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Sensitive PCR Detection of *Meloidogyne arenaria*, *M. incognita,* and *M. javanica* Extracted from Soil

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Abstract: We have developed a simple PCR assay protocol for detection of the root-knot nematode (RKN) species *Meloidogyne arenaria, M. incognita*, and *M. javanica* extracted from soil. Nematodes are extracted from soil using Baermann funnels and centrifugal flotation. The nematode-containing fraction is then digested with proteinase K, and a PCR assay is carried out with primers specific for this group of RKN and with universal primers spanning the ITS of rRNA genes. The presence of RKN J2 can be detected among large numbers of other plant-parasitic and free-living nematodes. The procedure was tested with several soil types and crops from different locations and was found to be sensitive and accurate. Analysis of unknowns and spiked soil samples indicated that detection sensitivity was the same as or higher than by microscopic examination.

Key words: Detection, diagnosis, *Meloidogyne arenaria, Meloidogyne incognita, Meloidogyne javanica,* PCR, root-knot nematode, soil.

Determination of the presence of plant-parasitic nematodes in soil samples as well as information on their numbers and species is essential for many cropping decisions. Currently, identification and quantification require extraction of nematodes from soil samples and microscopic examination to determine the numbers and species of nematodes present. Microscopic identification of particular species or groups of species in soil samples can be difficult, especially when a large number of other plant parasites is present, and requires a well trained specialist. Nematodes generally are unevenly distributed in soil, and often it is prohibitively time consuming and expensive to examine the large number of samples required to adequately analyze a field. Molecular biology techniques have the potential to increase the efficiency and sensitivity of this process.

DNA analysis has been widely used in systematics and for identification of nematodes (Williamson et al., 1997; Zijlstra et al., 2004; Powers et al., 2005). PCR assays have been used for identification of nematodes to species and are sensitive enough to identify the species of a single nematode. PCR amplification is generally carried out using purified nematode DNA or handpicked individual nematodes (Hübschen et al., 2004; Powers et al., 2005). However, using PCR to detect nematodes and other organisms in soil extracts has been difficult due to the presence of inhibitors of DNA polymerase (Hyman et al., 1990; Volossiouk et al., 1995; Miller et al., 1999; Roose-Amsaleg et al., 2001).

The RKN species *Meloidogyne arenaria, M. incognita, M. javanica*, and *M. hapla* are generally considered the most widespread and damaging plant-parasitic nematodes (Sasser and Carter, 1985). *Meloidogyne arenaria, M. incognita,* and *M. javanica* comprise a group of closely related species and are referred to in this paper as the *M. incognita* group or MIG. In many applications, it is sufficient to know whether any of these three species is present. For example, the nematode resistance gene *Mi* in tomato is able to confer resistance to all of these species (Williamson, 1998). Soil samples are frequently submitted to diagnostic laboratories to determine whether a species of MIG RKN is present. The predominant stage of RKN in soil is the J2, and limited morphological characters make it difficult to determine the species of this stage.

Our objectives were to develop and optimize procedures to detect RKN extracted from soil using a robust PCR assay. We have developed a protocol to separate the nematode-containing fraction from soil and to rapidly release DNA that is of sufficient quality that it can be amplified by PCR. We demonstrate that this protocol allows sensitive detection of MIG RKN from a variety of soil conditions and test the technique using field samples from several settings in California.

Materials and Methods

Nematode strains and DNA preparation: DNA was obtained from eggs of previously characterized cultures of the RKN species *M. arenaria* (strain EC2), *M. chitwoodi* (Race 1), *M. hapla* (strain VW1), *M. incognita* (strain VW6), and *M. javanica* (strain VW4) (Williamson et al., 1997). The species identity of each of these cultures was confirmed by female perineal patterns (Eisenback, 1985), isozyme electrophoresis (Esbenshade and Triantaphyllou, 1990), PCR-RFLP, and/or PCR analysis (Powers and Harris, 1993; Williamson et al., 1997). DNA was purified by phenol-chloroform extraction and ethanol precipitation, and its concentration was measured as previously described (Williamson et al., 1997).

Caenorhabditis elegans strain N2 from a petri dish culture (Brenner, 1974), *Heterodera schachtii* from greenhouse culture, *Pratylenchus vulnus* from carrot disk culture (Moody et al., 1973), and *M. javanica* J2 from hydroponic culture (Lambert et al., 1992) were included as negative and positive controls. DNA was released from nematodes using proteinase K (Williamson et al., 1997).

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Primers and DNA amplification conditions: Meloidogyne incognita-group primers, MIGF (5′-ACACAGGGGAAAGTTTGCCA-3′) and MIGR (5′-GAGTAAGGCGAAGCATATCC-3′), were developed from a nematode DNA fragment obtained as a RAPD fragment in an attempt to produce species-specific primers for *M. javanica* by the same strategy used successfully to obtain *M. hapla* and *M. chitwoodi*-specific primers (Williamson et al., 1997; Williamson and Caswell-Chen, unpub. data). The DNA sequence of the MIG fragment does not have similarity to other sequences in Genbank. Preliminary experiments indicated that primers MIGF and MIGR developed from this genomic sequence amplify DNA from *M. incognita* and *M. arenaria* as well as from *M. javanica*. DNA primers (5′-TTGATTACGTCCCTGCCCTTT-3′ and 5′-TTTCACTCGCCGTTACTAAGG-3′) that span the ITS of most nematodes were used as a control for amplification quality of each sample (Vrain et al., 1992). These are referred to as ITS rDNA primers in this paper.

