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A PCR Assay to Identify and Distinguish Single Juveniles of *Meloidogyne hapla* and *M. chitwoodi*

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Abstract: Random amplified polymorphic DNA (RAPD) bands that distinguish *Meloidogyne hapla* and *M. chitwoodi* from each other, and from other root-knot nematode species, were identified using a series of random octamer primers. The species-specific amplified DNA fragments were cloned and sequenced, and then the sequences were used to design 20-mer primer pairs that specifically amplified a DNA fragment from each species. Using the primer pairs, successful amplifications from single juveniles were readily attained. A mixture of four primers in a single PCR reaction mixture was shown to identify single juveniles of *M. hapla* and *M. chitwoodi*. To confirm specificity, the primers were used to amplify DNA from several isolates of *M. hapla* that originated from different crops and locations in North America and also from isolates of *M. chitwoodi* that differed in host range. In characterizing the *M. hapla* isolates, it was noted that there was a mitochondrial DNA polymorphism among isolates for cleavage by the restriction endonuclease *DraI*.

Key words: diagnostic, DNA, *Meloidogyne chitwoodi, Meloidogyne hapla,* PCR, root-knot nematode, SCAR, STS.

Common techniques for species identification of root-knot nematodes (RKN; *Meloidogyne* spp.) include morphological characters, host range tests, and isozyme electrophoresis (Eisenback and Triantaphyllou, 1991). Morphological criteria have been problematic due to variability and the need for specialized personnel to perform the species determinations. Isozymes are highly reliable, allow species identification of single females, and are useful for routine species identification of RKN (Cenis et al., 1992; Esbenshade and Triantaphyllou, 1990); however, isozyme electrophoresis does not work well with single second-stage juveniles (J2) so samples subject to isozyme analysis must contain young, healthy females for successful identification. In practice, soil samples often contain only juveniles or eggs and thus require greenhouse culture to obtain females for isozyme analysis.

DNA variation is the basis of recent techniques for nematode identification (Baum et al., 1994; Castagnone-Sereno et al., 1993; Caswell-Chen et al., 1992; Cenis, 1993; Curran et al., 1986; Powers and Harris, 1993; Zijlstra et al., 1995). A PCR method for species identification of single *Meloidogyne* J2 was developed by Powers and Harris (1993). Identification is based on amplification of the intergenic spacer region between the cytochrome oxidase subunit II gene and the 16S rRNA gene in the mitochondrial genome of the nematode. Using a similar approach, Zijlstra et al. (1995) were able to differentiate *M. hapla* and *M. chitwoodi* by amplification of ribosomal DNA internal transcribed spacer. Because individual J2 can be assessed, the PCR techniques are useful for studies of mixed populations. Disadvantages of the techniques are the need for restriction enzyme digestion of the PCR products prior to electrophoresis.

Random amplified DNA (RAPD) patterns have been used to distinguish populations of the species *Heterodera schachtii* (Caswell-Chen et al., 1992) and species of RKN (Cenis, 1993). Cenis (1993) amplified DNA from single juveniles of RKN with 10-mer primers and saw some variability in minor bands though the major bands were reproducible. Our results suggest that RAPDs from single juveniles are too inconsistent for routine identification of single J2. The inconsistency in banding pattern likely is due to the variable amount of DNA extracted from single J2. Williams et al. (1993) have noted that too little DNA gives non-reproducible patterns under RAPD conditions.

RAPD markers can be converted into robust markers, SCAR (sequence character-

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ized amplified regions), or STS (sequence tagged sites) (Paran and Michelmore, 1993; Williamson et al., 1994) by cloning and sequencing selected RAPD fragments and then designing specific, long oligonucleotide primers that anneal at high temperatures and allow amplification of the target fragment. In this way, a single band representing one locus in the target genome can be amplified (Paran and Michelmore, 1993). With the higher annealing temperature used for the amplification, DNA template concentration is less critical, nonspecific amplification is minimized, and reproducible amplification of tiny and variable amounts of DNA is possible.

