DETECTION OF *ROTYLENCHULUS RENIFORMIS* FROM SOIL WITH REAL-TIME QUANTITATIVE PCR

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Abstract

A quantitative PCR procedure targeting the □-tubulin gene determined the number of *Rotylenchulus reniformis* Linford & Oliveira 1940 in metagenomic DNA samples isolated from soil (1). Of note, this outcome was in the presence of other soil-dwelling plant parasitic nematodes including its sister genus *Helicotylenchus* Steiner, 1945. The methodology provides a framework for molecular diagnostics of nematodes from metagenomic DNA isolated directly from soil.

Introduction

The reniform nematode, *Rotylenchulus reniformis*, is the limiting factor in cotton production in nematode infested fields in the southeast United States. The rapid spread and agronomic damage caused by the reniform nematode make its identification in soil samples of paramount importance for control and management practices. With the advent of new and quantitatively reliable molecular strategies, it is possible that the time required to diagnose the reniform nematode in soils could be reduced. Isozyme and DNA-based molecular tests (i.e. PCR) have been developed to determine and to confirm diagnoses of many plant parasitic nematodes (2-9). The PCR-based strategy is also useful because it can provide information on the presence of a particular organism in an environmental sample. Currently, little research has been conducted on the potential of qPCR for diagnostic nematology from environmental samples. The objective of this study was to determine, quantitatively, the number of *R. reniformis* in metagenomic samples isolated directly from the soil.

Materials and Methods

Soil samples from fields in crop production containing various species of nematodes, including *Helicotylenchus Mesocriconema, Pratylenchus*, *Tylenchorhynchus*, *Meloidogyne* and free living nematodes were collected from cotton and corn production fields at the North Plant Science Center at Mississippi State University. Four additional sites, determined to not contain *R. reniformis* were also sampled. At each site, metagenomic samples were collected in triplicate. Metagenomic DNA isolations were conducted by using the Powersoil® DNA extraction kit® with modifications. Beta tubulin (Rr-□-tub) (GT736478.1) was downloaded from GenBank. Areas of *R. reniformis* sequences from the ClustalW alignment that had very few matching bases were selected for *R. reniformis* primer generation. The sequences were trimmed to the divergent areas and imported into Primerselect® of the Lasergene® software package. Primer pairs were generated using Primerselect®. The Rr-□-tub primers (**Table 1**) were evaluated in reactions having three different levels of DNA sample complexity. Quantitative PCR (qPCR) Taqman® 6-carboxyfluorescein (6-FAM) probes were used. To generate a standard curve for the amount of *R. reniformis* in a soil sample, estimates of approximately 1,000 nematodes in 0.3 ml of water were placed into the Powersoil® DNA isolation kit® bead beating tubes and extracted as described previously. A 1:10 serial dilution of DNA extracted from approximately 1,000 nematodes was created and used for generation of the standard curve by qPCR. To evaluate accuracy of the standard curve, samples containing 100, 10, 1, and 0 vermiform *R. reniformis* were

generated by hand collecting using a stereoscope and isolating the DNA as described. To confirm that the amplification products from the qPCR conditions was indeed *R. reniformis* DNA, the amplicon was isolated, sequenced and compared to the original DNA sequence from which the Rr-□-TUB qPCR primers were designed.

Results and Discussion

The Rr-□-TUB qPCR primers were evaluated in standard PCR reactions with DNA having three different levels of sample complexity. The first level of complexity was pure DNA samples from greenhouse cultured *R. reniformis* (**Figure 1**). The second level was total populations of nematodes extracted from field soil samples (**Figure 2; Table 2)**. The third and most complex level of DNA were samples from metagenomic DNA isolated from the South Farm (SF), Cotton (Ct) and Corn field samples (**Figure 3)**. Rr-□-TUB primers failed to amplify DNA in 2 of 3 samples where a single nematode was used as the template (**Figure 1, lanes 8 and 9**), but did amplify in one sample containing DNA isolated from a single individual (**Figure 1, lane 10**). The Rr□□-TUB primer never yielded amplification products when no template DNA was provided (**Figure 1, lanes 11-13**). This demonstrates that the Rr-□-TUB gene would be useful for detecting nematodes with thresholds between 1 and 10 nematodes. The goal of the metagenomic analysis was to take soil samples directly into DNA isolation procedures and downstream qPCR analyses. Soil samples from sites that lacked *R. reniformis* and sites having *R. reniformis* were focused on in direct metagenomic DNA isolation procedures. The experiments began by using the Rr-□-TUB qPCR primers under standard PCR conditions as a quick screen to determine how well the primers amplify their target DNA on metagenomic samples. The Rr-□-TUB primed reactions exhibited no amplification in the SF samples (**Figure 3;** lanes 1 and 2). The three Ct sites that had 41, 8 and 12 nematodes, respectively, yielded strong amplification of 300 bp (**Figure 3;** lanes 3-5, respectively).