All PCR reactions were carried out with a Peltier Thermal Cycler (PTC-200, MJ Research, Inc., Waltham, MA). Each reaction was in a final volume of 25 µl in a 0.2 ml-tube and contained 2.5 µl 10X PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 0.1% Triton X-100) (Perkin Elmer, Foster City, CA), 0.8 µM each primer, 0.05 mM each dNTP, and 0.5 unit DNA polymerase (AmpliTaq, Perkin Elmer). In PCR reactions using purified DNA as template, 30 ng DNA was used in each reaction. For the MIG primers, the PCR program was: 94°C, 3 min; (94°C, 0.5 min; 58°C, 1 min; 72°C, 1 min) × 35; 72°C, 10 min. For the ITS rDNA primers, the program was: 94°C, 3 min; (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) × 35; 72°C, 8 min. Ten microliters of the amplified product from each reaction was loaded into each well, subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide, and photographed with a Gel Documentation System (UVP Inc., Upland, CA).

Optimization of conditions for nematode extraction from soil for PCR detection: Soil was collected in summer from a tomato field in Yolo County, CA, that was not infested by RKN. To compare PCR amplification products of soil extracts before and after sucrose flotation, two well mixed 100 cm^3 soil samples were mixed with 250 and two with 500 *M. javanica* J2 from hydroponic culture. Two samples with no J2 added were included as controls.

Nematodes were extracted from soil by the Baermann funnel method (Dropkin, 1989). Briefly, a 7-cm-diam. plastic tea strainer with a screen of about 710 µm pore size was suspended in a 10-cm-diam. glass funnel with a 10-cm length of rubber tubing attached to the bottom of the funnel. The tubing was sealed with a pinch clamp. Two layers of Kimwipes tissue paper (Kimberly-Clark Co., Roswell, GA) were placed on the screen, and 50 cm^3 of soil was placed on the Kimwipes. Two funnels were used for each 100 cm^3 sample. Water was added to the funnel to just cover the soil, and any trapped air in the funnel was removed. Nematodes were collected by draining 10 ml of extract from each funnel after two days at 25°C. The two 10-ml extracts of each soil sample were combined and mixed, then 10 ml of this solution was poured into a counting dish (60 × 15 mm tissue culture dish with 2-mm grid, Corning Glass Works, Corning, NY). Root-knot nematodes and free-living nematodes were counted in one quarter of the dish area using a dissecting microscope, and the solution was remixed with the remaining sample. The 20 ml of extract from each sample was concentrated by centrifugation at 900*g* for 5 min at 15°C and transferred to 1.5-ml microfuge tubes.

From a duplicate set of Baermann extracts, 20 ml of each sample was mixed with 20 ml of 70% sucrose in a 50-ml centrifuge tube, producing 40 ml of 35% sucrose solution. Two milliliters of water were carefully layered on top of the solution followed by centrifugation at 80*g* for 5 min. After centrifugation, the top 5 ml was immediately collected with a pipette and transferred to a new 50-ml centrifuge tube containing 40 ml water and mixed. Free-living and root-knot nematodes in one quarter area of the dish containing a 10-ml subsample of this mixture were counted, and then the subsample was poured back. Nematode fractions were concentrated and transferred into a 1.5-ml microfuge tube as described above.

Each nematode fraction in a 1.5-ml microfuge tube was concentrated by spinning at 12,000*g* for 10 min at 4°C. The supernatant was removed with a pipette to leave 32 µl at the bottom of the tube. Next, 4 µl of 10X PCR buffer and 4 µl of proteinase K (600 µg/ml; Promega, Madison, WI) were added to and mixed with the nematode fraction. Samples were stored at −80°C overnight, incubated at 60°C for 1 hr, followed by boiling for 5 min and then used immediately or stored at −80°C. PCR reactions were carried out using the procedure described above for the purified nematode DNA. One microliter of the proteinase K-digested extract was used as DNA template in the final 25 µl PCR reaction.

Optimization of nematode recovery from centrifugal flotation: Four thousand *M. javanica* J2 collected from hydroponic culture were mixed in 40 ml of 35% sucrose solution and overlaid with 2 ml water in a 50-ml centrifuge tube. After centrifugation at indicated centrifugal forces and times, nematodes were counted in the top 5 ml, second 5 ml, third 5 ml, and the remaining 27 ml of solution.

