A SPECIES-SPECIFIC PCR ASSAY FOR THE DETECTION OF *ROTYLENCHULUS RENIFORMIS*

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Abstract

The reniform nematode (*Rotylenchus reniformis*) is a major crop pest in cotton, sweet potato, and soybean in Mississippi. Management for the reniform nematode begins with nematode identification. Until now identification of the nematode has been conducted by morphological examination of extracted individuals by a trained technician or nematologist. Molecular methods have provided a way that eases the constraints of identification of the reniform nematode. The objectives of this research are 1) create species-specific primers that only amplify reniform nematode DNA, 2) evaluate the species specific primers on DNA isolated from individual vermiform nematodes and metagenomic nematode samples, 3) evaluate the primers from soil samples from multiple locations and states, and 4) create a real-time PCR assay that can identify the presence and quantify the numbers of reniform nematodes from soil samples. The preliminary results for the first three objectives are reported here.

Introduction

Rotylenchus reniformis is commonly known as the reniform nematode. This nematode has been found to infest 32.4% of cotton acres in Mississippi. The Cotton Disease Loss Estimate Committee estimated that in 2009 the reniform nematode caused a 5.5% loss in cotton production in Mississippi. State extension recommendations suggest that soils suspected of being infested with the reniform nematode as with all plant-parasitic nematodes, have soil samples taken and sent to the state nematode diagnostic labs for identification and enumeration. Diagnostics of the vermiform reniform juvenile is difficult in that it is morphologically similar to the spiral nematode, and identification is done best by technicians the routinely see the nematode. In areas where the reniform is not known to occur and the technician is unaccustomed to the reniform nematode it may be miss identified possibly as a spiral nematode.

Molecular diagnostic test have been described for several soil borne pathogens including plant-parasitic nematodes. Through PCR assays with species specific primers on metagenomic DNA isolated from the soil researchers can identify pathogens without morphological examination. Molecular diagnostics can be performed at a lower variable cost per sample, and requires little or no technical knowledge of the pathogen. Real-time PCR provides a way of estimating the number of nematodes that are present in a soil sample. Metagenomic DNA is extracted from the soil and contains DNA from most of the organisms present in the soil. Nematode DNA is readily obtained from metagenomic samples. Individual nematodes can be lysed and the lysate serves as template DNA in a PCR assay.

Few molecular studies have been reported on the reniform nematode. Agudelo et. al. 2005, in assaying the interspecies variation of the reniform from 27 populations from various locations in North and South America, reported a conserved first internal transcribed spacer region (ITS1) sequence for amphimitic reproducing reniform. The sequence differed only from a parthenogenic strain from Japan. The reniform was also included in a phylogenetic study in which the expansion region of the large subunit of the ribosome was sequenced, for the order tylenchida.

Methods

Species-specific primers where designed based on the conserved ITS1 sequence for the reniform nematode. Other ITS1 sequences, from plant-parasitic species including the spiral and root–knot nematodes were downloaded from GenBank and aligned using lasergene software. Places in which sequences differed from the reniform nematode were selected as potential primer binding sites.

Nematode populations used in this study included, the Southern Root-knot and reniform nematodes maintained in the Mississippi State Nematology Research Program's green house and soil samples collected from eight different locations; Calhoun, Chickasaw, Oktibbeha, and Carroll counties in Mississippi, Mississippi and Scott counties

Missouri, and Massac and Pope counties Illinois. Nematodes were extracted from the soil by the gravity sieving and sucrose centrifugal floatation technique. Nematodes were centrifuged at 2,000 X g for 6 minutes afterwards the supernatant was removed leaving 5 ml of extractant. Nematodes were brought back into suspension by agitation. Aliquots of the suspension were used in the individual and metagenomic DNA extractions.

