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A Polymerase Chain Reaction Method for Identification of Five Major *Meloidogyne* Species[1]

T. O. POWERS AND T. S. HARRIS[2]

Abstract: A polymerase chain reaction (PCR) method for discriminating *Meloidogyne incognita, M. arenaria, M. javanica, M. hapla,* and *M. chitwoodi* was developed. Single juveniles were ruptured in a drop of water and added directly to a PCR reaction mixture in a microcentrifuge tube. Primer annealing sites were located in the 3′ portion of the mitochondrial gene coding for cytochrome oxidase subunit II and in the 16S rRNA gene. Following PCR amplification, fragments of three sizes were detected. The *M. incognita* and *M. javanica* reactions produced a 1.7-kb fragment; the *M. arenaria* reaction, a 1.1-kb fragment; and the *M. hapla* and *M. chitwoodi* reactions resulted in a 0.52-kb fragment. Digestion of the amplified product with restriction endonucleases allowed discrimination among species with identically sized amplification products. Dra I digestions of the 0.52-kb amplification product produced a characteristic three-banded pattern in *M. chitwoodi,* versus a two-banded pattern in *M. hapla.* Hinf I digestion of the 1.7-kb fragment produced a two-banded pattern in *M. javanica,* versus a three-banded pattern in *M. incognita.* Amplification and digestion of DNA from juveniles from single isolates of *M. marylandi, M. naasi,* and *M. nataliei* indicated that the diagnostic application of this primer set may extend to less frequently encountered *Meloidogyne* species.

Key words: DNA, *Meloidogyne arenaria, M. chitwoodi, M. hapla, M. incognita, M. javanica,* nematode, polymerase chain reaction.

Species identification in *Meloidogyne* has been a major component of taxonomic research in nematology. Although there are approximately 60 described species of root-knot nematodes, most taxonomic attention has focused on less than the dozen that are typically associated with diseases of agronomically important plant species. *Meloidogyne incognita, M. javanica, M. arenaria,* and *M. hapla* account for the majority of crop losses caused by root-knot nematodes. An extensive survey of about 1,300 *Meloidogyne* populations from over 70 countries representing the primary food production regions of the world found at least one of these four species in 95% of the samples (4). The four species are worldwide in distribution, but the first three are generally limited by temperature to a region between 40°N and 33°S latitude. *Meloidogyne hapla* is more common than the others in colder latitudes and in higher elevations of the tropics (15). *Meloidogyne chitwoodi* is also found in colder latitudes and is widespread in the Pacific Northwest of the United States (14). The discrimination of *M. chitwoodi* and *M. hapla* is important because of the host range and physiological differences between them (12).

Identification of *Meloidogyne* spp. is based on adult female morphology (5). Because agricultural soils do not contain *Meloidogyne* adult females, identification of eggs or second-stage juveniles (J2) would improve crop management decisions. Identification of juveniles typically involves a combination of painstaking examination of morphological characters (5) and time-consuming reproduction on a set of differential host plants. In recent years, species-specific DNA hybridization probes have been sought as a replacement or complement for these procedures (3,10,13).

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[1] Journal Series No. 9897, Agricultural Research Division, University of Nebraska.

[2] Associate Professor and Research Technologist, Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583–0722.

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Although many of these probes appear promising, it is unlikely that any hybridization method involving radioactive isotopes will be accepted as a general diagnostic protocol. Nonisotopic labeling methods are available, but their lower sensitivity may fail to detect low numbers of eggs or second-stage juveniles.

The polymerase chain reaction (PCR) provides a highly sensitive method for DNA amplification and identification. Previously, we utilized PCR to amplify mitochondrial DNA (mtDNA) from individual *Meloidogyne* J2 and eggs (8). We now report a modification of the protocol, its molecular basis, and its usefulness in identification of five major *Meloidogyne* species.

Materials and Methods

Primer construction: PCR primer #C2F3, 5′-GGTCAATGTTCAGAAATT-TGTGG-3′, was designed by alignment of all invertebrate mitochondrial nucleotide sequences for the cytochrome oxidase subunit II gene (COII) available in GenBank. Primer #1108, 5′-TACCTTTGACCAAT-CACGCT-3′, was designed by nucleotide sequencing a cloned fragment of the large subunit of the ribosomal RNA gene (lrRNA) from *M. incognita* (7). Primers were synthesized at the DNA Synthesis Facility of the University of Nebraska, Center for Biotechnology.