Assay protocol for RKN J2 from field samples: For each sample, soil was mixed well, and a 100 cm^3 aliquot was extracted using a Baermann funnel as described above. RKN J2, other plant-parasitic nematodes and all free-living nematodes as a group were counted in a 2.5-ml aliquot of the 20-ml extracts using a dissecting microscope. The extracts were subjected to sucrose flotation

with centrifugation at 80*g* for 5 min, then collected and concentrated to 20 µl by centrifugation in microfuge tube. 10X PCR buffer and proteinase K were added, and the sample was processed as described above. For each PCR reaction, 2.5 µl of digested nematode solution was used in a final volume of 25 µl.

Blind testing for detection of RKN extracted from soil by PCR: Soil samples were obtained from several locations and soil types in California. Colleagues were asked to provide two soil samples, one that was thought to be infested with RKN and one thought to be free of RKN. These samples were coded upon receipt and information on RKN levels was not available until assays had been completed. Extraction, microscopic examination, and PCR assays were carried out in triplicate following the protocol described above.

Tests of assay sensitivity: Soil was obtained from a walnut orchard in Sutter County, CA. This field was infested with root-lesion nematode *Pratylenchus vulnus*, but not with RKN. Four sets of 100 cm^3 soil were spiked and mixed with 0, 25, 100 and 500 *M. javanica* J2 from hydroponic culture. Nematodes were extracted with Baermann funnels and counted in 5 ml of each 20-ml sample. Twenty-five microliters of the final extracts were prepared for PCR assay following the protocol described above. One microliter of these preparations was used in a final volume of 25 µl for each PCR reaction. The experiment was repeated two times.

Statistical methods and soil analysis: Numbers of nematodes from multiple counts for a sample were used to calculate means and standard errors. Significant differences among samples were conducted with Duncan new multiple range test at $P = 0.05$ by using SuperANOVA program (Abacus Concepts, 1989). Soil samples were analyzed for texture and organic matter by the DANR Analytical Lab, University of California (UC) Cooperative Extension at UC Davis.

Results

Characterization of MIG primers using nematode DNA: Amplification of DNA extracted from previously characterized cultures of the RKN species *M. arenaria*, *M. incognita*, *M. javanica*, *M. hapla*, and *M. chitwoodi* was carried out with the MIG primers. A single band of 500 bp was produced for *M. arenaria*, *M. incognita*, and *M. javanica*, but no bands were amplified from *M. chitwoodi* or *M. hapla* (Fig. 1A). When DNA from the same preparations was subjected to PCR amplification using the ITS rDNA primers, a single band of 760 bp was amplified from each of the five species (Fig. 1B). The same results were obtained from these assays using single J2 of the five species of RKN (data not shown). With MIG primers, DNA was not amplified in controls without extract or with *C. elegans, Heterodera schachtii*, or *Pratylenchus vulnus* DNA (Fig. 1A). PCR amplification with ITS primers produced 1,300, 1,250, and 925 bp bands from *C. elegans*, *H. schachtii*, and *P. vulnus* DNA, respectively (Fig. 1B).

Optimization of conditions for nematode extraction from soil for PCR detection: Microscopic examination of Baermann funnel extracts of a soil sample from a tomato field revealed the presence of a diverse community of free-living nematodes including bacterial feeders, fungal feeders and algae feeders, as well as other microscopic invertebrates and microbes. RKN J2 were not observed in this sample. Aliquots of the soil sample were spiked with J2 as described in Materials and Methods. PCR assays with either MIG or ITS primers on proteinase K-digested fractions taken directly after Baermann funnel extraction did not produce detectable bands when the soil sample contained the equivalent of 3 *M. javanica* J2/PCR reaction (this number was derived by counting RKN in the Baermann funnel extract). A very faint band of the expected size was ob-