Here we present species-specific PCR primers for identification of *M. hapla* and *M. chitwoodi*. Both species are important potato pathogens in the west-coast states of the United States, and there are differences in their host range on rotation crops (Pinkerton et al., 1987). Roots, and hence the adult females used for isozyme analyses, are typically not present in soil samples received for nematode species identification.

Materials and Methods

Nematode populations: M. hapla (strain VW1), *M. javanica* (strain VW4), *M. incognita* (strain EC1), and *M. arenaria* (strain EC2) were derived from greenhouse cultures maintained on UC82 or VFNT cherry tomato. Six additional *M. hapla* isolates from various locales and host origins were investigated (Table 1). Fourteen *M. chitwoodi* isolates were also investigated (Table 2).

Isozyme electrophoresis: The species identity of all the RKN isolates used in these studies were confirmed by electrophoresis of extracts of individual females using a Phastsystem electrophoresis apparatus (Pharmacia, Uppsala, Sweden). Gels were stained for malate dehydrogenase and esterase isozyme phenotypes to facilitate identification (Esbenshade and Triantaphyllou, 1991).

DNA extraction: Nematode eggs were obtained from greenhouse cultures and cleaned by sugar flotation (Jenkins, 1964). Eggs were frozen in liquid nitrogen and then ground to a powder. The powder was incubated in extraction buffer (100 mM Tris HCl, pH8, 200 mM NaCl, 1% SDS, 50 µg/ml proteinase K) for 2 hours at 50 °C. The sample was further purified by phenol-chloroform extraction and ethanol precipitation. DNA concentration was measured using a fluorometer (Hoefer TKO, San Francisco).

PCR of nematodes: All amplifications were carried out with a Twin Block thermocycler (Ericomp, San Diego). DNA amplification using random decamer primers (Operon Technologies, Alameda, CA) was carried out under previously described conditions (Caswell-Chen et al., 1992). Amplification with mitochondrial DNA primers was as described in Powers and Harris (1993) except that individual J2 were picked with an insect minuten pin and mechanically disrupted by using the pin to press the J2 against the wall of the PCR tube.

For species-specific PCR amplification, J2 were picked and transferred to individual 0.5-ml microfuge tubes containing 2.5 µl PCR buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl, 15 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin) with 90 µg/ml proteinase K, and crushed using a minuten pin. Samples were then frozen for 10 minutes at –70 °C. After freezing, samples were used immediately or stored at –70 °C for later use. Before amplification, samples were overlaid with mineral oil and incubated for 1 hour at 60 °C and 15 minutes at 95 °C. Amplification was carried out in 25 µl PCR Buffer with 0.1 mM each dNTP, 0.1 µM each primer, and 1.25 units *Taq* polymerase (Promega, Madison, WI) under the following program: 2 minutes at 94 °C followed by 35 cycles of 30 seconds at 94 °C, 1 minute at 58 °C, and 1 minute at 72 °C followed by 5 minutes at 72 °C.

Generation of species-specific primers: RAPD bands were eluted from the gel using an Ultrafree-MC Filter Unit (Millipore, Bedford, MA) and then cloned using a TA cloning kit (Invitrogen Corp., San Diego, CA). Plasmid DNA from insert-containing clones was extracted using QIA prep-spin Plasmid Kit (Qiagen, Chatsworth, CA). The DNA se-

quence of clones was determined by automated sequencing carried out by the Iowa State University DNA Sequencing and Synthesis Facility, Ames, Iowa.

The DNA sequence was used to design forward and reverse 20-mer primers. The primers did not include the original RAPD primer sequence, and were chosen to have at least 50% GC content and to lack obvious secondary structure. Multiplex reaction conditions were the same as for single primer pairs.

To assess the diagnostic utility of the *M. hapla* and *M. chitwoodi* specific primers, several J2 of each species isolate were subjected to PCR amplification using our RKN-specific primers. Prior to the assessment, individual J2 were subjected to PCR amplification using mitochondrial DNA primers (Powers and Harris, 1993) for comparative purposes.

