

Amplified fragment length polymorphisms of *Meloidogyne* spp. using oligonucleotide primers

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Summary – The polymerase chain reaction (PCR) method was applied to determine the identity and infraspecific forms of the root-knot nematodes *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. Amplified fragment length polymorphisms (AFLP) from primers Cyto 1, Cyto 2, Rpmc 62 and Rpmt 63 showed that the *Meloidogyne* populations tested had genome heterogeneity. With the Cyto 1 and Cyto 2 primers, the amplified regions at 0.54, 0.95, 1.10, and 1.20 kb were highly conserved between tested populations. The bands at 2.00 kb and 2.50 kb were species specific for *M. incognita* and the bands at 1.65 kb differentiated *M. hapla* and *M. javanica* from *M. incognita* and *M. arenaria*. The band at 0.85 kb, amplified from primer Rpmc 62 and Rpmt 63, could be a specific band for *M. incognita* race 4. The diversified sequences that were showed by species-specific and race-specific DNA fragments could have practical application as diagnostic probe sequences and in the study of phylogenetic relationships.

Résumé – Polymorphismes de longueurs de fragments d'ADN amplifiés avec des amorces oligonucléotidiques chez *Meloidogyne* spp. – La réaction d'amplification en chaîne par la polymérase (PCR) a été utilisée pour identifier les espèces et les formes sub-spécifiques des nématodes cécidogènes *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*. La longueur des fragments amplifiés en utilisant les amorces Cyto 1, Cyto 2, Rpmc 62 et Rpmt 63, était polymorphique (AFLP) chez les différentes populations de *Meloidogyne*, suggérant une hétérogénéité au niveau du génome. Les amorces Cyto 1 et Cyto 2 ont amplifié des fragments de 0,54, 0,95, 1,10, et 1,20 Kb, très fortement conservées chez les populations testées. Les bandes à 2,00 et à 2,50 Kb étaient spécifiques de *M. incognita*, et les bandes à 1,65 Kb ont permis de séparer *M. hapla* et *M. javanica* de *M. incognita* et *M. arenaria*. La bande à 0,85 Kb, amplifiée en utilisant les amorces Rpmc 62 et Rpmt 63, pourrait être spécifique à la race 4 de *M. incognita*. Les séquences divergentes des fragments d'ADN spécifiques d'espèces ou de races, pourraient être utilisées comme sondes de diagnostic, et pour les études de relations phylogéniques.

Key-words : AFLP, *Meloidogyne*.

Management strategies for *Meloidogyne* in a sustainable agricultural system include the use of resistant cultivars and of crop rotation. The success of these methods depends on the ability to identify species and races of these nematode pathogens.

Conventional identification of *Meloidogyne* is based on morphological characters and, more recently, on biochemical discrimination (Esbenshade & Triantaphyllou, 1985, 1990; Hussey, 1985; Fargette, 1987; Schots *et al.*, 1990; Venkatachari *et al.*, 1991). However, the diagnostic characters have certain limitations because they often vary considerably within a population and there is overlap of characters among species and among races. To overcome some of these problems for *Meloidogyne* populations molecular genetic differentiation has been utilized (Curran *et al.*, 1985, 1986; Hyman *et al.*, 1990; Castagnone-Sereno *et al.*, 1991; Garate *et al.*, 1991; Xue *et al.*, 1992). The polymerase chain reaction (PCR),

using fairly conserved primers, is a powerful technique and amplifies specific DNA sequences very rapidly. The amplified fragment length polymorphisms (AFLP) from genomic DNA can be used directly in diagnostics to differentiate nematodes from small samples in a short period.

The objective of the research was to differentiate the species and races of root-knot nematode by employing DNA amplification using oligonucleotide primers and direct viewing AFLP's from genomic DNA.

Materials and methods

NEMATODE SOURCES AND CULTURES

Populations of *Meloidogyne incognita* races 1, 2, 3 and 4, *M. arenaria* races 1 and 2, *M. javanica* and *M. hapla* were kindly provided by Dr. M. A. McClure (University

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of Arizona, Tucson, USA), Dr. D. Orion (Volcani Center, Bet Dagan, Israel), and Dr. K. R. Barker, Dr. J. N. Sasser and Dr. A. C. Triantaphyllou (North Carolina State University, Raleigh, USA) (See Table 1). The identities of the *Meloidogyne* species were confirmed morphologically by Dr. B. A. Ebsary (Research Station, Agriculture Canada, Ottawa).

