

A SPECIES-SPECIFIC DNA PROBE FOR THE IDENTIFICATION OF *MELOIDOGYNE HAPLA*[†]

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ABSTRACT

Dong, K., R. A. Dean, B. A. Fortnum, and S. A. Lewis. 2001. A species-specific DNA probe for the identification of *Meloidogyne hapla*. *Nematropica* 31:17-23.

Here we report the development of a DNA probe specific to *Meloidogyne hapla*, among four species in the genus that we have studied. A small-insert genomic library from an isolate of *M. hapla* was constructed in the pBluescript vector. From the library, 3 200 clones were differentially screened against genomic DNA from *M. arenaria*, *M. incognita* and *M. javanica*. About 99% of the clones hybridized positively to the three nematode species. The clones that hybridized specifically to the genomic DNA from different *M. hapla* isolates were selected and further characterized by slot-blot and Southern blot analyses. Sequence analysis from one of the clones reveals a 171 bp A and T-rich DNA insert, and a 55-base DNA oligonucleotide specific to *M. hapla* isolates.

Key words: diagnosis, DNA probe, genomic differential hybridization, molecular identification, *Meloidogyne arenaria*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Meloidogyne javanica*, nematode, root-knot, species.

RESUMEN

Dong, K., R. A. Dean, B. A. Fortnum, and S. A. Lewis. 2001. Una sonda específica de ADN para la identificación de *Meloidogyne hapla*. *Nematrópica* 31:17-23.

Reportamos el desarrollo de una sonda de ADN específica para *Meloidogyne hapla*, como resultado del análisis de cuatro especies del género en estudio. Una librería genómica de pequeños insertos obtenido de un aislado de *M. hapla* se construyó en el vector pBluescript. De la librería, 3 200 clones se seleccionaron diferencialmente al compararlos con ADNs genómicos de *M. arenaria*, *M. incognita* and *M. javanica*. Alrededor del 99% de los clones hibridaron positivamente con los tres especies de nematodos. Los clones que hibridaron específicamente con el ADN genómico de diferentes aislados *M. hapla* se seleccionaron y posteriormente caracterizaron a través de los análisis de slot-blot y Southern blot. El análisis de la secuencia de uno de los clones revela un inserto de ADN de 171 bp rico en A y T, y un oligonucleótido de 55 bases específico de aislados de *M. hapla*.

Palabras claves: diagnóstico, diferencial hibridación, especies, hibridación genómica, identificación molecular, *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, *M. javanica*, nematodo, nematodo nodulador, sonda de ADN.

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INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are a group of sedentary endoparasitic nematodes composed of more than 80 species and subspecies (Eisenback, 1997). The majority of economic damage is attributable to four of the most common species, *M. arenaria* (Neal) Chitwood, *M. hapla* Chitwood, *M. incognita* (Kofoid and White) Chitwood, and *M. javanica* (Treub) Chitwood. Due to their worldwide distribution and their interactions with other plant pathogens in disease complexes, root-knot nematodes have been ranked as the top pest affecting the food supply worldwide (Sasser, 1980).

Development of a rapid and precise *Meloidogyne* species identification method is critical if the utilization of non-host rotation and cultivar resistance is considered. The traditional diagnosis of *Meloidogyne* spp. relies on morphological characteristics (Eisenback *et al.*, 1981). Host range testing and biotyping according to host resistance provide a straightforward practice to distinguish the nematode species, races, biotypes and even the virulence genes (Hartman and Sasser, 1985; Roberts, 1995). Protein analyses have been demonstrated to be reliable, and esterase isozyme patterns are probably the most widely accepted markers used at present to identify the four major root-knot species (Esbenshade and Triantaphyllou, 1985). DNA-based techniques for species identification have been pursued, including the development of restriction fragment length polymorphisms among species (Cenis *et al.*, 1992). PCR patterns were also used to distinguish the four common species of *Meloidogyne* (Cenis, 1993; Powers and Harris, 1993; Williamson *et al.*, 1997). Mitochondrial DNA restriction site polymorphisms and internal transcribed spacer (ITS) nucleotide sequences also have been used to analyze the DNA-based

specificity (Powers *et al.*, 1986). Cloning specific DNA to generate probes is another practical approach to identifying these species. We have identified a DNA clone specific to *M. hapla* by using differential screening of a DNA library produced from genomic DNA.