Individual vermiform nematode extractions were conducted as described by Floyd et. al. 2002 modified only by aspirating the nematodes with a micropipette to transfer them into a tube in which they where lysed. After DNA extraction, 2 µl of the lysate was amplified with the species-specific primers. A separate PCR assay with the D2-D3 primers described by Subbotin et. al. 2006 was conducted to verify that the DNA extraction was successful verifying sufficient template DNA was present.

Metagenomic DNA was isolated using the Powersoil DNA Isolation Kit from MoBio labs. Since the kit calls for adding 0.25 grams of soil a sight modification was made. One ml of the nematode suspension was added to a bead-beating tube in which the beads and solution had been removed. The nematodes where centrifuged at 2,000 rpm X g for 6 minutes. This was followed by removing 800 µl of supernatant then adding beads and solutions back into the tub. The manufactures protocol was then followed to completion.

Results

Ten species-specific primers were designed of which two were selected and evaluated in this study. The primers produced a band near the 100 bp marker. The specificity of the primers on individual vermiform nematode was good producing a bright band on the reniform nematode samples and a very light band on all other samples including the water control not containing any nematodes. Unexpected amplification did occur in samples that did not have the reniform nematode in them. At this point the cause of the non-specific amplification was unclear. Amplification may have arisen from contamination, or non-specific amplification. The primers readily produced bands with template DNA isolated from individual vermiform reniform nematodes and metagenomic samples containing the reniform nematode.

Metagenomic samples that contained the reniform produced a bright band around the 100 bp marker. As in the individual samples a light band appeared in all the samples. Samples that did not contain the reniform nematode did not produce a bright band. Samples not containing the reniform nematode did have other plant-parasitic nematodes including the root-knot, soybean cyst, and stunt nematodes. All samples contained various free-living nematode trophic groups.

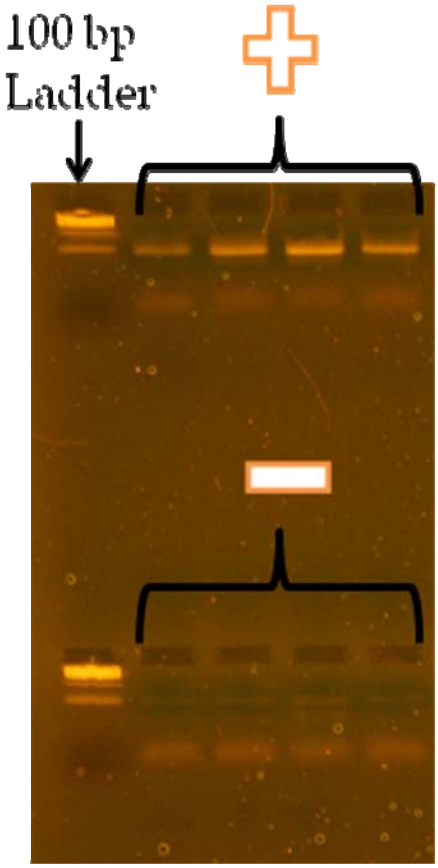

Figure 1. A 4% agarose gel of a metagenomic PCR using the species-specific primers designed in this study. The far left rows are a 100 bp ladder. The top row samples are samples that contained the reniform nematode from cotton fields in Calhoun, Chickasaw, Oktibbeha, and Carroll counties Mississippi, respectfully. The bottom row samples are samples that did not contain the reniform nematode from Mississippi and Scott counties Missouri, and Massac and Pope counties Illinois, respectfully.

Conclusion

The species-specific primers showed specificity to the reniform nematode by readily amplifying reniform DNA. The primers developed in our study are suitable for use in real-time assays because they produce a single amplicon near the 100 bp marker. The light bands and the non-specific band will be cloned and sequenced to determine the origin.

References

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Disclaimer

The interpretation of data may change with additional experimentation. Information is not to be constructed as a recommendation for use or as an endorsement of a specific product by Mississippi State University or the Mississippi Agricultural and Forestry Experiment Station.