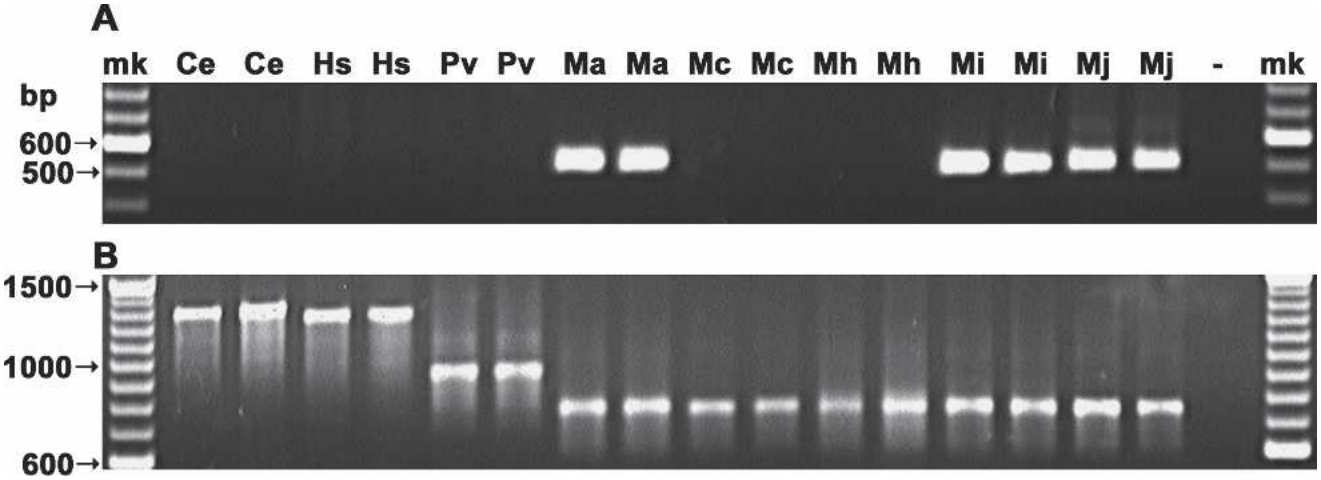

Fig. 1. PCR amplification of nematode DNA with MIG primers. MIG (A) or ITS rDNA (B) primers were used to amplify 30 ng DNA prepared from eggs of *Meloidogyne arenaria* (Ma), *M. chitwoodi* (Mc), *M. hapla* (Mh), *M. incognita* (Mi), and *M. javanica* (Mj), and from nematodes of *Caenorhabditis elegans* (Ce), *Heterodera schachtii* (Hs), and *Pratylenchus vulnus* (Pv). Lanes marked "mk" contain DNA molecular size markers, and lane "–" is a negative control, a PCR reaction without DNA template. Duplicate PCR reactions are shown for each species.

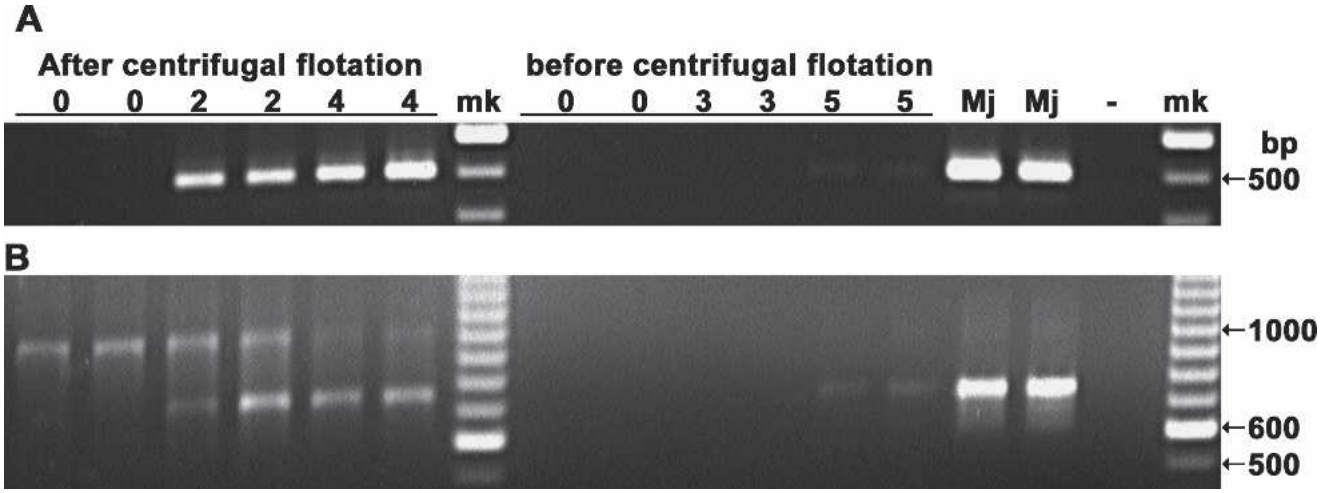

FIG. 2. Comparison of PCR amplification of Baermann funnel extracts before and after centrifugal flotation. Amplification was carried out using MIG primers (A) or ITS rDNA primers (B) with DNA template from Baermann funnel extracts before or after centrifugal flotation. The numbers above the lines correspond to the number of RKN J2 in the PCR reaction as deduced from microscopic counting before or after centrifugal flotation. Lanes marked Mj contain amplification products obtained using DNA template from *Meloidogyne javanica* J2. Lane "mk" contains DNA molecular size marker, and lane "–" contains the PCR reaction without DNA template.

served from the soil sample containing the equivalent of 5 J2/PCR reaction (Fig. 2). However, when the nematode fraction was further purified by sucrose centrifugal flotation, PCR with MIG primers amplified a 500 bp band in samples that contained extracts corresponding to 2 and 4 J2/reaction (Fig. 2A). With the same primers, no DNA was amplified in controls without extract or in the soil sample without added *M. javanica* J2 (Fig. 2A). PCR amplification with ITS rDNA primers amplified a 1,050 bp band in soil extracts, presumably due to DNA from the dominant species of free-living nematodes or other invertebrates in the soil (Fig. 2B). Spiked extracts also showed amplification of a 760 bp band, the expected size for the *M. javanica* ITS region. Together, these results suggested that the Baermann funnel extract contained inhibitors of DNA amplification and that sucrose centrifugal flotation reduced the inhibition.

