Ribosomal Intergenic Spacer: A Polymerase Chain Reaction Diagnostic for *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla*

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ABSTRACT

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Polymerase chain reaction amplification of the intergenic spacer region between the 5S and 18S genes from *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla* enabled these three important temperate species to be differentiated. Length polymorphism was found between *M. chitwoodi* and *M. fallax* as a result of differing numbers of short repeats located between the 5S and 18S genes. The presence of the 5S gene within the rDNA cistrons was confirmed in the *Meloidogyne* spp. included in this study. The region between the 28S and 5S genes for *M. chitwoodi* and *M. fallax* was short and lacked variability in repeated sequences compared with the main tropical *Meloidogyne* spp. and *M. hapla*. Differences in the number of these repeats resulted in intraspecific length polymorphism for *M. hapla*.

Additional keywords: repeated DNA sequences, root-knot nematodes.

Root-knot nematodes from the genus *Meloidogyne* occur throughout the world, infect all major crop plants, and cause substantial reduction in crop yield and quality (29). *M. chitwoodi* is a major pest on potato in North America and is found in the Netherlands, Portugal, Belgium, and Germany (D. Mugniéry, *personal communication*) and has been reported in South Africa (9). The closely related *M. fallax* has been recently identified in the Netherlands and France (17) as well as in New Zealand (18), Australia (22), and South Africa (9). Damage caused in the Netherlands by *M. hapla* has increased because of the reduction in the use of soil pesticides and because less cereals are being grown, reducing the effectiveness of crop rotation. However, *M. chitwoodi* and *M. fallax* are of particular concern. Their host range is wider than that of *M. hapla* and includes monocotyledonous crops, legumes, and root crops, and they are now quarantine organisms in Europe and Canada. Host races of *M. chitwoodi* have been described (20,21, 40) with considerable variation in virulence observed on resistant hosts (15). *M. chitwoodi* and *M. fallax* differ in their response to resistant genotypes of *Solanum* spp., and considerable variation in virulence has been found with populations of *M. hapla* (16).

The differences between *M. chitwoodi* and *M. fallax* compared with *M. hapla*, in terms of host ranges and therefore management strategies, combined with morphological and cytological similarity (17), emphasizes the importance of and need for an efficient, simple, molecular diagnostic for distinguishing clearly among the three species at any life cycle stage and in mixtures. The aim of this work was to develop a polymerase chain reaction (PCR)-based diagnostic as part of a EU-funded collaborative project to develop management tools for controlling *M. chitwoodi* and *M. fallax* in Europe.

Reliable diagnostic approaches, which directly target polymorphisms in the genomic DNA using the PCR, avoid the effect of life stage or environmental factors. DNA from a single nematode juvenile may be amplified by PCR. The amplified DNA obtained may then be digested and restriction fragment length polymorphisms (RFLPs) identified (2,44,45). More recent PCR-based methods have used primers that distinguish species without the requirement for a subsequent digestion step. Williamson et al. (41) synthesized PCR primers derived from the sequences of randomly amplified polymorphic DNA (RAPD) fragments that distinguished single juveniles of *M. chitwoodi* from *M. hapla*. This technique was explored further by Zijlstra (43), who identified and sequenced RAPD fragments from *M. chitwoodi*, *M. fallax*, and *M. hapla*, and designed primers to complement the terminal sequences, producing sequence characterized amplified regions. A multiplex PCR using three sets of primers was able to produce species-specific bands for *M. chitwoodi*, *M. fallax*, and *M. hapla*; however, for use with single juvenile nematodes, a subsequent PCR using nested primers was required. Other PCR methods for distinguishing some of these species have been reported using mitochondrial DNA (26,30), satellite DNA (3), and ribosomal DNA, including the internal transcribed spacer (ITS) region (44,45) and the intergenic spacer (IGS) region (24,25). At this time, no simple, single-step PCR capable of amplifying from and specifically identifying single nematodes of all three important temperate species (*M. chitwoodi*, *M. fallax*, and *M. hapla*) has been described.

