
20-30	15	3	20.0
30-40	14	0	0.0
40-50	10	1	10.0
50-60	6	0	0.0

Fig. 5. Representation of the external and internal features of the rhizocephalan, *Sacculina carcini*, showing the root system of the parasitic barnacle extending into the periopods of the host crab, *Carcinus maenas* from Boas (1920, p. 302).

This suggests that smaller size at parasitic anecdysis is influenced more by the initial success of the infection rather than subsequent factors such as predation that affect host survival. The lack of successful infection of larger juvenile crabs and the overall low rates of infection following penetration by the stylet of the kentrogon larvae (as well as low infection rates seen in field populations) suggests that the host crab may be able to mount some type of immune response against the parasite during the initial stages of infection. The fact that successful infection by rhizocephalans involves more than mere access to hosts is reinforced by data from Ritchie and Høeg (1981) who reported that over 75% of a group of hosts ($n = 210$) did not become infected following exposure to infective larvae, even though the vulnerability of the potential hosts had been increased by removal of their cleaning appendages.

Permeation of the crab host by the mature parasite is unclear. Eye-catching illustrations of the ramified root system of sacculinids extending out into the walking appendages of the hosts (Fig. 5) have appeared in so many invertebrate zoology (Kaestner, 1970; Barnes et al., 1993), parasitology (Noble et al., 1989; Roberts and Janovy, 2005), and marine biology textbooks (Nicol, 1967; Levinton, 2001) that the original drawing (Boas, 1920) could be considered a classic. Although we have detected the presence of *Loxothylacus* in the chelipeds and walking appendages of a blue crab bearing an adult externa, we were unable to detect it in the posterior periopods or "paddles" of that crab. Either the full development of the root system of the parasite takes more time than the maturation of the gonads in the externa or not all host legs are penetrated by the rhizocephalan.

Use of molecular techniques for taxonomic identification of organisms is now an approach widely used by systematists and population geneticists. Particularly in animals, highly variable, but phylogenetically informative DNA sequences are being used to "barcode" individual taxa (Paul et al., 2003; Moritz and Cicero, 2004; John, 2007). Using this approach, cytochrome oxidase subunit I (COI), 18S DNA, and 16S DNA have been used in crustaceans to provide information about phylogenetic relationships (Glenner et al., 2003; Gurney et al., 2006) and to determine the identity of closely related parasites that infect individual crabs (Tsuchida et al., 2006). While DNA barcoding is considered by some to be the best approach for absolute taxonomic identification, it would be cost prohibitive for use in some types of ecological studies. For example, screening water samples collected from plankton hauls for the presence of *L. texanus* would require a great deal of molecular cloning and DNA sequencing. This is because hundreds to thousands of individuals are likely to be present in each sample and PCR products of each individual would have to be sequenced. If not, then the PCR products would include a population of DNA fragments that could not be resolved after sequencing. If a subset of organisms in the sample were used to reduce the time of cost of analysis, then samples with low abundance of the parasite would not be likely to identify the target organisms in the samples. The high-stringency PCR and PCR-RFLP methods presented here will be not be quantitative (with respect to the numbers of *L. texanus* in a samples) but will allow for cost-effective ways to detect the presence of this parasite in a large sampling area. Thus, it could be used to look for spatial and temporal distributions of parasite larvae in the field and allow for investigation of the ecology of this organism, which has been impossible in the past.

There are numerous interesting and important unanswered questions about sacculinids, and *L. texanus* in particular, remaining to be investigated that can now be addressed with the help of the PCR-based techniques described here. For example, factors underlying the seasonal abundance of *Loxothylacus* larvae in the Gulf of Mexico are unclear (O'Brien et al., 1993). A diagnostic tool to screen planktonic samples would help to determine the temporal and spatial abundance of the parasite in the water column. Additionally, the prevalence of *L. texanus* in populations of blue crabs reported in the literature varies considerably [< 1%, Florida (Hochberg, 1992); 50%, Texas (Christmas, 1969)]. These values are based on crabs captured with parasitic externae present. It remains to be determined if numbers of parasitized crabs without externae are similar to these values previously reported, thus confirming high variability in different crab populations or if many infected individuals have gone undetected. Numbers of infected individuals may be underestimated as well, as young crabs may die before reaching the stage where the parasitic externae is visible. It is likely that *L. texanus* has a significant negative impact upon the blue crab fishery in the Gulf of Mexico, since sacculinids prevent the maturation of host gonads (Kuris, 1974) and adult hosts parasitized by many, if not most, species of sacculinids do not molt [This was first observed by Giard (1886) and was experimentally tested by O'Brien and Skinner (1990), but see Takahashi and Lützen (1998)]. Furthermore, since mean size of infected adult *C. sapidus* in the northern Gulf of Mexico is significantly smaller than uninfected adults (Overstreet et al., 1983),

commercial trapping may increase the relative abundance of parasitized hosts, a situation that may increase the deleterious impact of the parasite on future yields (Kuris and Lafferty, 1992). In 2002, the blue crab industry brought in 172.2 million pounds valued at over $129 million dollars in the U.S. alone (NMFS, 2002). Because of the crab's economic value, it is important that we understand the impact that *Loxothylacus texanus* has on the blue crab population to determine its economic and ecological impact. The utilization of the PCR-based techniques we outline here will help to facilitate the quantification of the impact *L. texanus* has upon the blue crab fishery in the Gulf of Mexico, which at this time is simply unknown.

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