Table 1. Source and biological origin of *Meloidogyne* species and races used in the experiments.

Species and race number	Locality, host of origin and/or strain No.	Laboratory source
<i>M. incognita</i>		
Mi1M	Arizona	M. A. McClure
Mi1B	Asparagus, NC	K. R. Barker
Mi2B	NC No. 632	K. R. Barker
Mi3M	Arizona	M. A. McClure
Mi3O	Israel	D. Orion
Mi3S	Tomato	J. N. Sasser
Mi4B	Tobacco, NC	K. R. Barker
<i>M. arenaria</i>		
Ma1S	Tomato	J. N. Sasser
Ma2T	Georgia No. 3145	A. C. Triantaphyllou
<i>M. javanica</i>		
MjT	NC No. 7	A. C. Triantaphyllou
MjS	Tomato	J. N. Sasser
<i>M. hapla</i>		
Mh1-2S	Tomato	J. N. Sasser

The nematodes were maintained in potted (sand : silt loam = 1 : 1) greenhouse tomato plants (*Lycopersicon esculentum* Mill) cv. Roma (Pacific Northwest Seed Co. Inc., Vernon, British Columbia) and cv. Bonny Best (Dominion Seed House, Georgetown, Ontario), fertilized (N.P.K. = 4.16.16) every 3 weeks and watered with tap water on alternate days or as required depending on the soil moisture.

Three week-old tomato seedlings were inoculated with one of the *Meloidogyne* isolates by adding nematode eggs or freshly hatched juveniles to the soil. The inoculated plants were allowed to develop for 6-15 weeks before use. *M. incognita* race 3, *M. arenaria* race 1 and *M. javanica* were each increased from single egg masses on cv. Bonny Best, and subsequently on cv. Rutgers.

NEMATODE EXTRACTION, DNA ISOLATION AND QUANTIFICATION

Meloidogyne eggs were extracted from tomato roots by the modified sodium hypochlorite-differential cen-

trifugation-sieve procedure (McClure *et al.*, 1973) using 0.5 % sodium hypochlorite solution, stirred with an electric blender at maximum speed for 40 s and sieved through nested 425 μ m, 75 μ m and 26 μ m sieves. The eggs were washed and stored and the DNA extracted as described in Xue *et al.* (1992). The DNA solution was extracted with chloroform-isoamyl alcohol, and precipitated with 8 M ammonium acetate and 95 % ethanol (Maniatis *et al.*, 1982). The concentration and purity of the DNA were determined using spectrophotometric measurement at 260 nm and comparing the intensity of fluorescence emitted by ethidium bromide with that of DNA standards (Sambrook *et al.*, 1989).

OLIGONUCLEOTIDE PRIMERS AND PURIFICATION

Several primers were tested to amplify *Meloidogyne* DNA. The following five regions were tested with the primers, the COII and 12s gene in the mitochondrial genome (primers for both obtained from Dr. A. Wilson, University of California, Berkeley), the heat shock 70 A gene, hsp 70 A-B (determined by homology with *Caenorhabditis elegans*) and the 18s ribosomal genes and the intergenic transcribed spacer in the nuclear genome (primers for both obtained from Dr. T. Vrain, Agriculture Canada, Vancouver). The sequences for primers that amplify the heat shock 70 A gene are in Beckenbach *et al.* (1992). One pair of the primers, Cyto 1 and Cyto 2, was designed based on a comparison of sequences in mitochondrial DNA fragments (Clary & Wolstenholme, 1985). The primer Cyto 1 has 21 bp nucleotides with sequence from 5' to 3' of GAT CGC AGA TTA GTG CAA TGG. The primer Cyto 2 has 20 bp nucleotides with sequence from 5' to 3' of GAT CAA GAG ACC AGT ACT TG. Primers Rpmc 62 and Rpmt 63 were designed as random primers for amplifying populations of *Meloidogyne*; primer Rpmc 62 has 11 bp nucleotides with sequence from 5' to 3' of GAC TCC ACG AC and the primer Rpmt 63 is 11 bp nucleotides with sequence from 5' to 3' of CGA GCC CCT GT. The primers were synthesized on a 391 DNA synthesizer, PCRmate EP (Applied Biosystems Co.).