Nuclear ribosomal genes are recognized as providing a useful target for phylogenetic information across diverse phyla as well as between closely related species. In eukaryotes, two ITS regions (ITS1 and ITS2) separate the 18S, 5.8S, and 28S genes. Adjacent copies of the rDNA repeat unit are separated by the IGS, a region generally more variable than the ITS and often containing subrepeating elements that are thought to serve as enhancers of transcription (5,11). Although concerted evolution may act to homogenize multicopy genes and associated spacers, the homogeneity of the rDNA copies from any organism must be empirically determined for each application of these sequences for phylogenetic or diagnostic purposes (32). This has become more apparent because there are now many reports that demonstrate intraspecific heterogeneity in the rDNA arrays of a diverse range of organisms (4,6,27).

In a study of the IGS of *M. arenaria*, Vahidi and Honda (36) reported variation due to changes in internal repeat numbers, as well as the presence, unusual in eukaryotes, of a functional 5S gene in the IGS of this species (35,37). Blok et al. (1) confirmed the pres-

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ence of the 5S gene in the IGS of other tropical root-knot nematodes (*M. javanica*, *M. incognita*, and *M. mayaguensis*) as well as in *M. hapla*.

This study evaluated rDNA homogenization in *M. chitwoodi*, *M. fallax*, and *M. hapla*, utilizing the presence of the 5S gene to examine the IGS in two parts (Fig. 1). It reports a simple one-step PCR that is able to distinguish the three temperate species, *M. chitwoodi*, *M. fallax*, and *M. hapla*, from single nematodes as well as bulk DNA. Variability in the IGS of these species is discussed.

MATERIALS AND METHODS

Root-knot nematode cultures. The isolates used were maintained on susceptible tomato plants (*Lycopersicon esculentum* L. cv. Moneymaker). The populations used are given in Table 1. Eggs were collected as described in Sasser and Carter (29) using NaOCl solution and sucrose floatation and stored at –70°C or hatched at 25°C and juveniles were collected.

DNA extraction. DNA was extracted from eggs according to the method of Pastrik et al. (23) with the addition of phenol/chloroform and chloroform extraction steps (28). DNA was extracted from single juvenile nematodes following the method described in Stanton et al. (31). Single females were ground in 30 µl of sterile distilled water (SDW) in a 1-ml glass homogenizer (Burkard Scientific, Uxbridge, UK), and single males were ground in 20 µl of SDW.

PCR. PCR amplification reactions were carried out using nematode DNA extracted from eggs (2.5 µl of 2 ng/µl) or from single juveniles according to the method described in Stanton et al. (31) (0.5 µl per reaction) or single females or males (2.5 µl per reaction). In 25-µl reactions, 5 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100, 1.5 mM $MgCl_2$, 2 mM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of *Taq* polymerase (Promega, Southampton, UK) were added to template DNA. Primers (Table 2) were used at 200 nM. Amplifications were for 35 cycles (40 cycles for single nematodes) of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s followed by 72°C for 10 min using a thermal cycler (Perkin-Elmer 2400; Applied Biosystems, Warrington, UK). An annealing temperature of 55°C was used for the JMV species-specific primers. The products from these PCR reactions were separated by electrophoresis in Tris-borate-EDTA (TBE) buffer with 1% agarose gel (28) and were visualized with UV illumination after staining with ethidium bromide.

Southern blots. One microgram of genomic DNA was digested with restriction enzyme *Mbo*I in 30 µl of reaction volume with 20 units of enzyme (New England Biolabs, Hitchin, UK) and 1× New England buffer incubated at 37°C overnight. The digested DNA was separated overnight by electrophoresis at 15 V in TBE-buffered 2% agarose gels (28) and visualized with UV illumination after staining with ethidium bromide to confirm complete digestion of DNA. DNA was transferred from the agarose gel to Hybond N^+ membrane by alkali blotting according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, UK). Prehybridization and hybridization were performed as described in the manufacturer's instructions, with the final wash at 65°C with 0.1× SSC (15 mM NaCl plus 1.5 mM sodium citrate) and (0.1%, wt/vol) sodium dodecyl sulfate. The membrane was then exposed to autoradiography film with intensifying screens at –70°C for 1 h and subsequent exposures increased if required.

