DEVELOPMENT OF PCR PRIMERS TO IDENTIFY SPECIES OF ROOT-KNOT NEMATODES: *MELOIDOGYNE ARENARIA*, *M. HAPLA*, *M. INCOGNITA* AND *M. JAVANICA*[†]

K. Dong, R. A. Dean, B. A. Fortnum, and S. A. Lewis

Department of Plant Pathology and Physiology, Clemson University, Clemson, SC 29634-0377, U.S.A. Present addresses of first and second authors are, respectively, California Department of Food and Agriculture, Plant Pest Diagnostics Center, 3294 Meadowview Road, Building E, Sacramento, CA 95832-1448, U.S.A., and Department of Plant Pathology, Box 7616, North Carolina State University, Raleigh, NC 27695-7616, U.S.A.

ABSTRACT

Dong, K., R. A. Dean, B. A. Fortnum, and S. A. Lewis. 2001. Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*. Nematropica 31:273-282.

DNA from twenty-six different single-egg-mass nematode isolates, including seven *Meloidogyne arenaria,* three *M. hapla*, eleven *M. incognita* and five *M. javanica*, were used to identify species-specific sequence tagged sites. RAPD-PCR was tested to detect species-specific DNA fragments. Nematode isolates of the same species could be grouped unambiguously by most of the polymorphic RAPD patterns. Variations among isolates of each species were also observed, especially within *M. arenaria* and *M. hapla*. Potential species-specific DNA fragments from RAPD-PCR were cloned and sequenced. Species-specific PCR primer pairs for *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* were developed.

Key words: diagnostic, DNA, identification, *Meloidogyne*, *M. arenaria*, *M. hapla*, *M. incognita*, *M. javanica*, PCR, RAPD, root-knot nematode, sequence tagged site, species-specific.

RESUMEN

Dong, K., R. A. Dean, B. A. Fortnum y S. A. Lewis. 2001. Desarrollo de de cebadores de PCR para identificar especies de nematodos agalladores de raíces: *Meloidogyne arenaria*, *M. hapla*, *M. incognita* y *M. javanica*. Nematrópica 31:273-282.

ADN de veintiseis diferentes aislados de nematodos de masa de un solo huevo que incluyó siete especies de *Meloidogyne arenaria,* tres *M. hapla*, once *M. incognita* y cinco *M. javanica,* fueron usados para identificar marcadores específicos. RAPD-PCR se evaluaó para detectar fragmentos de ADN específico para cada especie. Aislados de nematodos de la misma especie podrian ser agrupados inequivocamente por la mayoria de los patrones polimorficos de RAPD. También se observaron variaciones entre aislados de cada especie, especialmente dentro de *M. arenaria* y *M. hapla*. Se clonaron y secuenciaron fragmentos de ADN obtenidos a través de RAPD-PCR que presentaron potencial como especie específico. Se desarrollaron pares de marcadores especie específico de PCR para *M. arenaria, M. hapla, M. incognita* y *M. javanica*.

Palabras claves: ADN, diagnóstico, especies-específica, identificación, marcador específico, *Meloidogyne*, *M. arenaria*, *M. hapla*, *M. incognita, M. javanica*, nematodo agallador de raíces, PCR, RAPD.