Results

RAPD PCR of RKN species: The species identities of greenhouse cultures of *M. hapla, M. javanica, M. arenaria,* and *M. incognita* were confirmed by analysis of isozyme patterns for malate dehydrogenase and esterase in individual females. DNA was extracted from eggs of each species and screened for polymorphisms using RAPD PCR. Seven 10-mer primers (OPA-01, -03; OPK-01, -12, -15, -20; OPV-18) were tested for their ability to prime amplification of DNA from the four RKN species, *M. arenaria, M. hapla, M. javanica,* and *M. incognita.* All primers produced species-specific patterns (see Fig. 1A for examples). *M. arenaria, M. javanica,* and *M. incognita* RAPD patterns often shared one or more bands of the same size. In most cases, *M. hapla* did

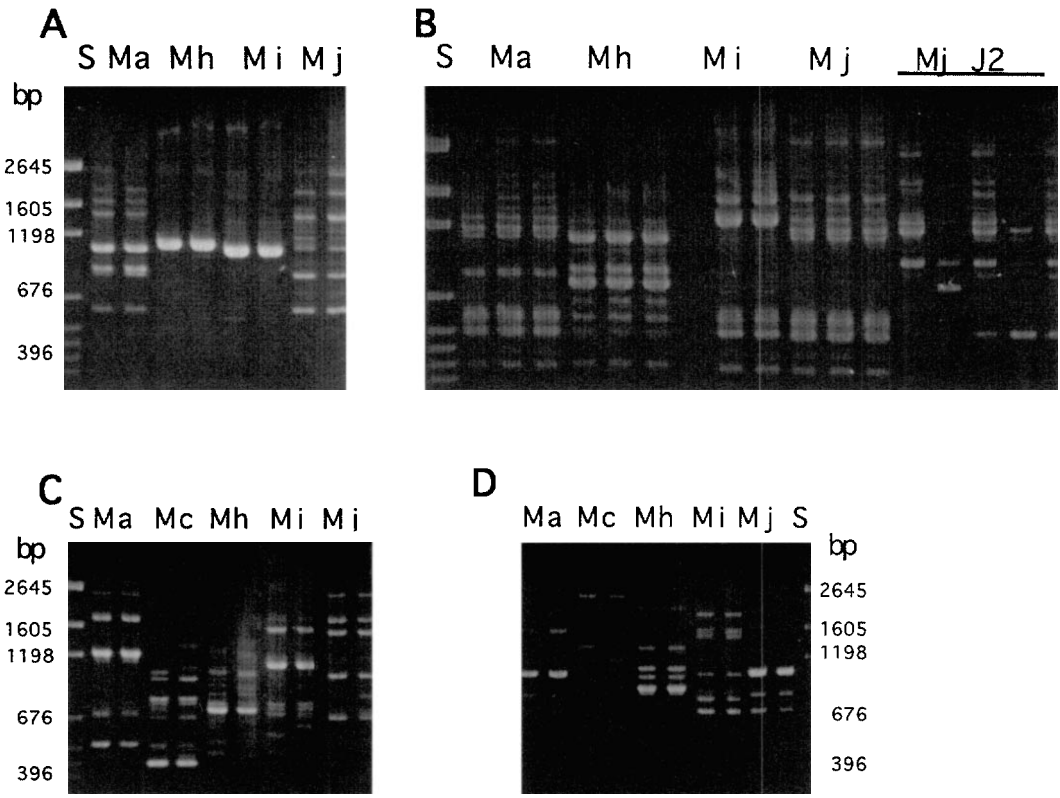

FIG. 1. RAPD patterns of root-knot nematode DNA. Primers OPA-01 (A), OPA-03 (B), OPG-02 (C), or OPG-08 (D) were used to amplify DNA prepared from eggs of *M. arenaria* (Ma), *M. chitwoodi* (Mc), *M. hapla* (Mh), *M. javanica* (Mj), and *M. incognita* (Mi) and from single *M. javanica* J2 (Mj J2). Each sample was run in duplicate (A, C, D) or triplicate (B, except Mi in duplicate). Lanes marked S contain size markers.

not share bands with the other four species. An additional seven primers (OPG-02, -03, -04, -05, -06, -08; OPK-15) were used to screen *M. chitwoodi* DNA and the four other species. With each primer *M. chitwoodi* produced a pattern distinctive from the other species (Fig. 1C,D).

