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

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A B S T R A C T

We describe techniques that enable the preservation of tissues from the rhizocephalan barnacle, , inside the body cavities of blue crab hosts, , 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 . In the first approach, a set of primers specific for was used to specifically amplify 18S sequence in a background of crab DNA or the DNA of other barnacle species. In the second approach, a set of general primers was used to amplify 18S sequence from , 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 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 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

gion t the hos-22 the host through the (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 cyprid 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 devel

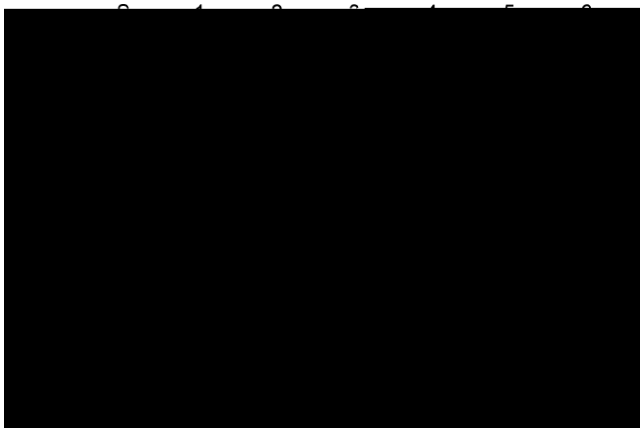

Fig. 1. Loxo3 primer specificity. PCR was conducted on , , -infected , , , externa, and the sessile barnacle , , , . PCR was conducted in the presence of the general primer pair (HI and 329) (lanes 1-3) or the , , species-specific primer pair (lanes 4-6). [lanes 1 & 4 - , , DNA, lanes 2 & 5 - DNA from 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 , , 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 , , . 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 I (Fermentas International Inc., Glen Burnie, MD, USA) as one of several enzymes that had a RFLP pattern specific to , , . 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 , (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 we33.9 (witp326.6 (ce)]TJT*9 (maint7 (d3.83e7 2 (m,2D(ce)]TJT*9 (mai3.82wa(an)6.2sed)-125.8 (to)-125q.9 (Size)-

Fig. 4. Detection limits of the assay. A. Various amounts of , , DNA were combined with 100 ng of , 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. , , 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 - , , 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 , , , Darwin, 1854, and , , (Ranzani, 1818) attach to the carapace (Williams, 1984) while , , , (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 , , , , .

Fig. 5. Representation of the external and internal features of the rhizocephalan, , , showing the root system of the parasitic barnacle extending into the periopods of the host crab, , , 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 (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

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