Diagnostic assay: The diagnostic assay consisted of three basic steps: nematode isolation, DNA amplification, and evaluation of amplified products. First, nematodes were isolated from soil by a combination of sieving and density gradient centrifugation (1). Individual *Meloidogyne* J2 were hand-picked and placed in a 15-μl drop of sterile water on a cover slip. The nematode was then ruptured by gentle pressure of a yellow, flat-tipped micropipet tip (Outpatient Services, Inc., Petaluma, CA), which is sufficiently translucent to allow viewing the nematode to verify lysis. At this point, nematode lysate can be frozen for future analysis or processed immediately for PCR. For DNA amplification, a mixture of Taq reaction buffer (Perkin Elmer Cetus, Norwalk, CT), four deoxynucleotides (200 μM each), amplification primers #C2F3 and #1108 (0.8 μM each), 1.0 U Taq polymerase (Perkin Elmer Cetus), and sterile water was added to the nematode lysate to bring the reaction volume to ca. 25 μl under a mineral oil overlay. This mixture was placed in a DNA thermal cycler (Perkin Elmer TC-1) already heated at 94 C for a modified "hot start." PCR amplification conditions were as follows: denaturation at 94 C for 1 minute, annealing at 48 C for 1 minute, and extension at 70 C for 2 minutes, repeated for 45 cycles. A 5-minute incubation period at 72 C followed the last cycle in order to complete any partially synthesized second strands.

Following DNA amplification, the products were screened on a 1.0% agarose gel, stained with ethidium bromide, and visualized on a midrange UV box. Usually 8–10 μl of amplified product were sufficient for easily scored gel patterns. Standard restriction digestion of the amplified products was conducted on 5 μl of product and evaluated on 1.4–2.0% agarose gels. Dra I and Hinf I (Promega Corp., Madison, MI) digestions were conducted for 2–4 hours at 37 C.

Nematode isolates: The protocol was applied to nematode isolates from 15 states in the western, southwestern, central, and southeastern regions of the United States (Table 1). Juveniles from 14 *M. arenaria* race 1 isolates from Georgia, Florida, and North Carolina were hatched from egg masses on peanut (*Arachis hypogaea*), which permits reproduction of only *M. arenaria* race 1 and *M. hapla* (9). Some *M. javanica* (JHF, Jav610, Jav525, PRJC17, E781) and *M. incognita* (NCSU #E1135, JC1, NCSU #108) isolates were partially characterized by esterase patterns (6) and observations of perineal patterns of adult females (9). Most isolates were received from source laboratories as juveniles and eggs in water and processed directly for PCR analysis. A minimum of five individuals were assayed from each isolate.

TABLE 1. Isolates and sources of species of *Meloidogyne* characterized by polymerase chain reaction (PCR) amplification of DNA.

Species	Isolate	Location	Source
Meloidogyne arenaria	#1–12 (race 1)	Georgia	James Noe
	Pelion (race 2)	South Carolina	Stephen Lewis
	Govan (race 2)	South Carolina	Stephen Lewis
	Florence (race 2)	South Carolina	Stephen Lewis
	SC-1 (race 1)	South Carolina	Stephen Lewis
	Live Oak (race 1)	Florida	Robert McSorley
	NCSU#64	North Carolina	Charles Opperman
	A 436	North Carolina	Charles Opperman
	82-4	Texas	James Starr
Meloidogyne chitwoodi	WA1 (race 1)	Washington	Gerald Santo
	OR (race 2)	Oregon	Gerald Santo
	PA (race 2)	Washington	Gerald Santo
	PEA (race 1)	Washington	Gerald Santo
	Davis	California	Valerie Williamson
Meloidogyne hapla	WA	Washington	Gerald Santo
	UT	Utah	Gerald Santo
	AZ	Arizona	Gerald Santo
	Dod	Washington	Gerald Santo
	Minden	Nebraska	Thomas Powers
	Parsnip	Nebraska	Thomas Powers
	E20	Wisconsin	An McGuidwin
	Madison	Wisconsin	An McGuidwin
	Berdo	California	David Bird
	KRB	North Carolina	Kenneth Barker
	HAZ	Arizona	Michael McClure
Meloidogyne incognita	NCSU #E1135	California	Charles Opperman
	JC1	California	Thomas Powers
	NCSU #108	Alabama	Charles Opperman
	Lubbock	Texas	Stephen Thomas
	Az Cuke	Arizona	Michael McClure
	Az Pepper	Arizona	Michael McClure
	UGA 1–3	Georgia	James Noe
	San Juan	Dominican Republic	Graciela Godoy
	Hoke	North Carolina	Kenneth Barker
	Cumberland	North Carolina	Kenneth Barker
	82-2	Texas	James Starr
Meloidogyne javanica	JHF	California	Thomas Powers
	Jav 610	California	Thomas Powers
	#10	California	Thomas Powers
	Jav 525	California	Thomas Powers
	PRJC 17	California	Thomas Powers
	Biosphere II	Arizona	Michael McClure
	UGA	Georgia	James Noe
	Jav 1	California	Valerie Williamson
	E 781	California	Thomas Powers
Meloidogyne marylandi	Turf	Missouri	Terry Niblack
Meloidogyne naasi	Sorghum	Kansas	Timothy Todd
Meloidogyne nataliei	Grape	Michigan	George Bird