Synthetic oligonucleotide primers were purified using C-18 Sep-Pak Cartridges following the instruction from Waters Associates. The oligonucleotide primers were eluted with 2 ml of 20 % acetonitrile, evaporated to dryness, rinsed with 90 % ethanol and re-dried with a Speed Vac drying machine (SVC 100, Savant Instrument Co.). Purified primers were dissolved in 100 μ l water and further quantified and used for DNA amplification.

AMPLIFICATION CONDITIONS

PCR amplification was performed using the thermostable AmpliTaq recombinant Taq DNA polymerase. The working solution included 0.1 μ g of DNA, 1 X Taq reaction buffer, 200 μ M each of dATP, dGTP, dTTP and dCTP, 1.30 μ M of each primer, and 0.3 units of AmpliTaq DNA polymerase. The 1 X Taq reaction

buffer was composed of 10 mM Tris HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂ and 0.01 % (w/v) gelatin (Sigma).

The reactions were performed with 50 µl of reaction mixture in 0.5 ml polypropylene microcentrifuge tubes. The tubes were siliconized by 5 % dichlorodimethylsilane in chloroform. Before the reactions were processed, the tubes were dry-sterilized to eliminate nucleases and minimize contamination.

Amplification was conducted in the programmable cyclic reactor model PCR TCX 15. Optimum amplification conditions were determined by varying the denaturation, annealing, and extension temperatures and the number of cycles. The first step was initial melting at 94 °C for 80 s, annealing at 40-47 °C for 2 min and extending at 72 °C for 2 min (one cycle); second step melting at 92 °C for 30 s, annealing at 40-47 °C for 1 min and extending at 72 °C for 1-2 min (35 cycles); third step melting at 92 °C for 30 s, annealing at 40-47 °C for 1 min and extending at 72 °C for 10 min (one cycle). The above annealing temperatures were changed to 40 °C and 47 °C for primers Rpmc 62-3 and Cyto 1-2, respectively.

The amplified DNA fragments were separated on a 1.2 % agarose gel (Agarose low EEO, Boehringer Mannheim Biochemicals) with standard one kb markers (BRL), stained with ethidium bromide and viewed under 260 nm UV light.

Results

The primers, CoIIA-B, Hsp70A1-Hsp70A2, 12sA-12sB, and 18XR1-18XR2 were used to amplify the regions of COII and 12s gene in the mitochondrial genome, the heat shock 70A, 18s ribosomal genes and the intergenic transcribed spacer in the nuclear genome. These primers amplified only one or two bands, and are

not very promising for the differentiation of species (Fig. 1). The primers 18XRA and 18XRB, which originated from *Xiphinema* nematodes, gave a band at 0.95 kb. This band was digested with different enzymes such as EcoRI, HindIII and Xba I, and showed no differences between species. The sequence close to the end of 26s ribosome DNA was obtained by the method of primer-directed sequencing, but there were no nucleotide differences.

The primers Cyto 1-Cyto 2 and Rpmc 62-Rpmt 63 amplified *Meloidogyne* DNA, and several bands were produced (Figs 2, 4). The concentration of magnesium (3 mM MgCl₂) and the annealing temperatures were critical for obtaining multiple bands in this and subsequent experiments. The optimum annealing temperatures were 40 °C for primers Rpmc 62 and Rpmt 63 and 47 °C for primers Cyto 1 and Cyto 2.

Under optimum amplification conditions DNA from different species and races was amplified using the primers Cyto 1 and Cyto 2 and the results are shown in Figure 3. The major bands at 0.95 kb and the weak bands at 1.10 and 1.20 kb were identical for all the species and races. Strong bands at 2.00 kb and 2.50 kb were identical for all populations of *M. incognita*, but were weak or absent for the populations of *M. arenaria*, *M. hapla*, and *M. javanica*. The band pattern for *M. incognita* was distinctive and differentiated this species from the other *Meloidogyne* species tested. The bands at 0.70 kb, present in *M. arenaria*, *M. hapla* and *M. javanica*, were not present in *M. incognita*. The bands at 0.65 kb and 0.60 kb differentiated the species of *M. arenaria*, *M. hapla* and *M. javanica*, in that the 0.60 kb band was species specific to *M. javanica* and the 0.65 kb band was specific to *M. arenaria* and *M. hapla*. These latter two species could be differentiated on the basis of a band at 2.60 kb in *M. arenaria*.