When using the qPCR primers under qPCR conditions that low concentrations of *R. reniformis* can be quantified (**Table 3**). Results also show that low numbers of *R. reniformis* can be quantified from field extracted nematodes **(Table 4**) and metagenomic DNA isolated directly from soil samples at three different sites know to either have or lack *R. reniformis*. The results show a close relationship between the qPCR outcome of hand-counted and DNA extracted directly from soil (**Table 4**). The amplicon was isolated, sequenced and compared to the original DNA sequence to confirm that the amplification products from the qPCR conditions were indeed *R. reniformis*.

Figure 1. Specificity of the Rr- β-TUB qPCR primers under standard PCR conditions from known numbers of *R. reniformis*. *R. reniformis* DNA was isolated from vermiform J2s serving as the template. The Rr-□-TUB-primed reactions. Abbreviation, R.r. - *R. reniformis*. Lane 1, 1000 R.r.; Lane 2, 1000 R.r.; Lane 3, 1000 R.r.; Lane 4, 100 R.r.; Lane 5, 100 R.r.; Lane 6, 100 R.r.; Lane 7, 10 R.r.; Lane 8, 10 R.r.; Lane 9, 10 R.r.; Lane 10, 1 R.r.; Lane 11, 1 R.r.; Lane 12, 1 R.r.; Lane 13, 1 R.r.; Lane 14, No DNA; Lane 15, No primers.

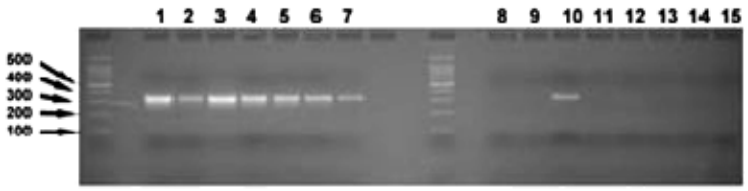

Figure 2. Amplification characteristics of the Rr-β-TUB qPCR primer on DNA isolated from *R. reniformis* extracted from the SF, Corn and Cotton sites under standard PCR conditions. The field sites are SF, Corn and Cotton. Each site was replicated in triplicate. The number of nematodes whose DNA was isolated is provided below the amplicon in each reaction. Lane 1, SF1; Lane 2, SF1; Lane 3, SF1; Lane 4, SF2; Lane 5, SF2; Lane 6, SF2; Lane 7, SF3; Lane 8, SF3; Lane 9, SF3; Lane 10, Corn 1; Lane 11, Corn 1; Lane 12, Corn 1; Lane 13, Corn 2; Lane 14, Corn 2; Lane 15, Corn 2; Lane 16, Corn 3; Lane 17, Corn 3; Lane 18, Corn 3; Lane 19, Cotton 1; Lane 20, Cotton 1; Lane 21, Cotton 1; Lane 22, Cotton 2; Lane 23, Cotton 2; Lane 24, Cotton 2; Lane 25, Cotton 3; Lane 26, Cotton 3; Lane 27, Cotton 3; Lane 28, No DNA; Lane 29, No Primers but having DNA.