MATERIALS AND METHODS

Nematode isolates: Nematode isolates had the following origins: *M. arenaria*-Canada, *M. arenaria* race 1-Georgia (GA), *M. arenaria* race 2-North Carolina (NC)1, *M. arenaria* race 2-NC2, *M. arenaria* race 2-South Carolina (SC), *M. hapla*-NC, *M. hapla*-New York (NY)1, *M. hapla*-NY2, *M. hapla*-NY3, *M. hapla*-Virginia (VA), *M. hapla*-Washington (WA), *M. hapla*-Wisconsin (WI), *M. incognita*-Tennessee (TN), *M. incognita* race 1-NC, *M. incognita* race 2-NC, *M. incognita* race 3-NC, *M. incognita* race 4-NC, *M. javanica*-Arizona (AZ)1, *M. javanica*-AZ2, *M. javanica*-GA, and *M. javanica*-NC. The 21 isolates were grown on *Lycopersicon esculentum* L. cv. Rutgers. Single egg masses from each isolate were inoculated onto tomato seedlings to initiate pure nematode cultures. Species of the single-egg-mass cultures were further confirmed using perineal pattern characters and isozyme phenotypes. Esterase, malate dehydrogenase (Mdh) and superoxide dismutase (Sod) phenotypes were tested (Esbenshade and Triantaphyllou, 1985).

DNA extraction and genomic library: Nematode eggs were extracted from tomato roots, purified by sucrose gradient centrifugation, and frozen at -80°C. Eggs were resuspended in DNA isolation buffer (100 mM NaCl, 100 mM Tris-HCl pH8.5, 50 mM EDTA, 1% SDS, 1% β-mercaptoethanol, and 100 µg/ml Proteinase K), and incubated at 65°C for 1 h with occasional agitation. The DNA was extracted with phenol/chloroform and precipitated in isopropanol

at room temperature. The DNA pellet was then washed twice with 70% ice-cold ethanol, resuspended in 50 μ l H₂O and stored at -80°C.

The pBluescript KS vector (Stratagene, La Jolla, CA) was used to construct a small-insert genomic library. The DNA sample extracted from the Wisconsin *M. hapla* isolate was digested to completion with *Eco*RI and ligated into *Eco*RI cloning site in pBluescript KS. The ligation mixture was electroporated into DH10B cells. All molecular techniques were performed as described in Sambrook *et al.* (1989).

Colony hybridization and DNA blots to detect candidate clones: 3 200 clones from the *M. hapla* WI library were randomly picked, and the differential screening was sequentially conducted with genomic DNAs from *M. arenaria*, *M. incognita* and *M. javanica*. About 200 ng nematode DNA from each species was labeled with [α ³²P] dCTP (Multi-prime DNA Labeling Systems, Amersham, Arlington Heights, IL). Candidate *M. hapla* specific clones that did not hybridize were selected and re-screened against a mixed DNA probe of *M. arenaria* race 2-NC2, *M. incognita* race 1-NC and *M. javanica* AZ1. A ³²P-labeled North Carolina *M. hapla* DNA sample was further used to select the *M. hapla* positive clones. Colony transfer and lysis on Hybond+ nylon membranes, colony hybridization, membranes washing, and autoradiography was performed as described in Sambrook *et al.* (1989).

The candidate clones selected from colony hybridization were restriction analyzed. The clones that contained different inserts were tested on a Slot-blot to determine *M. hapla* specificity. The plasmids with *M. hapla* DNA inserts were mini-prepared (Promega Wizard™ Plus DNA Purification System, Promega, Madison, WI), and labeled with [α ³²P] dCTP as probes. The 16 nematode DNA samples used for the slot-blot test were from *M. arenaria*-