Dot blots. One microgram of DNA in a 10-µl volume was heated to 95°C and chilled on ice. Ten microliters of 20× SSC was added and 2-µl aliquots were dotted onto Hybond N^+ membrane, denatured, neutralized, and dried according to the manufacturer's instructions. Prehybridization and hybridization were performed as described previously.

Root blots. Infected (8 weeks after infection) and uninfected root samples were laid between two pieces of Hybond N^+ membrane (prewetted with 10× SSC) and pressed with a 1-kg weight. The root tissue was removed and denaturing, neutralizing, and drying were carried out according to the instructions. Prehybridization and hybridization were performed as described previously.

TABLE 1. *Meloidogyne* spp. and isolates used in this study

Species	Population[a]	Country of origin
M. chitwoodi	York 1, York 2	Intercepted potato
	Portugal	Portugal
M. chitwoodi (race 1)	Carg	Argentina
	Ca, Ccc, Ci, Cj, Ck, Cl, Co, Cx, Cy	The Netherlands
M. chitwoodi (race 2)	Cbd	Washington, USA
	Cbf	Oregon, USA
M. chitwoodi (race3)	Cbh	Washington, USA
M. fallax	Fa, Fb, Fc, Fd, Fe	The Netherlands
	Paimpol	France
M. hapla	L33, Ha, Ham, Hbq, Hf, Hi, Hk	The Netherlands
	Q48	Queensland
	Cyprus	Cyprus
M. hapla (race B)	Hh	The Netherlands
M. incognita	L11, L12, L27	USA
	L15	Thailand
	L19	French West Indies
M. javanica	L23	Burkino Faso
	L24	Spain
	L25	Portugal
M. mayaguensis	L3, L5	Ivory Coast

[a] *M. chitwoodi* populations York 1 and York 2 were obtained from S. Hockland, CSL, York, UK from intercepted potato; *M. fallax* population Paimpol from D. Mugneiry, INRA, Rennes, France; and the Cyprus population from E. Tzortzakakis, National Agricultural Research Foundation, Heraklion, Crete, Greece. All other *M. chitwoodi*, *M. fallax*, and *M. hapla* (except L33) isolates were provided by C. Zijlstra, L. Poleij, and H. van der Beek, Plant Research International, Wageningen. *M. incognita*, *M. javanica*, *M. mayaguensis*, and *M. hapla* L33 obtained from the Scottish Crop Research Institute collection.

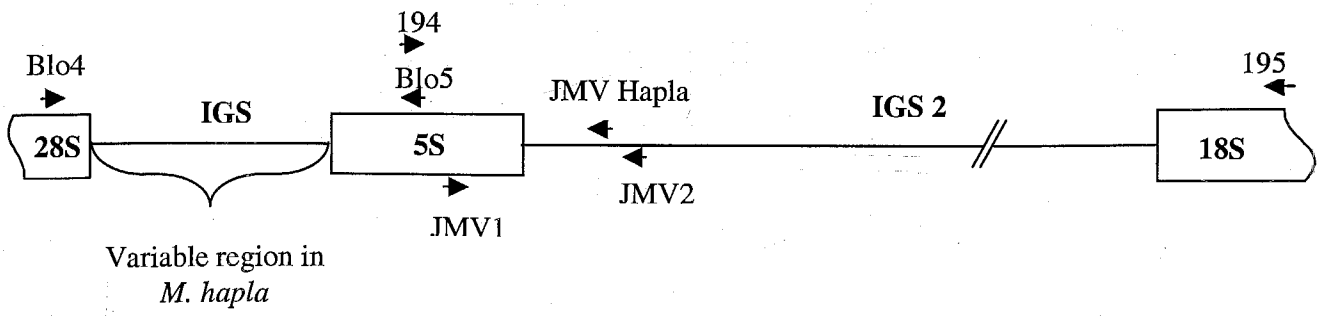

Fig. 1. Schematic diagram (not to scale) of the *Meloidogyne chitwoodi* and *M. fallax* rDNA showing the 5S gene within the intergenic spacer (IGS) region between rDNA cistrons. The annealing locations of oligonucleotide primers, and their orientations are indicated by arrows. The region of variability in *M. hapla* is identified.