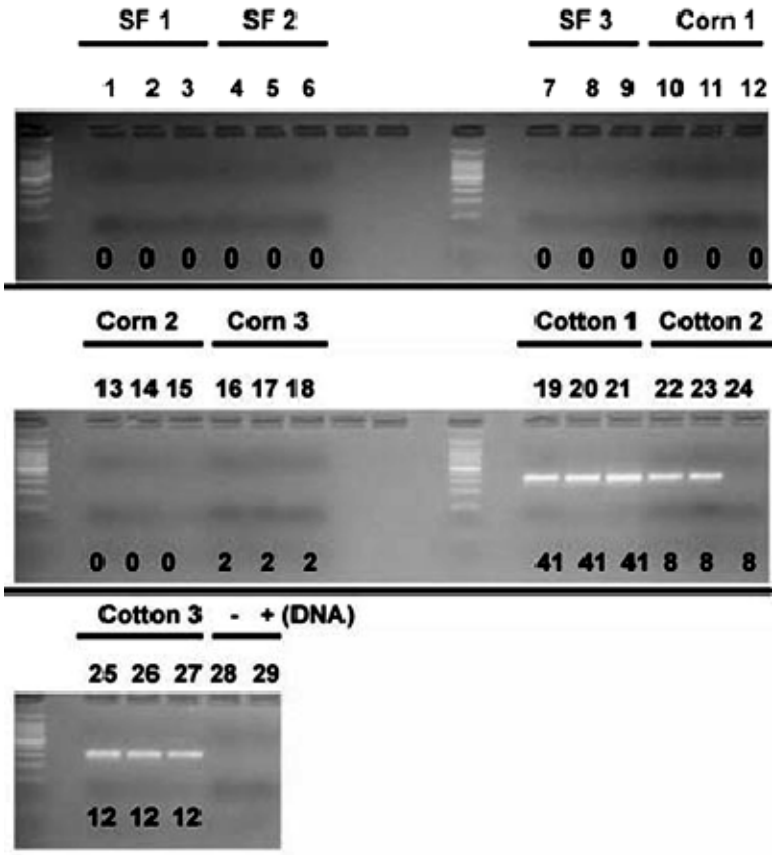

Figure 3. Amplification characteristics of the Rr-β-TUB qPCR primers under standard PCR conditions on metagenomic DNA isolated directly from soil collected at the South Farm (SF) and cotton (Ct) sites. The number of nematodes whose DNA was isolated is provided below the amplicon in each reaction. Rr-β-TUB primed reactions. Lane 1, SF1; Lane 2, SF2; Lane 3, Ct1 sample 1; Lane 4, Ct2 sample 2; Lane 5, Ct3 sample 3; Lane 6, No DNA; Lane 7, No Primers.

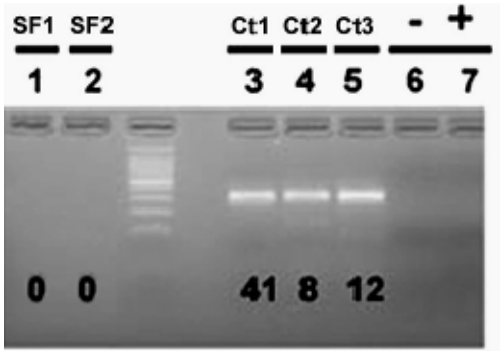

Table 1. *R. reniformis* β-tub primers for PCR assay

Name	Sequence
Hit.7. (Rr-β-TUB) F	5'-CAAAATGTCCGCCACCTTCGTT-3'
Hit.7. (Rr-β-TUB) R	5'-GTGCCGTCTCCTCAGCCTCGTA-3'
Hit.7. (Rr-β-TUB) probe	5'-ACGAGATGGAATTCACTGAGGCCGAA-3'

Table 2. Number of nematodes per 500 cm3 soil used in field assay

Field Sample Description								
Sample	Crop grown in field	*Rotylenchulus reniformis*	*Meloidogyne* spp.	*Helicotylechus* spp.	*Mesocriconema spp.*	*Pratylenchus* spp.	*Tylenchorhynchus* spp.	Free-Living
1	Fescue	0	0	86	258	0	0	1204
2	Fescue	0	0	86	0	86	0	3698
3	Fescue	0	0	344	0	0	0	0
4	Corn	0	0	86	0	0	0	1634
5	Corn	0	0	0	0	0	86	3526
6	Corn	1743	0	0	0	0	0	1032
7	Cotton	344	0	688	0	0	0	688
8	Cotton	5676	0	0	0	0	0	258
9	Cotton	516	0	0	0	0	0	516

Table 3. The Rr-β-TUB qPCR assay estimates of nematodes from a serial dilution of the nematode suspension

Sample ID	Number of *R. reniformis*	Ct Value Mean	Ct STD	qPCR mean estimate	qPCR STD
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	1	0	0	0	0
5	1	33.25	3.60	21.24	28.29
6	1	37.00	1.03	0.65	0.49
7	10	34.67	0.98	3.00	1.89
8	10	33.19	0.16	7.47	0.83
9	100	31.82	0.49	19.94	6.82
10	100	32.51	1.80	20.40	25.18
11	1000	25.28	0.07	1727.00	82.18
12	1000	25.26	0.20	1754.89	253.59

Table 4. Comparison of hand counted (estimated) and qPCR experimentally determined *R. reniformis* DNA extracted directly from soil.

Powersoil® DNA Isolation Samp ID	Estimated number of *R. reniformis*	STD of number of *R. reniformis*	qPCR mean determined *R. reniformis*	qPCR STD
SF-1	0	0	0	0
SF-2	0	0	0	0
SF-3	0	0	0	0
Corn-1	0	0	0	0
Corn-2	0	0	0	0
Corn-3	2.72	0.67	0	0
Cot-1	48.63	10.59	16.63	3.47
Cot-2	9.73	0.67	8.66	4.00
Cot-3	14.00	6.50	13.93	0.69

Conclusions

Quantitative methods for determining the thresholds of nematodes in soil are an important management tool for the reniform nematode because in a period of a few hours the number and species in a soil sample can be determined. A quantitative PCR procedure targeting the □-tubulin gene determined the number of *Rotylenchulus reniformis* Linford & Oliveira 1940 in metagenomic DNA samples isolated from soil. The methodology provides a framework for molecular diagnostics of nematodes from metagenomic DNA isolated directly from soil. Importantly, the procedure also allows for the monitoring of nematodes in near real time through the course of a growing season.

Disclaimer

The interpretation of data presented may change with additional experimentation. Information is not to be construed 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.

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