Canada, race 1-GA, race 2-NC1, race 2-NC2, race 2-SC; *M. hapla*-NC, WI; *M. incognita* race 1-NC, race 2-NC, race 3-NC, race 4-NC, TN; *M. javanica*-AZ1, AZ2, GA, and NC. About 500 ng DNA from each nematode isolate was slot-blotted onto the Hybond+ nylon membrane (Schleicher & Schuell Minifold II Slot-Blot System, Keene, NH). Furthermore, a candidate clone was also tested by genomic DNA hybridization analysis (Southern blot). About 10 μ g genomic DNA from *M. arenaria* race 2-NC1, *M. hapla*-WI, *M. incognita* race 2-NC, and *M. javanica*-NC was digested with *Eco*RI, resolved on a 1.0% agarose gel, and Southern blotted. DNA probe preparation, hybridization, membrane washing and autoradiography were conducted as described above (Sambrook *et al.*, 1989).

Oligonucleotide specificity and sensitivity analysis: A *M. hapla*-specific DNA fragment was sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. The reaction was conducted in 20 μ l total volume with 0.5 μ g template DNA, 1 μ l M13 forward or reverse primers (3.2 pmoles), and 8 μ l reaction mix. Thermal cycling conditions were 96°C 30 seconds, 50°C 15 seconds, and 60°C 4 minutes for 25 cycles. The reaction product was purified in a sephadex G50 column. The DNA sequence was read on ABI 373 (PE Applied Biosystems, Foster City, CA) and sequence similarity was compared by a BLASTN search of the GenBank database.

A DNA oligonucleotide of 55 bases was designed according to the sequence analysis result. The oligonucleotide was synthesized commercially (Integrated DNA Technologies, Coralville, IA). About 500 ng of the oligonucleotide DNA was 5' end labeled with [γ ³²P] ATP catalyzed by bacteriophage T4 polynucleotide kinase (Sambrook *et al.*, 1989).

The specificity of this oligonucleotide to *M. hapla* was tested on a slot-blot. Four

different *Meloidogyne* isolates: *M. arenaria* race 2-NC1, *M. hapla*-WI, *M. incognita* race 4-NC, and *M. javanica*-NC, were tested at 500 ng DNA per slot. Seven DNA samples from different *M. hapla* isolates: NC, NY1, NY2, NY3, VA, WA, WI, were also tested at 500 ng DNA per slot.

The sensitivity of this oligonucleotide to detect *M. hapla* was also evaluated. Different amounts (100 ng, 50 ng, 10 ng and 5 ng) of purified *M. hapla* DNA were applied on a slot-blot filter. In addition, different types of *M. hapla* samples that included: five females, one female, one female-gall, and a single-egg-mass were independently collected in 1.5 ml microcentrifuge tubes and lysed by grinding in water. The samples were then denatured in 0.4 N NaOH and applied on a slot-blot filter. Hybridization, membrane washing and autoradiography were conducted as described previously (Sambrook *et al.*, 1989).

RESULTS

Colony hybridization and DNA blots to detect candidate clones: After screening the *M. hapla*-WI genomic library, a total of 32 clones that did not strongly hybridize to the mixed DNA probe of the other three *Meloidogyne* species were selected. The same selected clones were also tested with the ³²P labeled genomic DNA from *M. hapla*-NC, and 22 clones were identified as hybridizing positively to that isolate. These clones were designated as pMh01-pMh22. Restriction analyses indicated that 20 out of the 22 clones, e.g. clones pMh01-pMh18 and pMh21-pMh22, contained the same size insert and implied a repetitive DNA fragment could be isolated.

The candidate clones pMh03, pMh19 and pMh20 with different inserts were chosen for further analysis on slot-blot analysis with genomic DNA from 16 different populations. The five *M. arenaria* populations

were Canada, GA, NC1, NC2, and SC; the two *M. hapla* were NC, and WI; the five *M. incognita* were NC1, NC2, NC3, NC4 and TN; and the four *M. javanica* were AZ1, AZ2, GA, and NC. The three clones tested all hybridized specifically to the two *M. hapla* isolates but not to the other *Meloidogyne* spp. The result from clone pMh03 is shown (Fig. 1). The specificity of clone pMh03 to *M. hapla* was further confirmed by Southern blot analysis with the genomic DNAs from the four species of *Meloidogyne*: *M. arenaria*-NC2, *M. hapla*-WI, *M. incognita*-NC2, and *M. javanica*-NC. The pMh03 clone hybridized specifically to an *Eco*RI fragment from *M. hapla* (Fig. 2).