Probes. *Southern blots.* Three probes were prepared by amplification of the IGS between the 5S-18S genes from *M. chitwoodi*, *M. fallax*, and *M. hapla* using primers 194 and 195. The PCR products were purified with a gel extraction kit (Qiagen, Crawley, UK) and radioactively labeled by random hexanucleotide priming (Roche, Welwyn Garden City, UK) with α-^{32}PdCTP (Amersham Pharmacia Biotech).

Dot blots and root blots. Three probes were prepared by amplification of the IGS from *M. chitwoodi*, *M. fallax*, and *M. hapla* using JMV1 and 2 (*M. chitwoodi* and *M. fallax*) and JMV1 and JMV hapla (*M. hapla*). PCR products were purified and radioactively labeled by random hexanucleotide priming as described previously.

Cloning and sequencing. Products to be sequenced were cut from the gel, purified, and ligated overnight at 4°C into a plasmid vector (pGEM-T easy vector; Promega, UK) that was then introduced into XL1-blue MRF1 cells (*E. coli* strain; Stratagene, La Jolla, CA) by the standard heat shock transformation method (28). DNA was prepared from recombinant plasmids for sequencing with the Wizard Plus Minipreps kit (Promega). Sequencing reactions were carried out with a DNA sequencing kit (Perkin-Elmer ABI PRISM Big Dye; Applied Biosystems) according to the manufacturer's instructions, and the sequence was obtained with an automatic sequencer (ABI 373 Stretch; Applied Biosystems).

Sequence for *M. chitwoodi* was obtained from populations Cbf and Ck (one clone from each) and Ca and Cbh (two clones from each). For *M. fallax*, populations Paimpol (one clone) and Fa (two clones) were sequenced. For *M. hapla*, populations Q48 (three clones) and Hh (four clones) were used. Sequences were obtained from both strands. Sequence comparisons were made using the GCG package on the Daresbury computer facility (7) (Daresbury Laboratory, Warrington, UK).

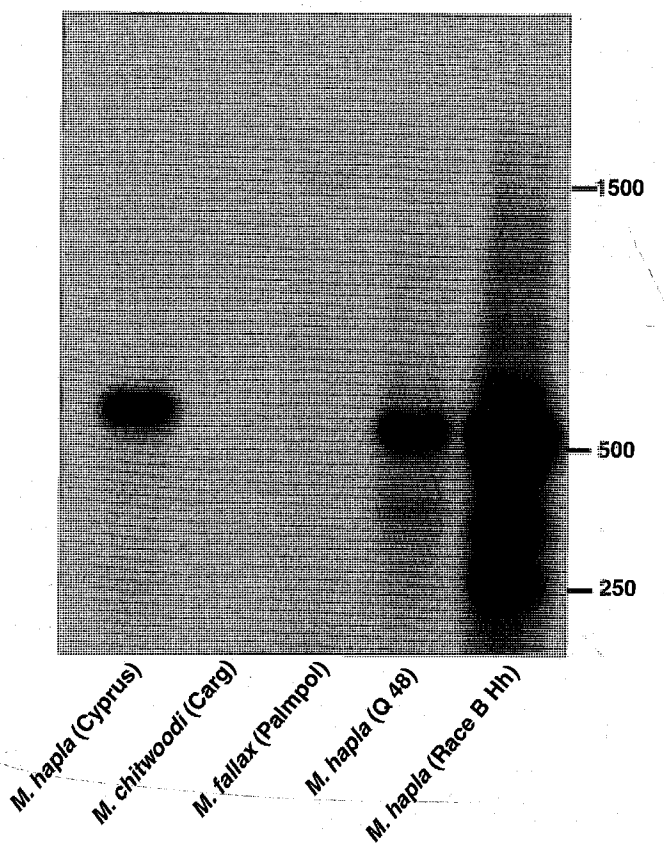

Fig. 3. Southern blot of restriction-digested total genomic DNA hybridized with the 5S-18S intergenic spacer region from *Meloidogyne hapla*.