Amplifications with the primers that yielded species-specific patterns were repeated to test pattern reproducibility. The patterns were reproducible, though there was some variability in minor bands from different amplification reactions. DNA extracts from individual *M. javanica* J2 hatched from a single egg mass gave variable amplification patterns (Fig. 1B). Because this species is parthenogenetic, the J2 were expected to be genetically identical. Failure to obtain consistent patterns from single J2 prompted us to obtain robust primers as described below.

Conversion of RAPD markers into diagnostic tools: A large-scale amplification was carried out with *M. hapla* (strain VC1R) DNA and primer OPA-01 to obtain a sufficient quantity of a 1.1-kb band to allow cloning. This 1.1-kb restriction fragment was selected because Cenis (1993) obtained a fragment of the same size from DNA of *M. hapla* race A and race B isolates from Virginia and Chile, respectively. Our observation of the same size fragment led us to consider that the fragment might be conserved in the species. The 1.1-kb band was isolated from the gel and cloned. DNA sequences for the ends of the *M. hapla* fragment were determined and used to design the 20-mer primers we designate MH0F (5′-CAGGCCCTTCCAGCTAAAGA-3′) and MH1R (5′-CTTCGTTGGGGAACTGAAGA-3′). Using primers MH0F and MH1R, a band of 960 bp was amplified from DNA extracted from *M. hapla* DNA but not from *M. incognita, M. javanica,* or *M. arenaria* DNA (Fig. 2A). These primers allowed amplification of DNA from single *M. hapla* J2 (Fig. 2A).

Similarly, an 850-bp RAPD band generated using primer OPG-08 with *M. chitwoodi* DNA was cloned. Two 20-mer primers we designate MC3F (5′-CCAATGATAGAGATAGGCAC-3′) and MC1R (5′-CTGGCTTCCTCTTGTCCAAA-3′) were constructed, and they amplified a 400-bp DNA fragment from *M. chitwoodi,* but not the other four