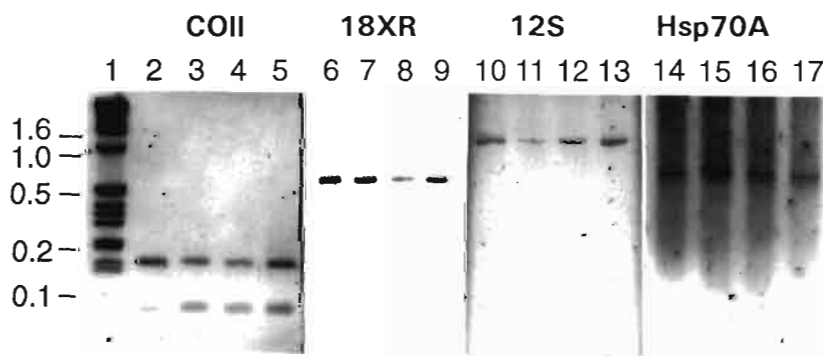


Fig. 1. Gel showing amplified DNA products with different primers; Col. 1, 1 kb marker; Cols. 2, 6, 10, and 14 were *Meloidogyne incognita* race 3; Cols. 3, 7, 11, and 15 were *M. arenaria* race 2; Cols. 4, 8, 12, and 16 were *M. hapla*; Cols. 5, 9, 13, and 17 were *M. javanica*; Cols. 2-5, 6-9, 10-13, and 14-17 were products amplified by the primers, COIIA-B, 18XR1-18XR2, 12sA-12sB, and Hsp70A1-Hsp70A2, respectively, with molecular weights of 0.17, 0.90, 1.50 and 0.80, respectively. The agarose gels were 1.2 % and loaded with 4 µl of amplified products and 1 µl of loading buffer.

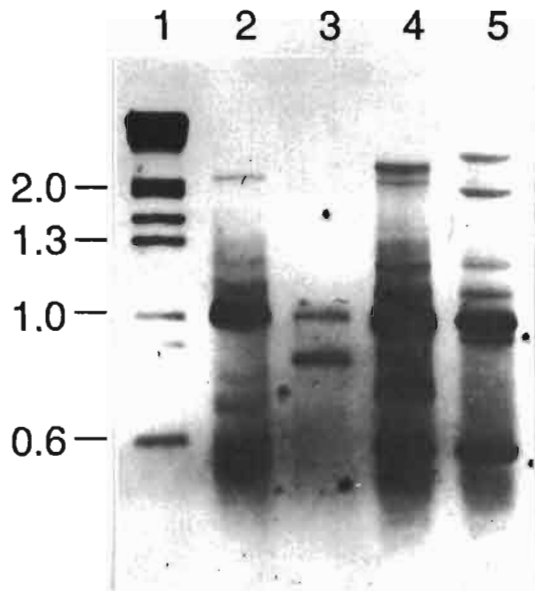


Fig. 2. Gel showing amplified DNA products with Cyto 1 and Cyto 2 primers. Col. 1, 1 kb marker; col. 2, *Meloidogyne javanica*; col. 3, *M. hapla*; col. 4, *M. arenaria* race 2; col. 5, *M. incognita* race 3. The agarose gels were 1.2% and loaded with 4 μ l of amplified products and 1 μ l of loading buffer.

From Figure 3, it is clear that there were both inter- and intra-specific band differences among the populations tested. The bands at 0.80 kb in cols 3 and 4 were present only in race 1, but not in other races of *M. incognita*. Repeated experiments showed that these bands were stable for populations of race 1 and were race specific. Race 2 had a weak band at 0.75 kb in col. 5, this band was also present in race 3. Two populations of race 3 had specific bands at 1.50 kb (cols 6, 7), but another population of race 3 (col. 8) did not have this band. Race 4 of *M. incognita* was similar to race 3 but lacked the distinctive bands at 0.75 and 1.50 kb. The two populations of *M. arenaria* in cols 11 and 12 had similar band patterns except that the highest molecular weight band had a slight shift and the bands in col. 12 were weak. One population of *M. hapla* had a band at 1.40 kb in col. 13 which did not appear in the population in col. 14. However, this band is not a specific population character because it was not stable between replications. The two populations of *M. javanica* in cols 15 and 16 had identical band patterns.