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INTRODUCTION

Traditional techniques for species identification of root-knot nematodes (*Meloidogyne* spp.) have relied on morphological characters (Eisenback, 1985), host range tests (Hartman and Sasser, 1985; Roberts, 1995) and isozyme phenotypes (Esbenshade and Triantaphyllou, 1985a, b). Recent publications about species-specific molecular markers, such as DNA probes and PCR primers, have been mainly focused on the species from colder latitudes, e.g., *M. chitwoodi*, *M. fallax* and *M. hapla* (Petersen and Vrain, 1996; Petersen *et al.*, 1997; Piotte *et al.*, 1995; Williamson *et al.*, 1997; Zijlstra, 1997; 2000; Zijlstra *et al.*, 1997). The root-knot species *M. arenaria*, *M. incognita* and *M. javanica* usually occur in the warmer or tropical areas and are responsible for major agronomic damage. However, due to their closely related evolutionary lineage and their polyploid genomic nature (Triantaphyllou, 1985), it is more difficult to develop molecular techniques for identification (Williamson *et al.*, 1997). Molecular identification reports for these species are relatively few (Baum *et al.*, 1994; Chacon *et al.*, 1991; Powers and Harris, 1993). Zijlstra *et al.* (2000) published three pairs of species-specific primers for the identifications of *M. arenaria*, *M. incognita*, and *M. javanica* using sequence characterized amplified region (SCAR). Here we report a similar research product and the species-specific PCR primer pairs developed in our study will provide additional information for species identifications.

MATERIALS AND METHODS

Species identification of nematode isolates based on morphological and isozyme phenotypes: The origins of root-knot nematode isolates for these experiments are listed in Table 1. The 26 populations developed from single-egg-masses (seven *M. arenaria*, three *M. hapla*, eleven *M. incognita*, and five *M. javanica*) were grown in the greenhouse on tomato (cv. Rutgers). Identification of each isolate was initially conducted by perineal pattern analysis of 8-10 females per isolate (Table 1). The non-specific esterase and superoxide dismutase (SOD) phenotypes were further tested according to Esbenshade and Triantaphyllou (1985b) (Table 1). Esterase activity was developed by staining the polyacrylamide gels at 37°C in the dark for 30 minutes with a solution of 0.3 mg/ml EDTA, 0.6 mg/ml fast Blue RR salt, and 0.4 mg/ml α-naphthyl acetate in 0.1 M phosphate buffer pH 7.4. The SOD activity was determined by staining in the dark at 37°C using the following solutions: A) 2.45×10^{-3} M nitro blue tetrazolium for 20 minutes; and B) 0.028 M tetramethylethylenediamine, 2.8×10^{-5} M riboflavin, and 0.036 M potassium phosphate at pH 7.8 for 15 minutes, respectively. The stained gels were then transferred on to a fluorescent light box and exposed at room temperature for 5 to 15 minutes to allow color development.

RAPD-PCR and polymorphism identification: Nematode eggs were extracted from infected roots and purified by sucrose gradient centrifugation. Eggs were re-suspended 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 hour with occasional agitation. DNA was extracted with phenol/chloroform and precipitated in isopropanol at room temperature, and the DNA pellet was then washed twice with 70% ice-cold ethanol, re-suspended in H_2O and stored at -80°C. PCR amplification reactions were performed in 15 µl volumes containing: 5.0 µl 4 ng/µl template DNA, 5.0 µl 5 ng/µl primer, 1.5 µl 10 × PCR buffer, 0.9 µl 25 mM $MgCl_2$, 1.2 µl 2.5 mM dNTP, 1.2 µl 10 µg/µl nonacetylated BSA, and 0.2 µl Taq polymerase (Promega, Inc., Madison, WI). The RAPD-

Table 1. *Meloidogyne* spp. isolates used in this experiment.