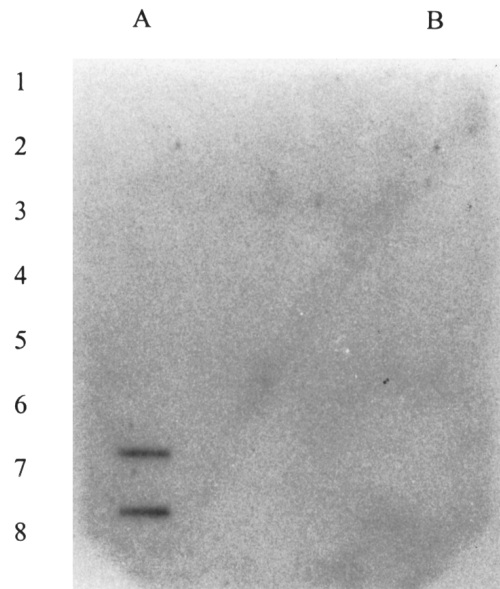


Fig. 1. Slot-blot analysis of species specificity exhibited by the cloned *M. hapla* genomic fragment pMh03. Top to bottom, column A: *M. arenaria* race 1-GA, *M. arenaria* race 2-SC, *M. arenaria* race 2-NC 1, *M. arenaria* race 2-NC 2, *M. incognita*-TN, *M. arenaria*-Canada, *M. hapla*-NC, and *M. hapla*-WI. Top to bottom, column B: *M. incognita* race 1-NC, *M. incognita* race 2-NC, *M. incognita* race 3-NC, *M. incognita* race 4-NC, *M. javanica*-AZ 1, *M. javanica*-AZ 2, *M. javanica*-NC and *M. javanica*-GA. Plasmid DNA of pMh03 was ³²P-labeled, and the probe was hybridized specifically to the *M. hapla* populations.

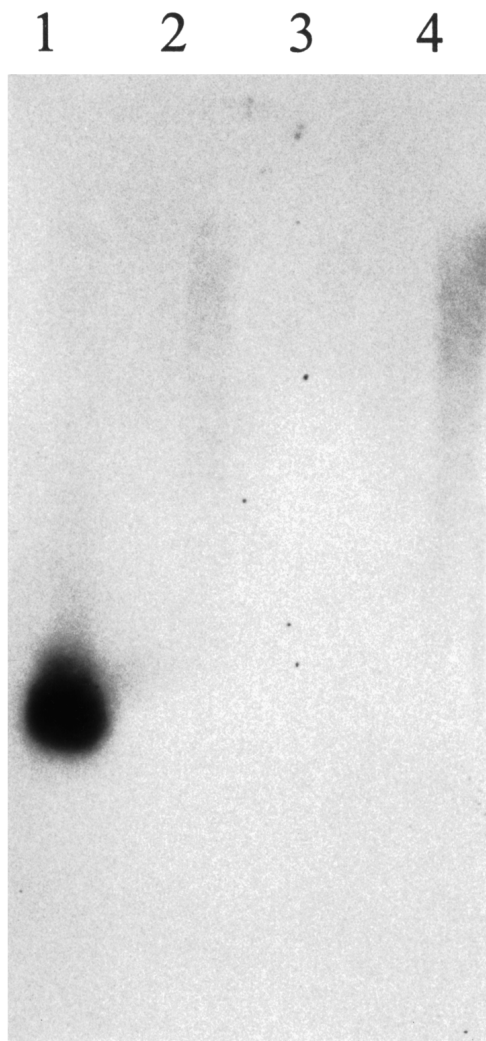


Fig. 2. Genomic Southern blot analysis: Lane (1) *M. hapla*-WI, (2) *M. arenaria* race 2-NC, (3) *M. incognita* race 2-NC, and (4) *M. javanica*-NC. The clone pMh03 was 32 P-labeled and used as a hybridization probe.