Consistent amplified fragments were obtained from a range of 0.50 μ g genomic DNA to 50 pg genomic DNA from *M. javanica* using primers Rpmc 62 and Rpmt 63 (Fig. 4). Figure 5 shows the amplified DNA fragments from different species and races using Rpmc 62 and Rpmt 63 primers. The weak bands at 0.60 and 0.70 kb were identical across all tested species and populations. The 1.40 kb band was identical for all populations of *M. incognita* and *M. arenaria* tested, but for *M. hapla*

and *M. javanica* 1.40 kb-band shifted slightly. Both *M. hapla* and *M. javanica* have a strong band at 1.65 kb which does not exist in *M. incognita* and *M. arenaria*. *M. javanica* has two bands at 0.85 kb and 0.30 kb that differentiate *M. javanica* populations from those of *M. hapla*. Both *M. hapla* and *M. arenaria* have one band that is less than 0.2 kb that is distinctive for these two species. Within *M. incognita*, the race 1 population has two bands close to 0.30 kb that are distinctive and are absent from *M. incognita* races 2, 3 and 4. *M. incognita* race 4 has a band at 0.85 kb that is not present in *M. incognita* races 1, 2 and 3.

Discussion

The discrimination of species and races among several *Meloidogyne* populations has been demonstrated using PCR amplification and analysis of the DNA fragment polymorphisms. Our study, using Cyto 1 and Cyto 2 primers, shows at the species level strong DNA fragment bands at 2.00 kb and 2.50 kb for all populations of *M. incognita*, that may be related to mtDNA, which clearly differentiate this species from the other *Meloidogyne* species tested. Moreover, the band at 0.70 kb, present in *M. arenaria*, *M. hapla* and *M. javanica*, is not present in *M. incognita*. The 0.60 kb band is specific to *M. javanica* and the 0.65 kb band specific to *M. arenaria* and *M. hapla*. Amplification using Rpmc 62 and Rpmt 63 primers shows a strong band at 1.65 kb for *M. hapla* and *M. javanica* that differentiates these two species from *M. incognita* and *M. arenaria*. Differences at the race level were shown by the amplified DNA fragment patterns. The 0.80 kb DNA fragment (Fig. 3) and the DNA fragments close to 0.30 kb (Fig. 5) in each of two populations of *M. incognita* race 1 differentiates this race from the other three *M. incognita* races. The 0.85 kb band, amplified from primers Rpmc 62 and Rpmt 63, is a specific band for *M. incognita* race 4.

Primers for use as in *Meloidogyne* differentiation can be chosen from heterologous or random DNA sequences (Williams *et al.*, 1990). Although the conserved sequences amplified by primers, such as COIIA-B, Hsp70A1-Hsp70A2, 12sA-12sB, and 18XR1-18XR2 used in the study, did not show amplified fragment polymorphisms sufficient for species and race differentiations, such results could probably be obtained from sequencing their amplified products. The pair of primers, Cyto 1 and Cyto 2, was originally generated from comparison of mtDNA sequences (Clary & Wolstenholme, 1985), and the amplified products arising from this pair of primers showed that the *Meloidogyne* populations had some heterogeneity in their genomes (Fig. 3). The primer amplification showed major bands at 0.95 kb and 0.54 kb and weak bands at 1.10 kb and 1.20 kb that were highly conserved between the populations of *Meloidogyne*. There were no differences in the conserved bands between the four species, *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica*.

Consistent, repeatable and clear race differences are not as easy to distinguish as are those for species. At the race level in *M. incognita*, as shown in Figure 3, there are only minor band differences present in races. However, some of the minor bands are stable within the races, such as 0.80 kb band in race 1, which could be a race-specific band. Some minor bands are not stable within races, such as 1.50 kb band in race 3, which is present in two populations of race 3, but not in another population. The bands showing population differences within this race cannot be used alone as race-specific diagnostic characters, but can be used in combination with several other bands. The 0.85 kb band amplified from primer Rpmc 62 and Rpmt 63 appears as a specific band for *M. incognita* race 4 (Fig. 5). Further research is needed to provide race-specific primers for the several races within *Meloidogyne* species and to relate this to race virulence.