TABLE 2. Primer sequences

Code	Primer sequence	Site of annealing	Reference
194	5′-TTAACTTGCCAGATCGGACG-3′	5S gene	Blok et al. (1)
195	5′-TCTAATGAGCCGTACGC-3′	18S gene (5′ end)	Blok et al. (1)
Blo4	5′-CTGCGATCTGTTGAGACTT-3′	28S gene (3′ end)	V. C. Blok, *unpublished data*
Blo5	5′-TCCGATCTGGCAAGTTAAGC-3′	5S gene	V. C. Blok, *unpublished data*
JMV1	5′-GGATGGCGTGCTTTCAAC-3′	5S gene	...
JMV2	5′-TTTCCCCTTATGATGTTTACCC-3′	IGS (*M. chitwoodi* and *M. fallax*)	...
JMV hapla	5′-AAAAATCCCCTCGAAAAATCCACC-3′	IGS (*M. hapla*)	...
JMV tropical	5′-GCKGGTAATTAAGCTGTCA-3′	IGS (*M. incognita*, *M. arenaria*, *M. incognita*, and *M. mayaguensis*)	...

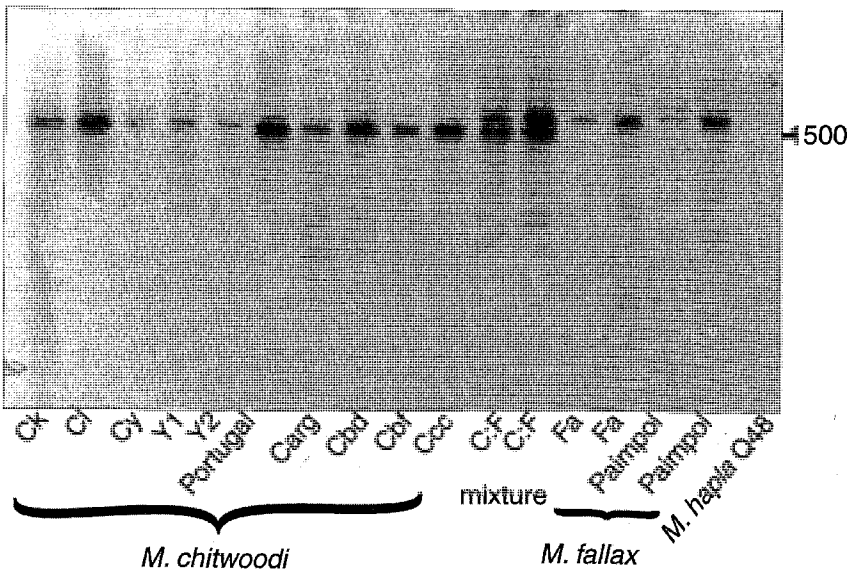

Fig. 2. Southern blot of restriction-digested total genomic DNA hybridized with the 5S-18S intergenic spacer region from *Meloidogyne chitwoodi*.

RESULTS

Southern hybridizations to detect cistron homogeneity. Southern blots of total genomic DNA from *M. chitwoodi* and *M. fallax* hybridized similarly to the *M. chitwoodi* probe (Fig. 2) and to the *M. fallax* probe (data not shown). A single hybridized band was observed differing by ≈100 bp between the two species (≈1,500 bp for *M. chitwoodi* and ≈1,600 bp for *M. fallax*). *M. hapla* did not hybridize to either probe under the conditions used, inferring low similarity with the IGS of *M. chitwoodi* and *M. fallax*. The hybridizations obtained for *M. chitwoodi* and *M. fallax* suggests that the IGS of these two species has diverged, but the single hybridized band obtained with both species indicated homogenization of the IGS amongst the ribosomal cistrons (lack of variability between paralogues) at the sensitivity achieved in this study. Variation in signal strength between the populations of *M. chitwoodi* may indicate variability in cistron number.

The *M. hapla* probe produced more complex patterns of hybridization, particularly race B population Hh (Fig. 3), showing variability among the IGS paralogues of *M. hapla*. There were faint hybridizations (single bands of ≈1,500 and ≈1,600 bp) with *M. chitwoodi* and *M. fallax*.