Optimization of nematode recovery by centrifugal flotation: Microscopic examination showed that only 10 to 20% of the nematodes were recovered in the top 5 ml of solution after flotation by centrifugation at 900*g* for 5 min in preliminary experiments. To optimize recovery, we tested a range of centrifugal forces and times. The highest recovery in the top 5 ml was 82% after centrifugation at 80*g* for 5 min ($P = 0.05$, Table 1). At this centrifugal force and time, the recovery in the top 5 ml was not significantly different at 30%, 35%, or 40% sucrose ($P = 0.14$). Therefore, centrifugation in our remaining experiments was carried out at 80*g* for 5 min in 35% sucrose.

Blind testing for detection of RKN in soil extracts by PCR: To test the utility of our assay for detecting RKN in soil, we obtained 12 soil samples from several sources in California (Table 2). The soil textures of the samples ranged from sandy loam to loamy sand, and the percentage of organic matter ranged from 0.35 to 1.79.

Because they were from a methyl bromide-treated area, samples 1 and 2 were expected to have no RKN, or a very low population density of RKN. In fact, no RKN were found by microscopic examination after Baermann funnel extraction. In contrast, because they were from control plots in the same trial, samples 3 and 4 were expected to have a high population density of RKN, and approximately 500 RKN J2/100 cm^3 were found in each of these two samples by microscopic analysis. More than 400 free-living nematodes were found per 100 cm^3 in each of the four samples. PCR with MIG primers amplified a band of the expected size for *M. javanica* in samples 3 and 4 (Fig. 3A) while with ITS primers DNA was amplified in all four samples (Fig. 3B, Table 2). Results were consistent for each of the three times that the extraction, microscopic examina-

TABLE 1. Recovery of RKN J2 after centrifugal flotation.

Centrifugal force (g)	Time (min)	Nematodes (%) in indicated fraction[a]			
		Top 5 ml	2nd 5 ml	3rd 5 ml	Final 27 ml
2,000	10	27 c	13 ab	11 abc	49 cd
1,400	10	23 bc	25 b	6 ab	47 cd
900	10	8 a	8 a	10 abc	74 e
225	10	41 d	16 ab	9 abc	34 bc
2,000	5	14 ab	17 ab	15 c	54 cde
1,400	5	15 abc	18 ab	14 bc	53 cde
900	5	13 ab	11 ab	14 c	62 de
225	5	41 d	12 ab	9 abc	38 bc
80	5	82 e	8 a	5 a	5 a
0	5	17 abc	43 c	17 c	23 ab

[a] Numbers represent the means of three replicates. The same letters within the same column indicate that the values are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

TABLE 2. Detection of RKN in soil samples by microscopic and PCR assays.

	Sample		Microscopic counts nems/100 cm^3 soil[a]		PCR assay		Soil properties	
#	Source	Description	RKN J2[b]	FLN	RKN[c]	Nem[d]	Texture[e]	% organic
1	SCREC[f]	Few or no RKN	0 ± 0	1,000	–	+	SL	0.57
2	SCREC	Few or no RKN	0 ± 0	720	–	+	SL	0.66
3	SCREC	Many RKN	530 ± 25	410	+	+	SL	0.75
4	SCREC	Many RKN	420 ± 38	480	+	+	SL	0.69
5	Orchard	RKN infested	77 ± 6	180	+	+	SL	0.64
6	Orchard	No RKN	0 ± 0	152	–	+	SL	0.35
7	Vineyard	RKN infested	250[g] ± 19	38	+	+	SL	0.71
8	Vineyard	No RKN	0 ± 0	190	–	+	SL	0.59
9	Tom. field	No RKN	0 ± 0	430	+	+	SL	0.63
10	Tom. field	RKN infested	144 ± 22	180	+	+	SL	0.71
11	Yard soil	No RKN	30 ± 5	2,000	+	+	LS	1.34
12	Yard soil	RKN infested	302 ± 29	3,400	+	+	LS	1.79
13	Orchard[h]	No RKN	0 ± 0	240	–	+	LS	0.5
14	Orchard[h]	Spiked 25 RKN	8 ± 0	428	+	+	LS	0.5
15	Orchard[h]	Spiked 100 RKN	36 ± 0.5	316	+	+	LS	0.5
16	Orchard[h]	Spiked 500 RKN	196 ± 1.5	228	+	+	LS	0.5

[a] Microscopic counts were conducted before centrifugal flotation, FLN = Free-living nematodes.
[b] Means followed by standard errors are calculated from three counts.
[c] Presence (+) or absence (–) of RKN detected by PCR using MIG primers.
[d] Presence or absence of nematodes or other organisms detected by PCR using ITS rDNA primers.
[e] SL = sandy loam; LS = loamy sand.
[f] The University of California South Coast Research and Extension Center, Irvine, CA.
[g] This sample was found to contain 1,958 citrus nematodes/100 cm^3. The first microscopic count failed to identify RKN due to the "masking" by the large number of citrus nematodes. After PCR result was obtained, the sample was recounted and RKN were identified as present.
[h] 100 cm^3 soil spiked with *Meloidogyne javanica* J2 was determined to contain 32, 20, 28, and 52 root-lesion nematodes, *Pratylenchus vulnus,* by microscopy after Baermann funnel extraction in samples 13, 14, 15, and 16, respectively.

tion, and PCR assays were conducted on these samples.