Oligonucleotide specificity and sensitivity analysis: The sequence obtained from the clone pMh03 is a 171 bp A and T-rich DNA fragment as follows: GAATTCATAAAAA-TTACAAATTGAAAAGGGGTACCCTTTT CGACTCGATAATTTCCGAAATTTATACC TAATTTAAAAATAAAAAATATAAAAATCG TCAGAATTCATTACTTGAATAATCATAC-

ATTCCCTACTAGGTACTTAACAAAATTAT ACTCAATTCGAATTC. No similar sequence was detected from the BLASTN search in the GenBank database.

A 55-base-oligonucleotide corresponding to nucleotides 1-54 of the above sequence was used as a probe. This probe has strong specificity to the *M. hapla*-WI population (Fig. 3A), and it positively hybridized to the *M. hapla* populations: NC, NY1, NY2, NY3, VA, WA, and WI (Fig. 3B). Sensitivity analysis indicated that the oligonucleotide DNA probe could positively detect as low as 5 ng purified *M. hapla* DNA (Fig. 3C). Furthermore, crude extracts of samples from a single female, a single-egg-mass, and one female in the gall were positively detected by the oligonucleotide probe (Fig. 3D).

DISCUSSION

Meloidogyne is a highly diversified group of plant-parasitic nematodes. DNA-based molecular markers are sufficiently sensitive to identify species using juveniles and eggs

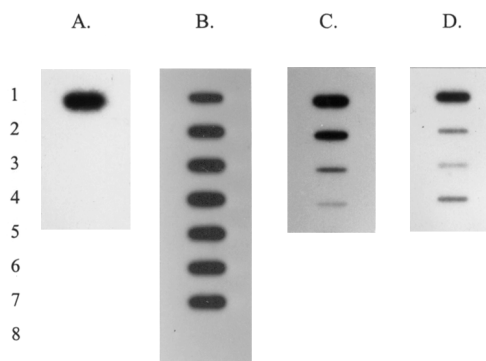


Fig. 3. Slot-blot analyses of specificity and sensitivity exhibited by the oligonucleotide as a probe. A). Top to bottom: (1) *M. hapla*, (2) *M. javanica*, (3) *M. incognita*, and (4) *M. arenaria*. B). Top to bottom: (1) *M. hapla*-NC, (2) *M. hapla*-WI, (3) *M. hapla*-VA, (4) *M. hapla*-NY1, (5) *M. hapla*-NY2, (6) *M. hapla*-NY3, (7) *M. hapla*-WA. C). Top to bottom: (1) 100 ng, (2) 50 ng, (3) 10 ng and (4) 5 ng of purified *M. hapla* DNA. D). *M. hapla* samples from different life stages, top to bottom: (1) five females, (2) one female, (3) one female and gall, and (4) a single egg mass.

tested directly. Practical applications of these molecular markers require specific PCR primers and specific DNA fragments (probes) to the target species. PCR methods for *Meloidogyne* species identification have been developed (Powers and Harris, 1993; Williamson *et al.*, 1997). An oligonucleotide DNA probe (~60 bp) was reported specific to *M. incognita* (Chacon *et al.*, 1991). The published *M. arenaria* species-specific DNA probe was a tandem repeat sequence of multiple copies of 28 bp subunits (Baum *et al.*, 1994). Abad (1994) reported a different repetitive DNA fragment isolated from *M. hapla* that can be used to distinguish *M. hapla* isolates from *M. chitwoodi* isolates.

Genetic backgrounds of *Meloidogyne* spp. are complex and variations among different isolates within a species are common (Carneiro *et al.*, 1998; Waeyenberge *et al.*, 2000; Whipple *et al.*, 1998). Additional information on identifying *Meloidogyne* spp. is always useful and has value because it can be complementary. The *M. hapla*-specific DNA probe reported in this work has been verified against multiple isolates of common species of *Meloidogyne*. It can be an additional resource to supplement and extend existing species specific probes. Practically, the DNA probe has been designed into a 55-oligonucleotide sequence and can be synthesized commercially at a reasonable price. The probe showed strong specificity and sensitivity to *M. hapla* and can positively detect this species from a root sample or a soil sample with eggs and juveniles. We are investigating the practical applications of *Meloidogyne* species probes in cropping sequence experiments in field plots that are infested with multiple root-knot nematode species.

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