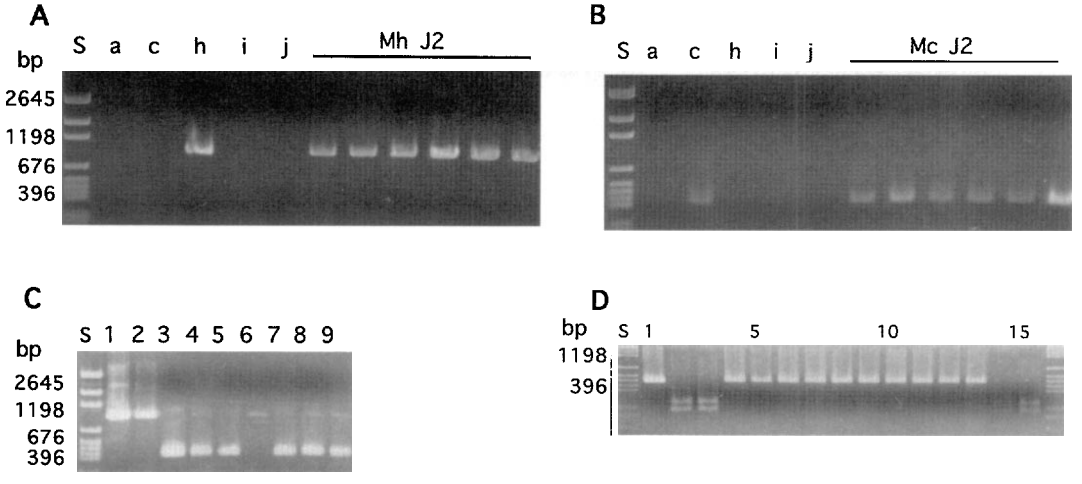

FIG. 2. PCR of bulk DNA and individual J2 using species-specific primers. DNA preparations from *M. arenaria* (a), *M. chitwoodi* (c), *M. hapla* (h), *M. incognita* (i), and *M. javanica* (j) were amplified using primers MH0F and MH1R (A) or primers MC3F and MC1R (B). Extracts of single J2 of *M. hapla* (A) or *M. chitwoodi* (B) were amplified with MH0F and MH1R or MC3F and MC1R, respectively. (C) Bulk DNA (lanes 1 and 3) or single J2 (lane 2, lanes 5–9) of *M. hapla* (lanes 1–2) or *M. chitwoodi* (lanes 3–9) amplified with a mixture of primers MC3F, MC1R, MH0F, and MH1R (lane 5 = WAMC24, lane 6–7 = WAMC25, lane 8 = ORMC8, lane 9 = ORMC12). (D) single J2 of *M. hapla* were amplified using mitochondrial DNA primers then cleaved with *DraI* before electrophoresis. Lane 1 contains DNA from California isolate of *M. hapla* (VW1), lanes 2 and 3 are from sample 2 (Table 1), lanes 4–7 are from sample 4, lanes 8–13 are from sample 5, and lanes 14–15 are from sample 3.

species (Fig. 2B). DNA from individual *M. chitwoodi* J2 was amplified with these primers (Fig. 2B).

Development and validation of a multiplex assay: The *M. chitwoodi* and *M. hapla* primers were designed to yield species-specific fragments of different sizes upon amplification. This allowed development of an assay to distinguish the species using a single PCR reaction. Oligomers MH0F, MH1R, MC3F, and MC1R were mixed in a single reaction and used to prime amplification of single *M. chitwoodi* and *M. hapla* J2. Typically, the anticipated PCR product characteristic for each species was obtained (Fig. 2C, Table 1, Table 2). In some cases amplification from single *M. chitwoodi* J2 did not yield the primary strong band expected (Fig. 2C, lane 6), indicating the need to assess multiple J2 in practical application of this technique. The weak band of size similar to the *M. hapla* band that appeared in some *M. chitwoodi* samples (Fig. 2C) was easily distinguished from the authentic *M. hapla* band by its relatively low abundance.

The PCR amplification of individual *M. hapla* J2 using primers specific for mitochondrial DNA yielded the expected 0.52-kb band (Powers and Harris, 1993) for all isolates. However, when these DNA fragments were cleaved with the restriction enzyme *DraI,* two different banding patterns were obtained (Fig. 2D). Amplified DNA of some

TABLE 2. *M. chitwoodi* identification.

Isolate[a]	Differential host plants[b] alfalfa	carrot	*S. bulbo*	Mt PCR on single J2[c]	Multiplex primers on single J2[d]
WAMC1	–	+	–	C	C
WAMC17	–	+	–	C	C
WAMC18	–	+	–	C	C
WAMC20	–	+	–	C	C
WAMC21	–	+	–	C	C
WAMC22	–	+	–	C	C
WAMC23	–	+	–	C	C
WAMC25	–	+	–	C	C
ORMC12	–	+	–	C	C
WAMC19	+	–	–	C	C
WAMC24	+	–	–	C	C
WAMC26	+	–	–	C	C
ORMC8	+	–	–	C	C
CAMC2	+	–	+	C	C

[a] Isolates were collected from Washington (WA), Oregon (OR), and California (CA) and numbered consecutively in IAREC nematode collection. They were raised on Stephens wheat for 55 days before increasing on Columbian tomato (G. Santo, pers. comm.). Eggs were extracted from tomato roots using 0.5% sodium hypochlorite.

[b] G. Santos pers. comm. "S. bulbo" is *Solanum bulbocastanum* (Brown et al., 1989).

[c] Pattern C is the three-banded pattern expected for *M. chitwoodi* after amplification with mitochondrial primers and digestion with *DraI.*

[d] Identification as *M. hapla* (H) or *M. chitwoodi* (C) after PCR with a mixture of the two species-specific primer pairs.

TABLE 1. *Meloidogyne hapla* identification.

Sample locale[a]	Host origin	Isozymes on females	Mt PCR,[b] single J2	Multiplex primers on J2[c]
WY	alfalfa, greenhouse	*M. hapla*	H2	H
Canada	potato, field	*M. hapla*	H2	H
NC	peanut, field	*M. hapla*	H2	H
MI	tomato, greenhouse	*M. hapla*	H1, H2	H
OH	soybean, field	*M. hapla*	H1	H
FL	strawberry, field	*M. hapla*	H2	H
CA	tomato, greenhouse	*M. hapla*	H1	H

[a] WY = Wyoming, NC = North Carolina, MI = Michigan, OH = Ohio, FL = Florida, CA = California.