Isolates	Perineal pattern	Esterase	Superoxide dismutase	Sources
M. arenaria				
Canada race 2	Ma[z]	Ma	Ma	J. W. Potter
GA race 1	Ma	Ma	Ma	R. S. Hussey
NC EM race 2	Ma	Ma	Ma	K. R. Barker
SC 83 race 2	Ma	Ma	Ma	S. A. Lewis
SC Florence race 2	Ma	Ma	Ma	B. A. Fortnum
SC Govan race 2	Ma	Ma	Ma	S. A. Lewis
SC Rawl race 2	Ma	Ma	Ma	S. A. Lewis
M. hapla				
NC	Mh	Mh	Mh	K. R. Barker
VA	Mh	Mh	Mh	E. L. Davis
WI	Mh	Mh	Mh	S. R. Koenning
M. incognita				
GA	Mi	Mi	Mi	R. S. Hussey
IA	Mi	Mi	Mi	E. L. Davis
NC 3-99	Mi	Mi	Mi	K. R. Barker
NC race 1	Mi	Mi	Mi	K. R. Barker
NC race 2	Mi	Mi	Mi	K. R. Barker
NC race 3	Mi	Mi	Mi	K. R. Barker
NC race 4	Mi	Mi	Mi	K. R. Barker
NC cotton	Mi	Mi	Mi	K. R. Barker
SC cotton	Mi	Mi	Mi	S. A. Lewis
SC Edisto	Mi	Mi	Mi	S. A. Lewis
TN	Mi	Mi	Mi	E. C. Bernard
M. javanica				
AZ a	Mj	Mj	Mj	M. A. McClure
AZ b	Mj	Mj	Mj	M. A. McClure
FL	Mj	Mj	Mj	D. W. Dickson
GA	Mj	Mj	Mj	R. S. Hussey
NC	Mj	Mj	Ma	K. R. Barker

[z]Phenotypes considered to be specific for the four species are designated with the letters, Ma-*M. arenaria*, Mh-*M. hapla*, Mi-*M. incognita*, and Mj-*M. javanica*.

PCR conditions were as follows: 96°C for 1 minute, 34°C for 1 minute, and 72°C for 2 minutes using 41 cycles. PCR amplification products were electrophoretically fractionated on 1.5% agarose gel, DNA bands were visualized by UV illumination after ethidium bromide staining. Approximately 120 random 10-mer primers (Operon Technologies, Inc., Alameda, CA) were tested on the 26 nematode isolates.

DNA sequencing and species-specific primer tests: Potential species-specific bands from RAPD reactions were cloned into pGEM-T easy vector (Promega, Inc., Madison, WI). Forward and reverse DNA sequencing were conducted using ABI PRISM BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Inc., Foster City, CA). We sequenced six clones from *M. arenaria*, nine clones from *M. hapla*, 26 clones from *M. incognita*, and 37 clones from *M. javanica*. Candidate primer pairs for each of the four *Meloidogyne* species and with melting temperatures of approximately 50°C were designed from the sequences obtained. Four primer pairs for *M. arenaria*, three primer pairs for *M. hapla*, 11 primer pairs for *M. incognita* and 15 primer pairs for *M. javanica* were commercially synthesized to test species specificity (Integrated DNA Technologies, Inc., Coralville, IA). In the PCR reactions using species-specific primers, the following conditions were used: 96°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes using 26 cycles. The reaction components and volumes were the same as those employed for RAPD-PCR.

RESULTS

Nematode isolates: Species identification was the same for perineal pattern analysis and esterase phenotyping in all isolates (Table 1). Identification using SOD and esterase isozymes were consistent with the above typing, with one exception: An NC isolate exhibited the *M. arenaria* phenotype with SOD and the *M. javanica* phenotype with esterase staining and perineal pattern analysis (Table 1).

RAPD-PCR and polymorphism identification: In some RAPD reactions, distinct polymorphic phenotypes were observed among the four common *Meloidogyne* species. Fig. 1A shows the polymorphisms among nematode isolates tested using the primer 5'-AGAATCCGCC-3'. Variations among different isolates of each species were also observed, e.g., the random primer 5'-AAAACCGGGC-3' produced variation in *M. arenaria* and *M. hapla* (Fig. 1B). Usually, the RAPD products from the three *M. hapla* isolates were the most variable. Variations among different isolates of *M. arenaria* were also detected. For example, the *M. arenaria* race 1 isolate from Georgia was distinct from the *M. arenaria* race 2 isolates in a RAPD reaction using 5'-AGAATCCGCC-3' primer (Fig. 1A).