The band pattern and signal strength of *Meloidogyne* AFLP from the primers Cyto 1, Cyto 2, Rpmc 62 Rpmt 63 showed considerable variation among the nematode species and races. As well, genetic variation exists within both species and races as shown in Figs 3 and 5. In Fig. 5, both *M. incognita* race 1 and *M. javanica* have the two bands close to 0.30 kb, which might show genetic or evolution trails between the populations. These AFLPs may be useful as genetic characters or markers for future studies of the species or of population relationships of root-knot nematodes.

When Harris *et al.* (1990) used the PCR method to amplify a specific 1.8 kb sequence of mitochondrial DNA from different *Meloidogyne* populations, the restriction digest of the amplified product with *Hinf* I discriminated four species. In the experiments we described here with direct viewing of the PCR-AFLP's, the discrimination of four species and several of their races was obtained from genomic DNA within one working day. Moreover, the direct viewing without using restriction endonucleases keeps the costs of analyzing samples to a minimum.

Use of the PCR technique for nematode identification and diagnosis has the advantage of being sufficiently sensitive to resolve differences between closely related populations from small samples of nematodes and to do so rapidly. Although the results of PCR amplification are greatly affected by the reaction conditions such as the temperature and the magnesium concentration, a PCR based approach to the identification of *Meloidogyne* species and races usefully complements traditional morphological methods and should be taxonomically and diagnostically practical.

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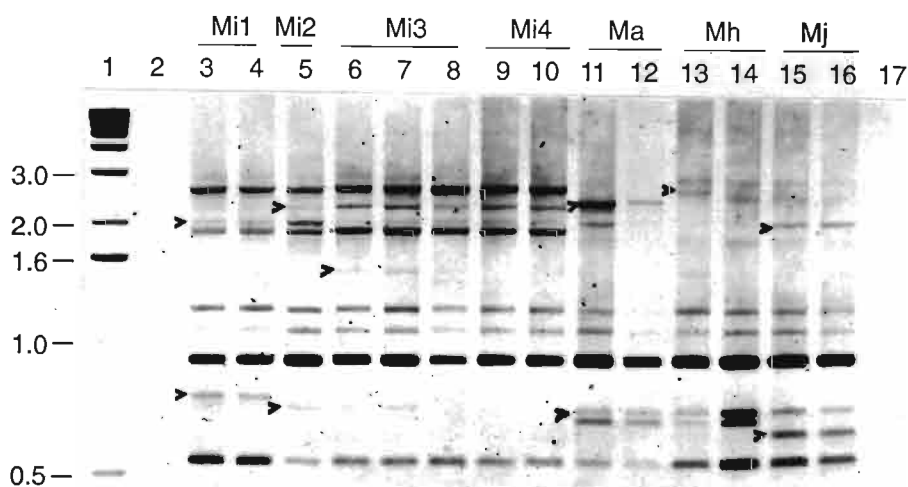


Fig. 3. Gel stained with ethidium bromide, showing amplified DNA products from different populations of *Meloidogyne* with Cyto 1 and Cyto 2 primers. Col. 1 was standard marker of one kb; cols. 2 and 17 were standard control without primers or DNA; cols. 3 and 4 were *M. incognita* race 1; col. 5 was *M. incognita* race 2; cols. 6 to 8 were *M. incognita* race 3; cols. 9 and 10 were *M. incognita* race 4; cols. 11 and 12 were *M. arenaria*; cols. 13 and 14 were *M. hapla*; cols. 15 and 16 were *M. javanica*. The arrows identify the bands that show major differences between the species and races. The numbers on the left-hand margin show molecule weight in kb. The agarose gels were 1.2 % and loaded with 4 μ l of amplified products and 1 μ l of loading buffer.