[b] Two different patterns have been found for *M. hapla* isolates. Type H1 is not cleaved by the enzyme *DraI.* H2 is cleaved into two fragments by *DraI.*

[c] Identification as *M. hapla* (H) or *M. chitwoodi* (C) after PCR with a mixture of the two species-specific primer pairs.

isolates was not cleaved by *DraI* (we refer to this pattern as Type H1), while DNA of other isolates was cleaved into two fragments (Type H2). Amplification of individual J2 from each *M. chitwoodi* isolate (Table 2) with mitochondrial PCR primers produced a 0.52-kb band, which was cleaved by *DraI* into the expected three bands (Powers and Harris, 1993).

Discussion

Several 10-mer primers were tested for their ability to prime amplification of RKN DNA. The RAPD PCR procedure accurately distinguished the five RKN species tested when bulk DNA was used as the template. With a number of primers, *M. javanica, M. arenaria,* and *M. incognita* gave similar patterns, whereas *M. hapla* and *M. chitwoodi* were easily distinguished from other species with most primers. Although RAPD conditions allowed us to successfully amplify DNA from individual J2, we concluded that the patterns were probably not consistent enough for this technique to be used for standard identification of J2. However, the RAPD bands from amplification of purified DNA provided material for development of STS primers that should be useful for routine use.

We have developed markers that can identify individual J2 of *M. hapla* and *M. chitwoodi* using a single reaction. The method is simple and, in our hands, results in successful amplification from 80–90% of J2 tested. This technique is faster and less expensive than the mitochondrial PCR assay as it does not involve a restriction digestion, and validation tests have shown that the primers work for a number of isolates from different geographic locations.

Using the procedure developed by Powers and Harris (1993), we found that the amplified mitochondrial band showed a polymorphism within the species *M. hapla.* To be sure that this was not an artifact due to incomplete digestion by the restriction endonuclease *DraI,* we carried out amplification and cleavage of several individuals of each of our isolates in at least three independent experiments. The H1 and H2 patterns were consistent from experiment to experiment for each isolate. Mitochondrial RFLP polymorphism among *M. hapla* isolates has been reported (Peloquin et al., 1993).

With the primers described, this assay will identify and distinguish isolates of *M. hapla* and *M. chitwoodi,* but J2 of other species will not be identified or distinguished. We examined a limited number of nematode isolates and did not assess other related species such as *M. graminicola* and *M. naasi,* so some caution may be necessary when using these primers as diagnostic aids. Many more isolates of *M. hapla* and *M. chitwoodi* need to be assessed with the primers described herein to determine if the primers are truly species-specific. The assay should be extended by developing species-specific probes for each of the major root-knot nematode species. If the primer pairs for each species amplify species-specific bands of different sizes, it should be possible to distinguish and identify several species using a single PCR reaction mix. A mixture of four primer pairs—each pair specific for one of the four major RKN species—could be used in a single PCR reaction. Gel electrophoresis to resolve product sizes would allow species identification. Such multiplex approaches have been used in human genetic studies (Cui et al., 1989).

The species-specific primers have potential for identification of root-knot nematodes (eggs, J2s) from soil samples or in root tissue, as the primers detailed herein should amplify DNA from *M. hapla* and *M. chitwoodi* but not other nematode species. One strategy would be to extract a nematode-containing fraction from soil using classical techniques (elutriation, sieves, sucrose flotation) and treat the extract to release the DNA, followed by PCR amplification. A major problem may be the presence of inhibitors in soil extracts (Hyman et al., 1990).

Within California, *M. hapla* has been identified in ca. 5–10% of RKN identifications by isozyme electrophoresis as conducted in the Extension Nematology Laboratory at UCD (Westerdahl, unpubl. data). Because commercially available tomato cultivars with

RKN resistance are susceptible to *M. hapla,* diagnosis of *M. hapla* from field samples is important. The PCR primers described herein offer a quick assay for identification of *M. hapla* J2 recovered from tomato fields, and may aid in preventing crop loss.

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