Species-specific PCR primers: The primer pairs intended for diagnosis of *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* were designed and screened for species specificity. The species-specific sequence tagged sites were selected with primer pairs listed in Table 2. Species-specific PCR products were produced by the designed primer pairs and resulted in unique band(s) for each of the four species (Fig. 2A-D). The species-specific primers produced a single band (~1 500 bp) for the *M. hapla* isolates (Fig. 2B). Among the seven *M. arenaria* isolates, a single ~950 bp band was amplified (Fig. 2A). In *M. incognita* reactions, there were doublet bands (both ~1 350 bp, differing by ~20 bp in size) produced from each of the eleven *M. incognita* isolates (Fig. 2C). For *M. javanica*, a single band (~1 650 bp) was produced in four out of the five *M. javanica* isolates. The NC nematode isolate did not amplify a PCR product with the chosen primer pair (Fig. 2D).

Fig. 1A.

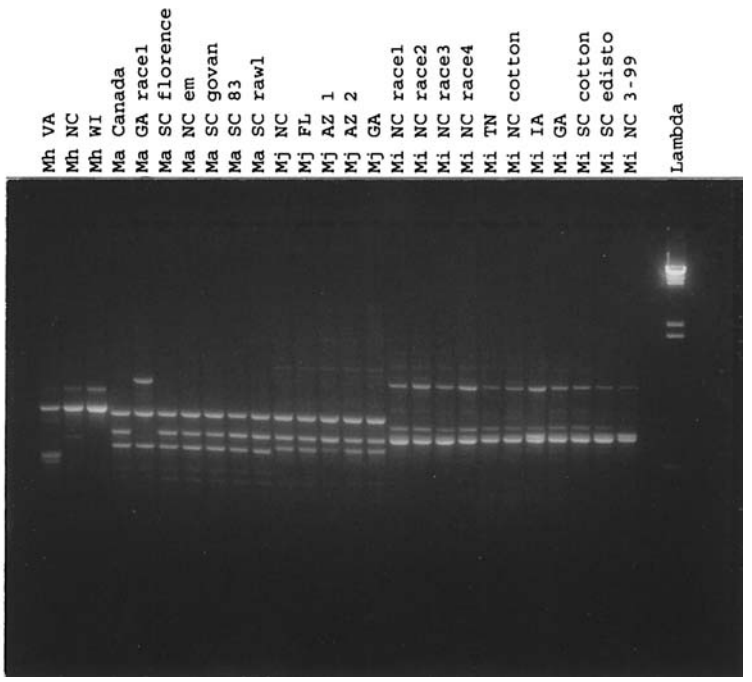

Fig. 1B.