RESULTS

Figure 1 illustrates the different size classes of amplification products in reactions with five *Meloidogyne* species. All isolates of both *M. incognita* and *M. javanica* produced a 1.7-kb fragment. Isolates of both races of *M. arenaria* produced a 1.1-kb fragment, and all isolates of *M. chitwoodi* and *M. hapla* produced 0.52-kb products. Three other species, *M. marylandi, M. nataliei,* and *M. naasi,* also produced 0.52-kb fragments. A diagrammatic representation of the amplification product size classes

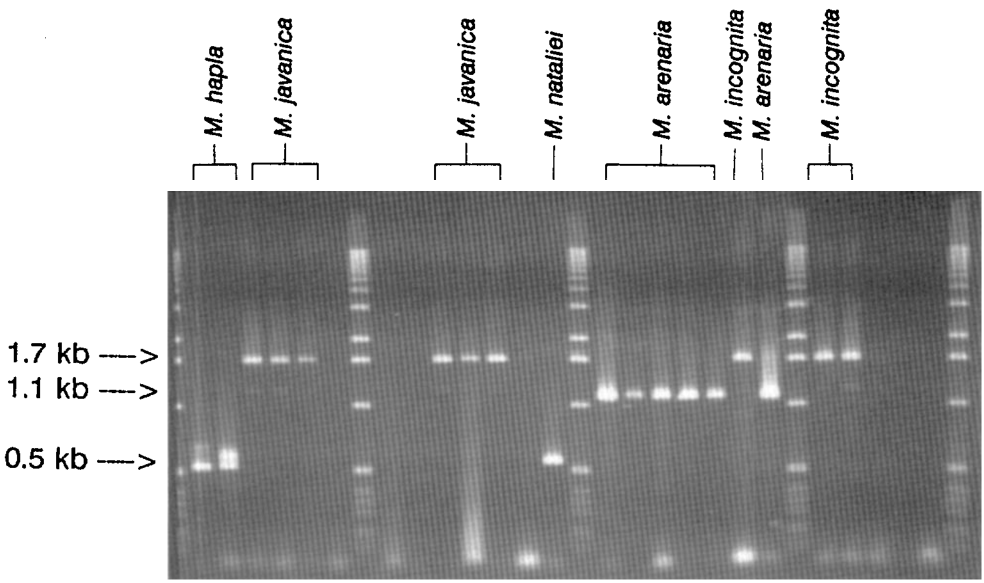

FIG. 1. Typical separation on a 1.0% agarose gel of products from PCR amplification of lysate from single *Meloidogyne* second-stage juveniles. The 1.7-kb product is characteristic of *Meloidogyne incognita* and *M. javanica;* the 1.1-kb product identifies *M. arenaria,* and the 0.52-kb product characterizes *M. chitwoodi, M. hapla, M. marylandi, M. naasi,* and *M. nataliei.* Empty lanes indicate failed reactions. Five lanes of 1 Kb DNA ladder (Gibco BRL, Gaithersburg, MD) are included as size standards.