PCR amplification of the IGS. PCR amplifications were carried out using primers (Table 2) located in different regions of the IGS (Fig. 1). The sizes of PCR products differed between *M. chitwoodi* and *M. fallax* over both IGS regions, and each PCR produced a single product of a discrete size, which separated these species by length polymorphism (Table 3). A PCR product

TABLE 3. Polymerase chain reaction products obtained from amplification of the intergenic spacer (IGS) from 28S-5S (Blo4/Blo5), 5S-18S (194/195), and species-specific IGS primers (JMV1 and 2 and JMV1 and JMV hapla)

Species	Populations tested	Approximate product size (bp)
28S-5S (Blo4/Blo5)		
Meloidogyne chitwoodi	Portugal, York 1, York 2, Carg, Ca, Ccc, Ci, Cj, Ck, Cl, Co, Cx, Cy Cbd, Cbf, Cbh	280
M. fallax	Fa, Fd, Fe, Paimpol	320
M. hapla	Hk, Q48	705
M. hapla	L33, Ha, Ham, Hbq, Hf, Hi, Cyprus	Multiple bands
M. hapla (race B)	Hh	Multiple bands
M. incognita	L11, L12, L27, L15, L19	Multiple bands
M. javanica	L23, L24, L25	Multiple bands
M. mayaguensis	L3, L5	Multiple bands
5S-18S (194/195)		
M. chitwoodi	Portugal, York 1, York 2, Carg, Ca, Ci, Cj, Ck, Cl, Co, Cx, Cy, Ccc, Cbd, Cbf, Cbh	1,600
M. fallax	Paimpol, Fa, Fb, Fc, Fd, Fe	1,700
M. hapla	L33, Ha, Hf, Hi, Hbq, Q48, Cyprus	700
M. hapla	Hh (race B)	700 + 200
M. incognita	L15, L19	720
M. javanica	L23, L25	720
M. mayaguensis	L3, L5	780
Species-specific IGS primers (JMV1 and 2 and JMV1 and JMV hapla)		
M. chitwoodi	Portugal, York 1, York 2, Carg, Cj, Ck, Cl, Co, Cy, Ccc, Cbd, Cbf, Cbh	540
M. fallax	Paimpol, Fa, Fb, Fc	670
M. hapla	L33, Ha, Ham, Hk, Q48, Cyprus	440
M. hapla	Hh (race B)	440
M. incognita	L15, L19, L27	None
M. javanica	L23, L24	None
M. mayaguensis	L3, L5	None

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                10         20         30         40         50         60
M.chitwoodi     CTGCGATCTG TTGAGACTTA GCCTTTGATC GGATGATTTG TCTTTTTTAT ATACTATAAA
M. fallax       .......... .......... .......... .......... .......... ..........

                70         80         90         100        110        120
M.chitwoodi     CTTGTTTTAT TTTTTGAATT TGGATTGATG GCAAAAAAAA -------AAT CCCCAACCA-
M. fallax       .......... .......... .......... .......... TCCCCCA... .......TGG

                130        140        150        160        170        180
M.chitwoodi     ---------- ----CTGGGT GGGAG--TCC CA----CAAG GTAGGGGG-G GTTTATGG--
M. fallax       GTAGTGGTCC CACT...... A....AG... ..ACCA.... .......TT. A.C.....TG

                190        200        210        220        230        240
M.chitwoodi     -AACGATCTA -TGGAAT--- -----GATCA ACTATGATCT ATGGCAGATG GTAT---GGA
M. fallax       G..T....A. C.A.G..CTA TGTCA..... .......... .........A ..GGCCG...

                250        260        270        280        290        300
M.chitwoodi     AAAAGGTTAC GACACTCCCG ATTCCCAGGA CGTCTCCGTT CCAAGTACTA AGGAAGCCCA
M. fallax       .......... .......... .......... .......... .......... ..........