Microscopic analysis of samples 5 and 6 from an orchard detected 77 and 0 RKN J2/100 cm^3 soil, respectively. PCR amplification with ITS primers amplified DNA in both samples (Fig. 3B), but MIG primers detected RKN in sample 5 only (Fig. 3A), consistent with the result from the microscopic assay. All three replicated extractions carried out on these samples produced the same PCR band profile.

Sample 7 from a vineyard thought to be infested with RKN was found to contain 1,958 juveniles of the citrus nematode, *Tylenchulus semipenetrans*, per 100 cm^3 soil by microscopic analysis, but RKN were not initially detected in either this sample or in sample 8 from a vineyard not thought to be infested with RKN. PCR amplification with MIG primers detected RKN in sample 7, but not in sample 8 (Fig. 3A). To determine whether MIG primers could amplify DNA from *T. semipenetrans*, we tested nematodes extracted from our greenhouse culture of citrus nematode and hand-picked citrus

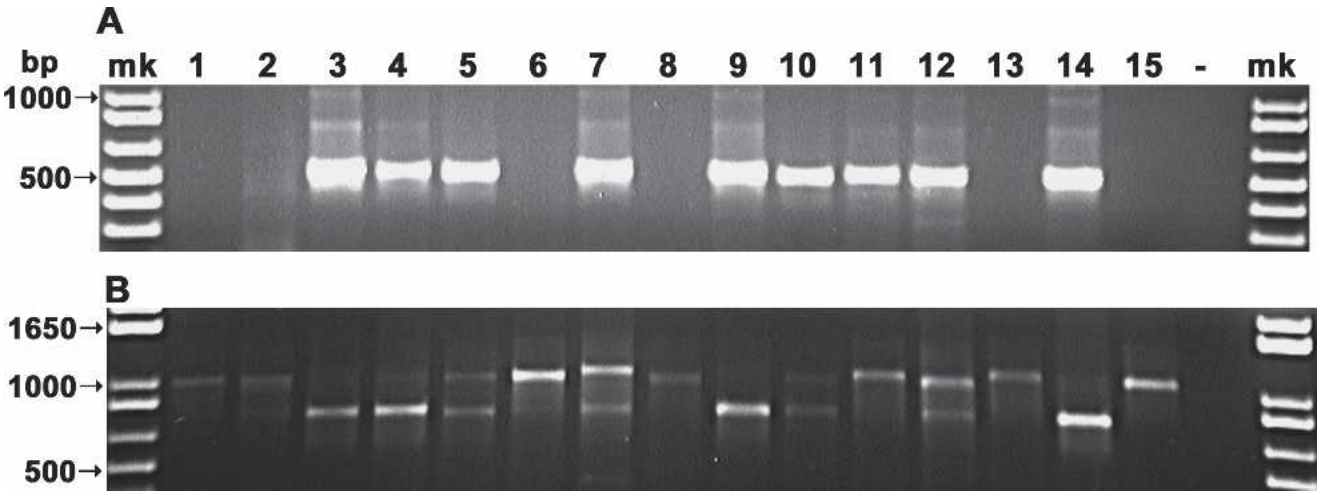

FIG. 3. PCR assays to detect RKN in field samples. Nematodes were extracted from soil as described in Materials and Methods and amplified using MIG primers (A) or ITS rDNA primers (B). Lane "mk" contains DNA size markers. Lanes 1–12 correspond to samples in Table 2. Lane 13 contains soil extract from a greenhouse culture of citrus nematode, *Tylenchulus semipenetrans*. Lane 14 contains extracts from hand-picked *Meloidogyne javanica* J2, and Lane 15 citrus nematode J2. Lane "–" is the PCR reaction without a DNA template.

nematodes. In neither case was a band amplified with the MIG primers (Fig. 3A), though the ITS primers did amplify DNA from these samples (Fig. 3B). Microscopic re-examination of sample 7 detected 250 RKN J2/100 cm^3 soil, about 1:8 ratio to the citrus nematodes. The similar appearance of juveniles of RKN and citrus nematodes had apparently resulted in our failure to identify them in the first microscopic study. The PCR banding patterns for these samples were consistent in all three replicate assays with both sets of primers.

Samples 9 and 11 were described by the provider as having no RKN infestation. Initial microscopic examination did not identify RKN in these samples, but RKN were abundant in samples 10 and 12 (Table 2). However, our PCR assay with MIG primers amplified a band in all four samples (Fig. 3A). All three replications of PCR amplification gave the same pattern. Recounting microscopic samples identified about 30 RKN J2 among 2,000 free-living nematodes in a 100 cm^3 soil extract in sample 11, but did not conclusively identify RKN in sample 9. However, a few plant-parasitic nematodes with poor morphology that may have been RKN J2 were seen in this sample.