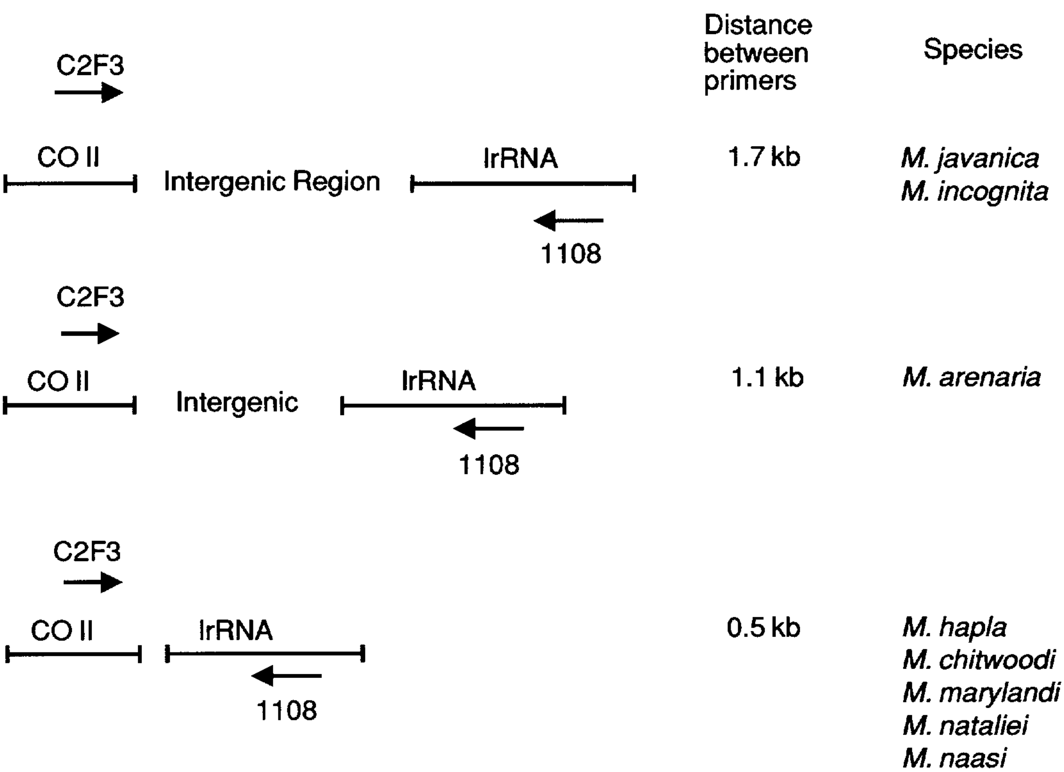

FIG. 2. Diagrammatic representation of primer binding sites on the *Meloidogyne* mitochondrial genome. Primer #C2F3 anneals to the coding strand of the cytochrome oxidase subunit II (COII) gene and primer #1108 anneals approximately 450 bp downstream from the start of the lrRNA gene. The intergenic region varies in size among the different *Meloidogyne* species.

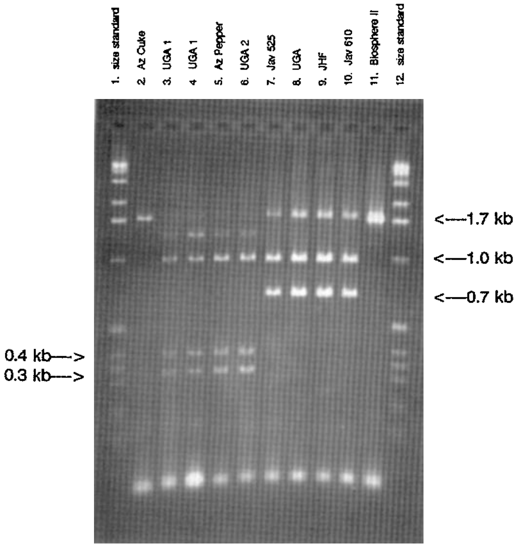

FIG. 3. Hinf I digestion products of the amplified 1.7-kb fragment separated on a 1.5% agarose gel. Lanes 2–6 and lanes 7–11 are products from *M. incognita* and *M. javanica*, respectively. Lanes 1 and 12 are size standards next to undigested product from each species (lanes 2 and 11). A small amount of undigested product remains in lanes 7–10, and a partially digested fragment is visible in lanes 3–6 at approximately 1.4 kb. The 1.0-kb digestion product is shared by both species, but an additional Hinf I restriction site in the 0.7-kb fragment produces two restriction fragments of 0.4 and 0.3 kb in *M. incognita*. The bright bands at the gel front are unincorporated primer.

and location of the primer-annealing sites is presented in Figure 2.