                310        320
M.chitwoodi     ACGTTGCTTA ACTTGCCAGA TCGGA
M. fallax       ....______ .......... .....
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Fig. 4. Alignment of the intergenic spacer 1 (IGS-1) for *Meloidogyne chitwoodi* (population Cbh) and *M. fallax*. Primer sites are underlined; 28S gene in italics; and 5S gene in bold italics.

of ≈700 bp was obtained for *M. hapla* when the 5S-18S region was amplified, except for the polyploid, mitotic population Hh (Table 3). Amplification of the 28S-5S IGS produced one main product for certain *M. hapla* populations, although other populations of *M. hapla* as well as the tropical *Meloidogyne* populations tested, produced complex patterns of multiple bands (Table 3).

Sequencing the PCR products. Consensus sequences were obtained for all three species for both regions of the IGS (EMBL Data Library accession nos. AJ421698 to AJ421708). The align-

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                    10         20         30         40         50         60
Q48 ------  CTGCGATCTG TTGAGACTTA GCCCTTGATC GGATGATTTG TCTTTTTTAT ATACTATAGA

                    70         80         90        100        110        120
Q48         CTTGTTTATC CACATTAAAG TGGCAAACCC AGGCATCATC TTGTTTTAAG GATTAGACTA

                   130        140        150        160        170        180
Q48         GATCCGACTT TTTTATAGCC TCAGGCAATA TTTGTCAAAT GTCCTTTGAC CCAATTGTCC

                   190        200        210        220        230        240
Q48         TTTGAACCAA TTGTGTCCTT TACTGTGTGT CCTTAATTGT CCTTAATACT GTGTGTCCTT

                   250        260        270        280        290        300
Q48         AATCTACAAC ATATATAATT TCCTCAGCAG CCTCGGTAAT TCCATAAATT AATTTACCTT

                   310        320        330        340        350        360
Q48         CAGGGGGTAT AATTTAATCT GTCTACAACA TATATAATTT CCTCAGCAGC CTCGGTATTA

                   370        380        390        400        410        420
Q48         CTTTACCTTC AGGGAATATA ATTTAATCTG TCTACAACAT ATATAATTTC CTCAGCAGCC

                   430        440        450        460        470        480
Q48         TCGGTAATTC CATAAATTAA TTTACCTTCA GGGGATAAAA GGGATTAATT TAGGATTAAA

                   490        500        510        520        530        540
Q48         GGGATTACTT TAAAGGGGAT TAAATTGGAG AGGGGATTAA AGGGTTTAAT TTAATTTATT

                   550        560        570        580        590        600
Q48         ATTTTAAATA GGAGAGGAGA TAAAGGATTA AATAGGAGAG GAGATAAAAG GGATTAATTT

                   610        620        630        640        650        660
Q48         AAAAAGGTAT TAATTTGGAA AAAGATTACG ACACCCCCGA TTCCCAGGAC GTCTCCGTTC

                   670        680        690        700        710        720
Q48         CAAGTACTAA GGAGCCAACG TTGCTTAACT TGCCAGATCG GACGGGATGG CGTGCTTTCA
                                                                 JMV1
                   730        740        750        760        770        780
Q48         ACGCGGTATG GTCGTAATCA TTTGACTCTT ACAATATTAA CATTGTGAAG GAAAAATCTT

                   790        800        810        820        830        840
Q48         GGGAGAGTTA CAGCGCTTCG CTATAATGAA ATAAAATGCT AGTTATTTTG AAGCGAACTA

                   850        860        870        880        890        900
Q48         AAAATTGTGA GAAGAACAGG AGGATTTGAA AAGATTTTTC AAAAGTTCGA AAGATTACAT

                   910        920        930        940        950        960
Q48         GATTAATGTA AATTTTGGAT TTCCTCGCTG GTTAATCTTA ATTCATTACA ACTACTGGTA

                   970        980        990       1000       1010       1020
Q48         GCATGmTGAA TCATCAGGAA TTTATGCCAG TACTCTGTTA GAAGTTGGTG AAGTGATTAA

                  1030       1040       1050       1060       1070       1080
Q48         TTTTCAATGA GATTAAAGCA TAATTTGTCT TGTGCAAAGG AGATTATAAT TTGCTGGCTT