Assay sensitivity: To address sensitivity of our PCR assay, we obtained soil from a walnut orchard previously determined to be infested with the root-lesion nematode *P. vulnus*, but not RKN (Table 2, samples 13–16). After Baermann funnel extraction of samples spiked with 0, 25, 100 and 500 *M. javanica* J2/100 cm^3 soil, microscopic counting detected 0, 8, 36 and 196 J2, respectively (Table 2). MIG primers produced bands only with the spiked samples (Fig. 4A) while PCR with ITS primers amplified DNA in all four samples (Fig. 4B). Similar results were obtained in two repeated experiments.

Diagnoses of RKN from extension samples by PCR with MIG primers: From 2001 to 2005, we diagnosed 57 soil samples from extension services for RKN in California. Of 28 soil samples from five different crops in six different counties, 20 were diagnosed as containing MIG RKN by both PCR assay with MIG primers and microscopic examination following the protocol described in this paper. The remaining eight were found not to have RKN either by microscopic examination or by PCR assays with MIG primers, but DNA from the extracts was amplified by PCR with ITS primers. Twenty-nine additional samples from nine different crops in eight different counties were diagnosed as containing MIG RKN by PCR assay on hand-picked single RKN J2. The RKN J2 in the 49 positive samples were identified as either *M. arenaria, M. incognita,* or *M. javanica* by PCR assay with species-specific primers (Zijlstra et al., 2000).

Discussion

In designing our assay, we considered disrupting the nematodes directly in the soil sample. However, DNA extraction directly from soil is inefficient and requires expensive purification procedures before the PCR assay (Volossiouk et al., 1995; Miller et al., 1999; Miller, 2001; Roose-Amsaleg et al., 2001). Furthermore, currently available soil DNA extraction kits generally use less than 10 grams of soil for each extraction (Atkins et al., 2005). Such small samples are inappropriate for nematode assays because nematodes are unevenly distributed in a field, and the cost and time for processing large numbers of soil samples is prohibitive. Our goal here was to develop a simple protocol to identify a few (<20) RKN in 100 cm^3 of soil.

Because of the sensitivity of PCR assays, we purposely

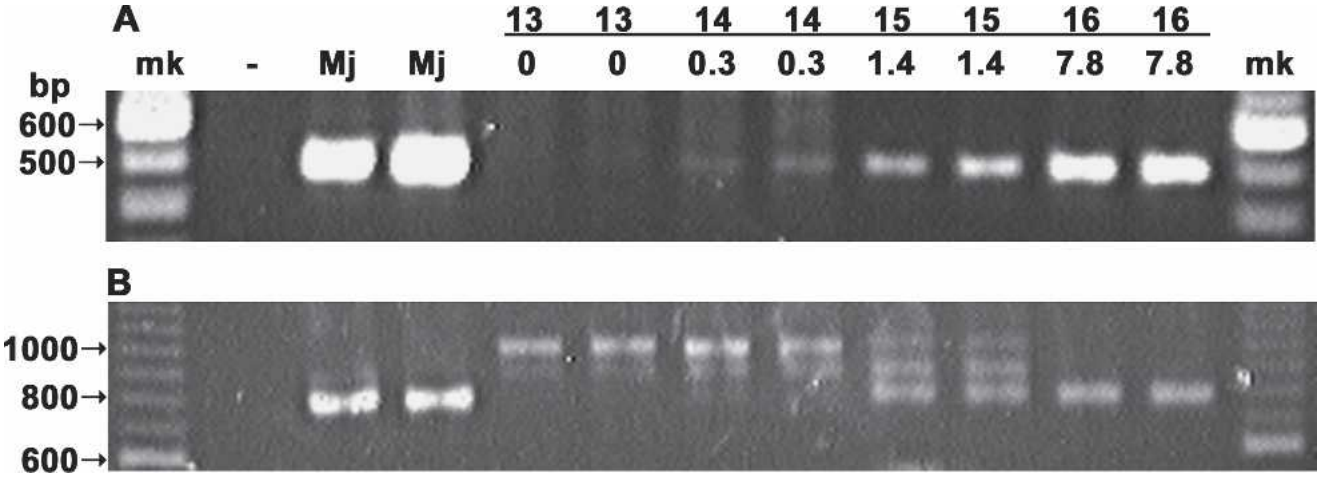

FIG. 4. Sensitivity of PCR assays of nematode extracts. Soil from an orchard was spiked with 0, 25, 100, and 500 RKN J2/100 cm^3 in samples 13, 14, 15, and 16, respectively. PCR amplification of DNA extracts was carried out with MIG primers (A) or ITS primers (B). The numbers over the line indicate sample number and those under the line correspond to the number of RKN J2 in the PCR reaction as deduced from microscopic counting after Baermann funnel extraction and before centrifugal flotation. Lanes marked Mj contain amplification products obtained using DNA template from one *Meloidogyne javanica* J2 digested with proteinase K. Lane "mk" contains DNA molecular size marker, and lane "–" contains the PCR reaction without DNA template.

avoided using sieving-funnel methods (Ayoub, 1980) as cross-contamination is more likely when processing multiple samples using common sieves even when these are cleaned between uses. Nematode fractions extracted by Baermann funnel followed by a centrifugal flotation step alleviated inhibition of PCR amplification and allowed sensitive detection of nematodes. It should be noted that only nematodes that are live and motile are detected in our assay due to the requirement that they migrate through Baermann funnels.