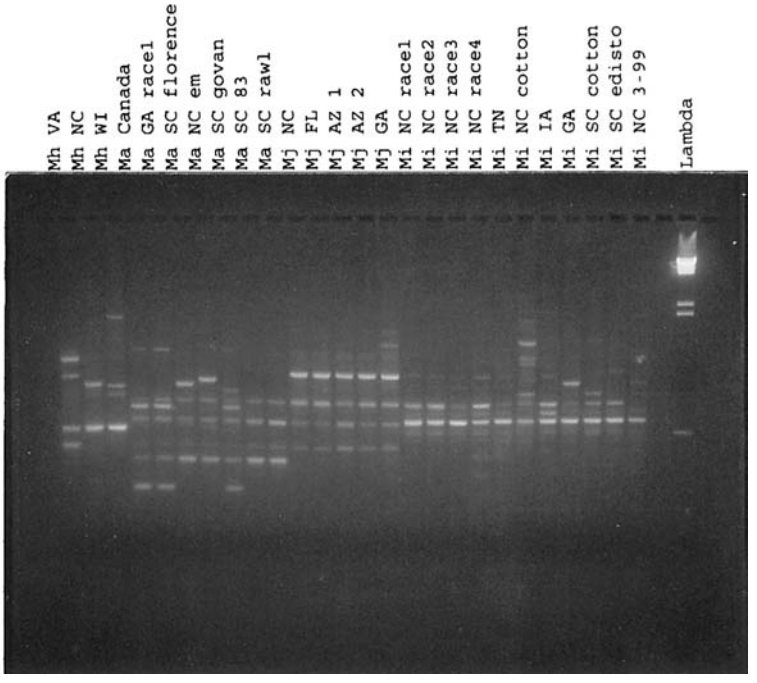

Fig. 1. (A) RAPD electrophoretic patterns of the 26 tested root-knot nematode isolates of Ma-*M. arenaria*, Mh-*M. hapla*, Mi-*M. incognita*, and Mj-*M. javanica* from primer AGAATCCGCC; (B) RAPD electrophoretic patterns of the 26 tested root-knot nematode isolates of Ma-*M. arenaria*, Mh-*M. hapla*, Mi-*M. incognita*, and Mj-*M. javanica* from primer AAAACCGGGC.

Table 2. The species-specific PCR primer pairs for the four *Meloidogyne* spp.

Species	Sequence of Primer Pairs (5'- 3')
M. arenaria	TCGAGGGCATCTAATAAAGG
	GGGCTGAATATTCAAAGGAA
M. hapla	GGCTGAGCATAGTAGATGATGTT
	ACCCATTAAAGAGGAGTTTTGC
M. incognita	TAGGCAGTAGGTTGTCGGG
	CAGATATCTCTGCATTGGTGC
M. javanica	CCTTAATGTCAACACTAGAGCC
	GGCCTTAACCGACAATTAGA

DISCUSSION

A PCR method for the identification of the major root-knot nematode species, *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*, is described in this report. Species-specific PCR primers were developed and each primer pair amplified discriminatory DNA fragment(s) for each species. Zijlstra *et al.* (2000) recently published details on three pairs of species-specific primers for the identification of *M. arenaria*, *M. incognita*, and *M. javanica* using sequence characterized amplified region (SCAR). The nematode isolates used in that study were mainly from European and African countries. Due to the variations observed among populations of these economically important nematodes, additional information for species identifications should be valuable. The different sets of primer pairs reported here have been tested on multiple isolates of each species, primarily based on North American nematode populations. These four PCR primer pairs will be useful for routine identification of these four *Meloidogyne* species in nematology laboratories.

Polymorphism within the species *M. arenaria* was detected in some RAPD reactions, with the nematode isolate *M. arenaria* GA race 1 isolate exhibiting some different phenotypes compared with other isolates of the same species. Because it was the only race 1 isolate included in this test, it will be necessary to examine a broad range of isolates of the two races in *M. arenaria* before the race level specificity can be confirmed.

Genetic variations within *M. javanica* have also been reported (Abdel-Momen *et al.*, 1998). Some *M. javanica* isolates are able to parasitize peanut (Carneiro *et al.*, 1998). Esbenshade and Triantaphyllou (1985a) reported that the phenotypes of esterase (J3) and SOD (JA2) correlate perfectly among different isolates of *M. javanica*, but within the *M. arenaria* isolates (esterase A1-A3), variations for SOD phenotypes are observed (JA2 and A4) (Esbenshade and Triantaphyllou, 1985a). Here, the five *M. javanica* isolates all showed typical esterase phenotypes (J3), but the SOD phenotype of the NC isolate was a *M. arenaria* (A4) type (gel not shown). Furthermore, primer pairs developed in this experiment for *M. javanica* and *M. arenaria* did not amplify a DNA fragment from this isolate. Based on most of the generated RAPD electrophoretic banding patterns, the NC isolate still would be grouped into *M. javanica* species. The variations among *M. javanica* await further study.

Combining several species-specific primer pairs in a single PCR reaction cocktail would be a highly valued technique for the field nematode isolate identification. Williamson *et al.* (1997) conducted a multiplex assay using a *M. chitwoodi* and *M. hapla* primer combination, and easily distinguished the two species. The four species-specific primer pairs developed in this study were also combined for a multiplex assay. However, the four primer pairs resulted in primer interactions and the species-specific PCR bands were not produced. Simplifying species-specific PCR identification will need further investigation.