Restriction digestion of the 1.7-kb amplification product with Hinf I produced diagnostic patterns for all *M. incognita* and *M. javanica* isolates (Fig. 3). One restriction site on the *M. javanica* products resulted in digestion products of 1.0 and 0.7 kb (Fig. 3, lanes 7–10). An additional restriction site in the *M. incognita* product resulted in cleavage of the 0.7-kb fragment to yield two fragments of ca. 0.4 and 0.3 kb (Fig. 3, lanes 3–6). *Meloidogyne chitwoodi* and *M. hapla* products were differentiated by digestion with the restriction enzyme Dra I (Fig. 4). Two digestion products of ca. 290 and 230 bp were generated in each isolate of *M. hapla*, whereas the 230-bp fragment from each *M. chitwoodi* isolate possessed an additional restriction site, resulting in two smaller fragments of 130 bp and 100 bp.

Dra I also produced unique patterns for *M. nataliei, M. naasi,* and *M. marylandi;* but because only a single isolate of each was examined, the specificity and consistency of the patterns could not be evaluated.

DISCUSSION

A rapid species identification method for *Meloidogyne* juveniles has been described. Benefits of the method are that it requires no radioactive isotopes and only simple nematode lysis before PCR, instead of complex extraction of DNA. The ability to identify soil J2 permits species determinations to be conducted before planting. The specificity of the assay may allow monitoring species shifts in mixed *Meloidogyne* populations with a single PCR amplification (Noe and Powers, unpublished).

This protocol is an improvement over a previously published method that also used PCR amplification of mtDNA (8). That method had the drawbacks of an inability to separate *M. hapla* from *M. javanica* and a lack of amplification of some *M. arenaria* isolates. The present method overcomes those problems and adds the important ability to discriminate *M. hapla* and *M. chitwoodi.* At least three other *Meloidogyne* species can be amplified with this primer set and differentiated from the

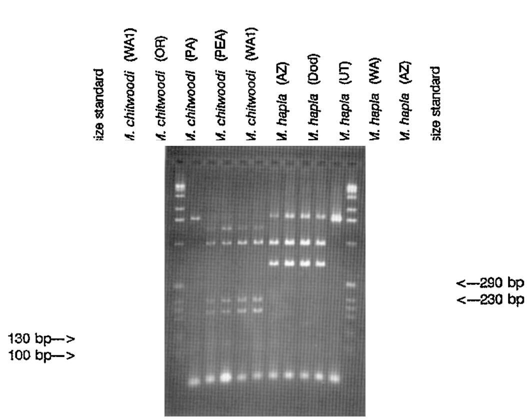

FIG. 4. Separation on a 2.0% agarose gel of Dra I digestion products of the 0.52-kb fragments of *M. chitwoodi* and *M. hapla* from different localities. Both species share the 290-bp digestion product, but an additional restriction site in the 230-bp fragment results in 130- and 100-bp fragments in *M. chitwoodi.*

other five species by endonuclease digestion of the amplified product; however, it will be necessary to examine a broad range of isolates of the "minor" species before species specificity can be determined. It should be noted that nearly all of the *Meloidogyne* isolates examined were North American. A survey of worldwide isolates should be conducted to confirm the general applicability of this method.

At present, no resolution below the species level has been achieved with this protocol. Intraspecific mitochondrial variation has been observed in a nematode parasite of cattle, *Ostertagia ostertagi* (2), and in a region of multiple nucleotide repeating units in *Meloidogyne* (11). The repeating units are located in a different region of the mitochondrial genome than the amplified region described in the present study; however, intraspecific variation suggests that more rapidly evolving portions of the mitochondrial genome may provide subspecific discrimination in *Meloidogyne*. We are currently sequencing the amplified products to determine the extent of genetic divergence among *Meloidogyne* species and to evaluate the feasibility of host race differentiation in *M. arenaria*, *M. chitwoodi*, and *M. incognita*.

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