The sensitivity of a PCR assay on nematodes from soil extracts depends not only on how free the extract is from PCR inhibitors, but also on recovery of nematodes during extraction. The efficiency of extraction of nematodes from soil can be highly variable and all methods used today have limitations. Based on our assays in which we spiked a soil sample with J2, recovery of RKN from Baermann funnel extraction is about 35%. Activity, mobility, and age of RKN J2 as well as the thickness of soil layer in the Baermann funnel may affect the recovery. We found that more than 80% of the nematodes were recovered in the top 5 ml of solution after centrifugation at 80*g* for 5 min in a 35% sucrose solution.

For all soil samples examined in this work, the nematode-containing fraction obtained after centrifugal flotation included a wide range of nematode species and other microscopic invertebrates. Since ITS rDNA primers are able to amplify DNA of a wide range of organisms (Powers et al., 1997), these primers provided a convenient control for the ability of DNA in the extracts to be amplified by PCR. The size of the ITS region is highly variable among nematode genera and even among some species in a genus (Powers et al., 1997). With primers spanning the ITS region, *C. elegans*, *H. schachtii*, *P. vulnus*, *T. semipenetrans*, and RKN produced PCR products with different sizes. The sizes of the ITS bands in individual soil extracts probably reflect those of the predominant nematode species present. All samples that we studied were amplified with these primers after centrifugal flotation.

Since the MIG RKN species are of primary concern in many applications in California, we chose to develop our assay to detect these species. Our experiments indicate that the MIG primers are robust for DNA amplification of *M. incognita*, *M. javanica*, and *M. arenaria*. These same primers did not amplify DNA of *M. hapla, M. chitwoodi*, *T. semipenetrans*, *C. elegans, H. schachtii, P. vulnus*, and *P. penetrans*. In this paper, we tested the assay with solicited samples (Table 2) as well as extension samples collected from multiple crops in 12 different counties in California and detected RKN in all cases in which they were detected by microscopic examination. All samples diagnosed microscopically as containing MIG RKN were amplified, indicating broad utility of these primers for use in California extension samples.

Among 15 samples tested that lacked RKN by microscopic examination, only one sample (sample 9, Table 2) was amplified with MIG primers, indicating that primers were highly specific. We feel that contamination is not a likely explanation for the amplification in this case because our control reactions without DNA template or with a soil extract from the greenhouse culture of citrus nematode did not amplify bands with MIG primers. It is possible that the MIG primers amplified a non-target organism in this sample. However, we think a more likely explanation is the limitation of microscopic counting. Optically, it is difficult to accurately identify nematodes around the outer edges of our counting dish, so we generally count only nematodes in a grid marked off in the center of a dish. Generally, we examined one-eighth of the sample. In addition, the presence of large numbers of free-living nematodes, other parasitic nematodes and small invertebrates can make accurate counting difficult. This is exemplified by sample 7, in which citrus nematode was present, and sample 11, which had high levels of free-living nematodes. In these samples, the initial failure to detect RKN was due to "masking" by the high levels of other nematodes present.

In a 100 cm^3-soil sample spiked with 25 *M. javanica* J2, eight J2 were counted after Baermann extraction. Since 1 out of 25 µl of this extract was used in the PCR reaction, the reaction contained the equivalent of 0.3 J2. In practice, even with losses due to Baermann funnel extraction (~35% recovery), centrifugal flotation (~80% recovery), or residual inhibition of PCR, we estimate that fewer than 25 RKN J2/100 cm^3 of soil would be detected in most cases. This is at least as sensitive as our detection by microscopic counting.

The protocol we developed here is sensitive, efficient, and amenable to processing large numbers of field samples. Detection of low numbers of RKN J2 in soil by microscopic examination is difficult and time consuming, especially when soil samples contain large numbers of non-target nematodes. Because PCR analysis is not affected by the presence of other nematodes, our method should permit wide field coverage by pooling Baermann extracts, or by analyzing large numbers of 100 cm^3 soil samples. It should also be possible in the future to use modifications of this method of soil extraction to estimate RKN numbers by using a dilution series or, with appropriate primers, by quantitative, real-time PCR (Bates et al., 2002; Cao et al., 2005; Madani et al., 2005; Subbotin et al., 2005).

The procedure presented here can be modified to detect other plant-parasitic groups. Species-specific primers have already been developed for a number of other RKN species (Williamson et al., 1997; Zijlstra et al., 2000) and for species of *Pratylenchus* (Al-Banna et al., 2004). We now routinely use this protocol with MIG primers to identify RKN in samples submitted by farm advisors and by other diagnostic laboratories to the Cooperative Extension laboratory at UC Davis. Although

we feel the assay has widespread validity, prior to adoption, each laboratory should validate the technique on representative samples collected from the local area they serve. The strategy described here could also be used to track specific species and groups of nematodes for studies of population biology, dynamics, and spatial structure.

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