Fig. 2A.

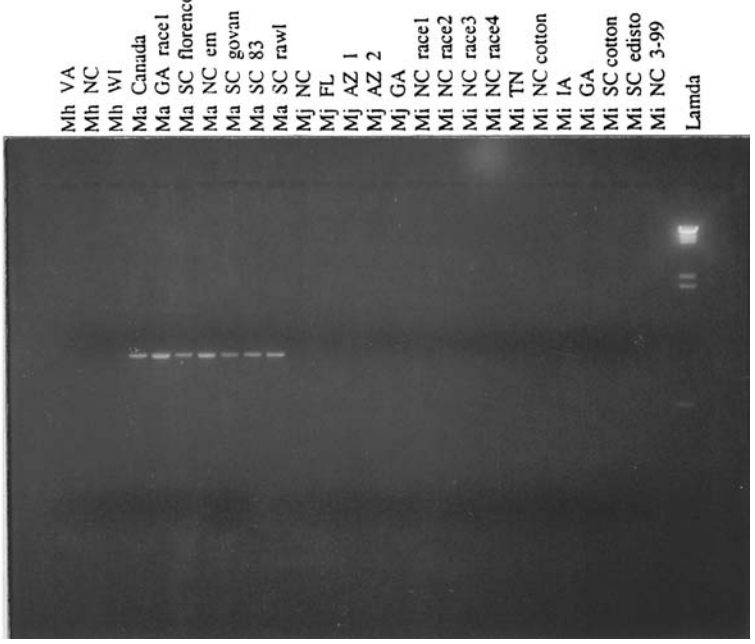

Fig. 2B.

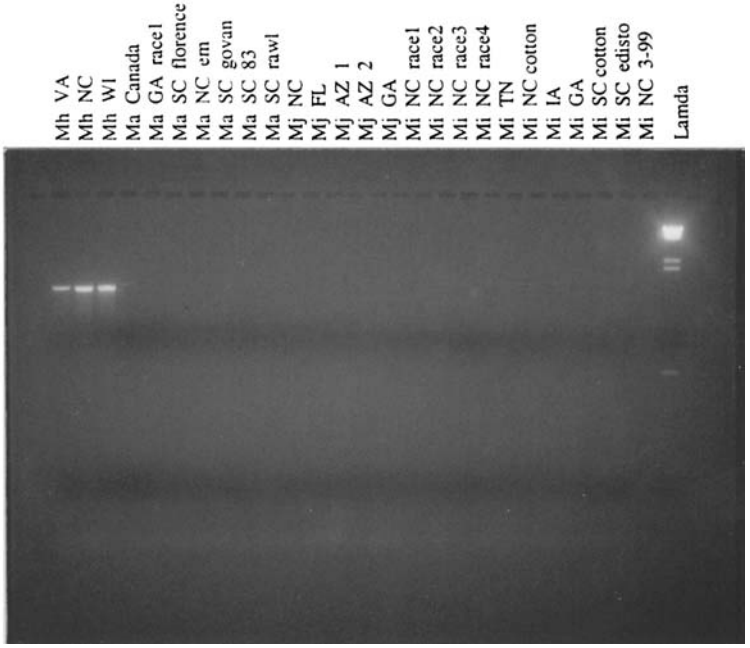

Fig. 2. (A) Species-specific PCR electrophoretic patterns of the 26 tested root-knot nematode isolates of Ma-*M. arenaria*, Mh-*M. hapla*, Mi-*M. incognita*, and Mj-*M. javanica* from the *M. arenaria* specific primer pair; (B) Species-specific PCR electrophoretic patterns of the 26 tested root-knot nematode isolates of Ma-*M. arenaria*, Mh-*M. hapla*, Mi-*M. incognita*, and Mj-*M. javanica* from the *M. hapla* specific primer pair.