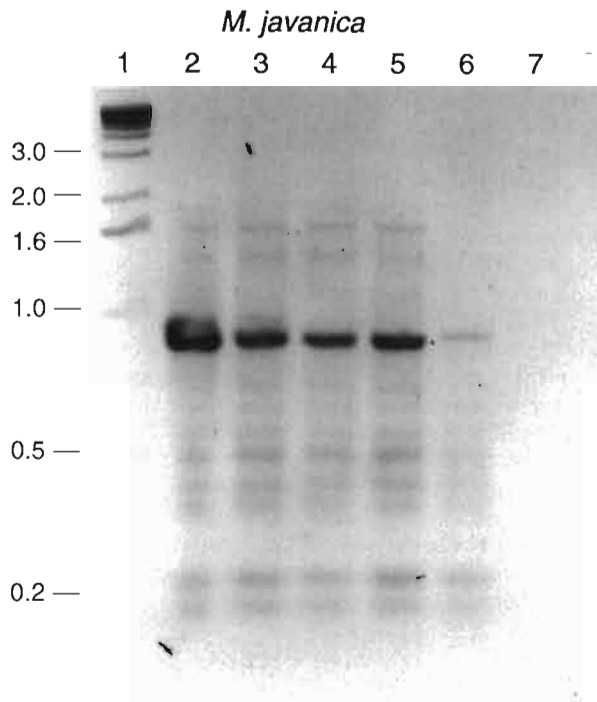


Fig. 4. Gel stained with ethidium bromide, showing amplified DNA products from *Meloidogyne javanica* with primer Rpmc 62 and Rpmt 63. Col. 1 was standard marker of one kb (BRL). Col. 2 was amplified product from about 0.50 µg of the original DNA of *M. javanica*. Cols. 3, 4, 5, 6 and 7 were amplified product from 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ dilution of the original DNA respectively. At an annealing temperature of 40 °C and standard conditions as shown in the text.

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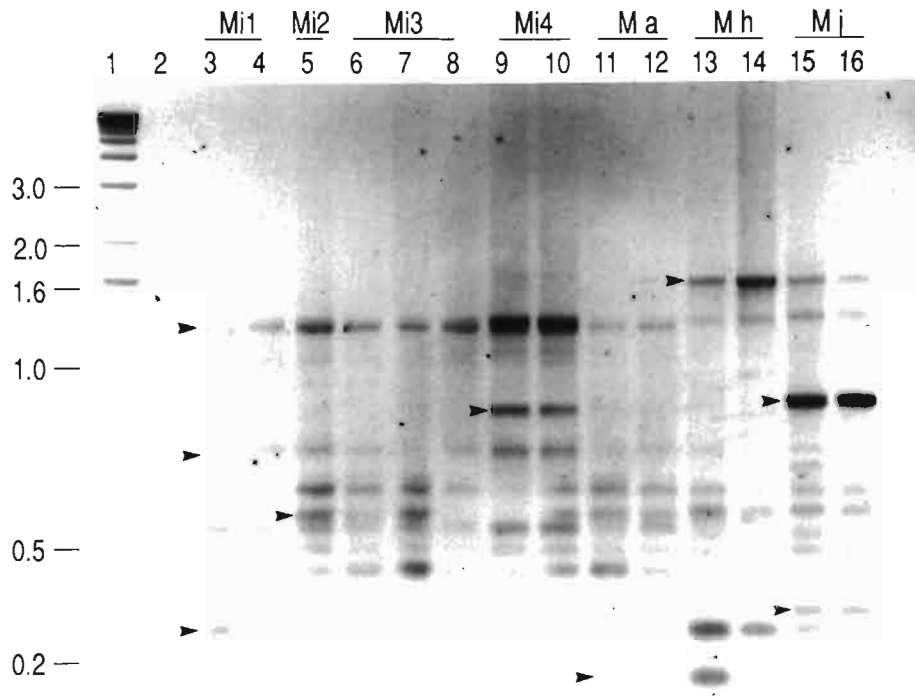


Fig. 5. Gel stained with ethidium bromide, showing amplified DNA fragment polymorphisms from different populations of *Meloidogyne* using Rpmc 62 and Rpmt 63 primers. Col. 1 was standard one kb markers. Col. 2 was standard control without target DNA; cols. 3 and 4 were *M. incognita* race 1; col. 5 was *M. incognita* race 2; cols. 6 to 8 were *M. incognita* race 3; cols. 9 and 10 were *M. incognita* race 4; cols. 11 and 12 were *M. arenaria*; cols. 13 and 14 were *M. hapla*; cols. 15 and 16 were *M. javanica*. The arrows show the major amplified fragment differences of tested species and races. The numbers on the left-hand margin show molecule length in kb.