Fig. 2C.

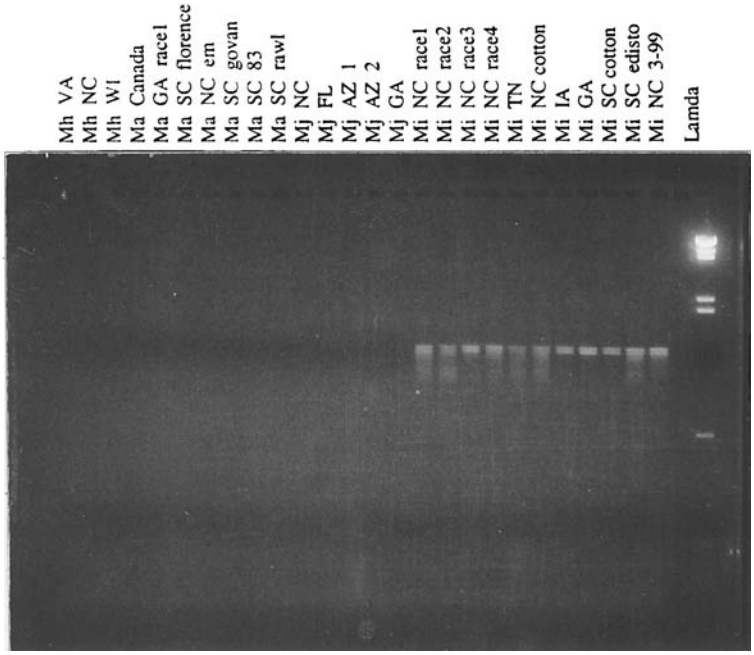

Fig. 2D.

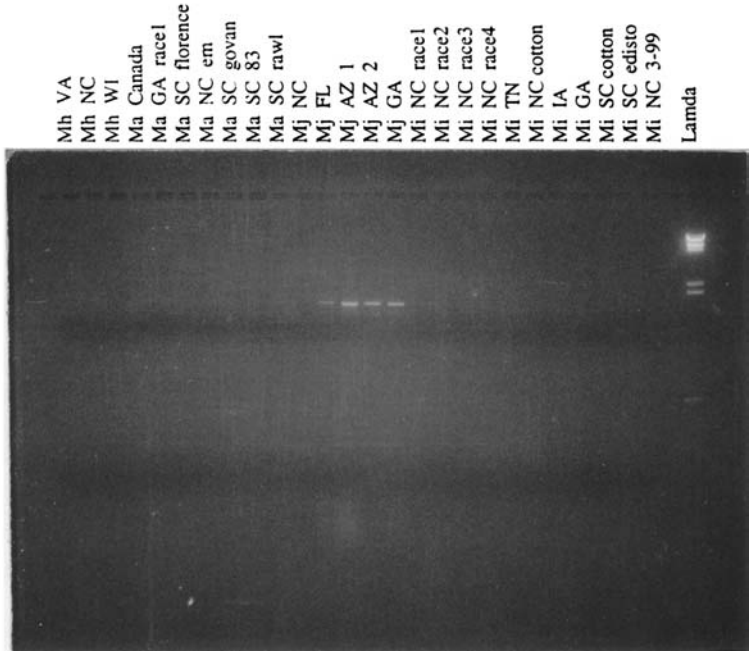

Fig. 2. (C) Species-specific PCR electrophoretic patterns of the 26 tested root-knot nematode isolates of Ma-*M. arenaria*, Mh-*M. hapla,* Mi-*M. incognita,* and Mj-*M. javanica* from the *M. incognita* specific primer pair; (D) Species-specific PCR electrophoretic patterns of the 26 tested root-knot nematode isolates of Ma-*M. arenaria*, Mh-*M. hapla*, Mi-*M. incognita,* and Mj-*M. javanica* from the *M. javanica* specific primer pair.

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