                  1090       1100       1110       1120       1130       1140
Q48         GTCATTTTAA TCTTTAATCA TATTTTCGCA AAGGGATTAA AATGGTGGAT TTTTCGAGGG
                                                                 JMV Hapla
                  1150       1160       1170       1180       1190       1200
Q48         GATTTTTCGG CATGTTTTTG TATTTTTCGA TGTTTAAAAA AAAAAAAAAA AAAAATTTCA

                  1210       1220       1230       1240       1250       1260
Q48         CTTGATTGAG ATGACAAGCT ATACGTTGTA TTTGATTAAT TAGTTAAAAA GATATCTGGT

                  1270       1280       1290       1300       1310       1320
Q48         TGATCCTGCC TGAACTGATG TGTTCATTTC AAAGATTAAG CCATGCATGT ATAAGTTTAA

                  1330       1340       1350
Q48         TCGTATTTAC GAGAAACCGC GTACGGCTCA TTAGA
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Fig. 5. Sequence of the intergenic spacer 1 and 2 (IGS-1 and IGS-2) for *Meloidogyne hapla* population Q48. Primer sites are underlined; 28S gene in italics; 5S gene in bold italics; and 18S gene from 1267. The three pairs of repetitive sequence are underlined and italics and bold.

ment of the sequence of the IGS-1 region, derived from consensus sequences from the *M. chitwoodi* population Cbh and *M. fallax* population Fa, is shown in Figure 4. Differences from this sequence were observed in some of the *M. chitwoodi* clones. One clone, from population Ca, had C instead of T at position 41 and another clone from population Cbh had an A missing at position 93. Another clone from population Ca had a TC missing at position 148/149, giving rise to a *Hin*f1 site polymorphism at that position. No sequence differences were found within the *M. fallax* sequences for IGS-1. Sequences were identical for IGS-2 within each species.

The sequence obtained from *M. hapla* showed differences between clones and populations. The sequence from one clone from Q48 is shown in Figure 5. The other clone from this population had a 69-bp deletion from position 355 to 425. At position 675 there was an extra G in all HH clones, making the IGS-1 sequence 704 bp long with Q48 clones 703 bp long (except for the clone with the 69-bp deletion). The sequences of IGS-2 were identical with no base changes (Fig. 5).

The IGS orthologues from *M. chitwoodi* and *M. fallax* were highly similar (similarity 95.8%), with the former being identical to that published by Peterson and Vrain (24). In the very short IGS-1 there was a size difference of 44 bp between *M. chitwoodi* and *M. fallax* resulting from short insertions in *M. fallax* in a region of short repeats (91% similarity) (Fig. 4). In IGS-2, a region of repeats gave rise to a size difference of 136 bp due to a higher number of repeats in *M. fallax* (95.9% similarity) (Fig. 6).

The IGS sequence for *M. hapla* differed from *M. chitwoodi* and *M. fallax*, having a repetitive region in IGS-1 with three pairs of imperfect repeats (Fig. 5). There was intraspecific size polymorphism detected due to two missing adjacent repeats (position 358 to 426; Fig. 5) (EMBL AJ421704). There was no repetitive sequence in the IGS-2 of the *M. hapla* populations sequenced.

Species-specific primers. Species-specific primers were designed with a common primer located in the conserved 5S gene and unique primers located in IGS-2. To distinguish *M. fallax* from *M. chitwoodi*, a primer was designed outside the region of repeats to produce different sizes of product for each species due to the increased number of repeats in *M. fallax* (Fig. 6). A third primer was designed in the IGS-2 of *M. hapla* to produce a product complementary in size to those produced by *M. fallax* and *M. chitwoodi*. A single step multiplex PCR with three primers was able to amplify from bulk DNA (Fig. 7A) single juveniles (Fig. 7B), single females (Fig. 7C), and males (data not shown) as well as detect <1% of a second species in mixtures (Fig. 7D). There was clear differentiation between these three important temperate root-knot nematode species. The JMV tropical primer was tested in a multiplex PCR (JMV1, JMV2, JMV hapla, and JMV tropical) and specifically amplified products of 549 and 615 bp for *M. mayaguensis* and *M. incognita*, respectively. The 549-bp product

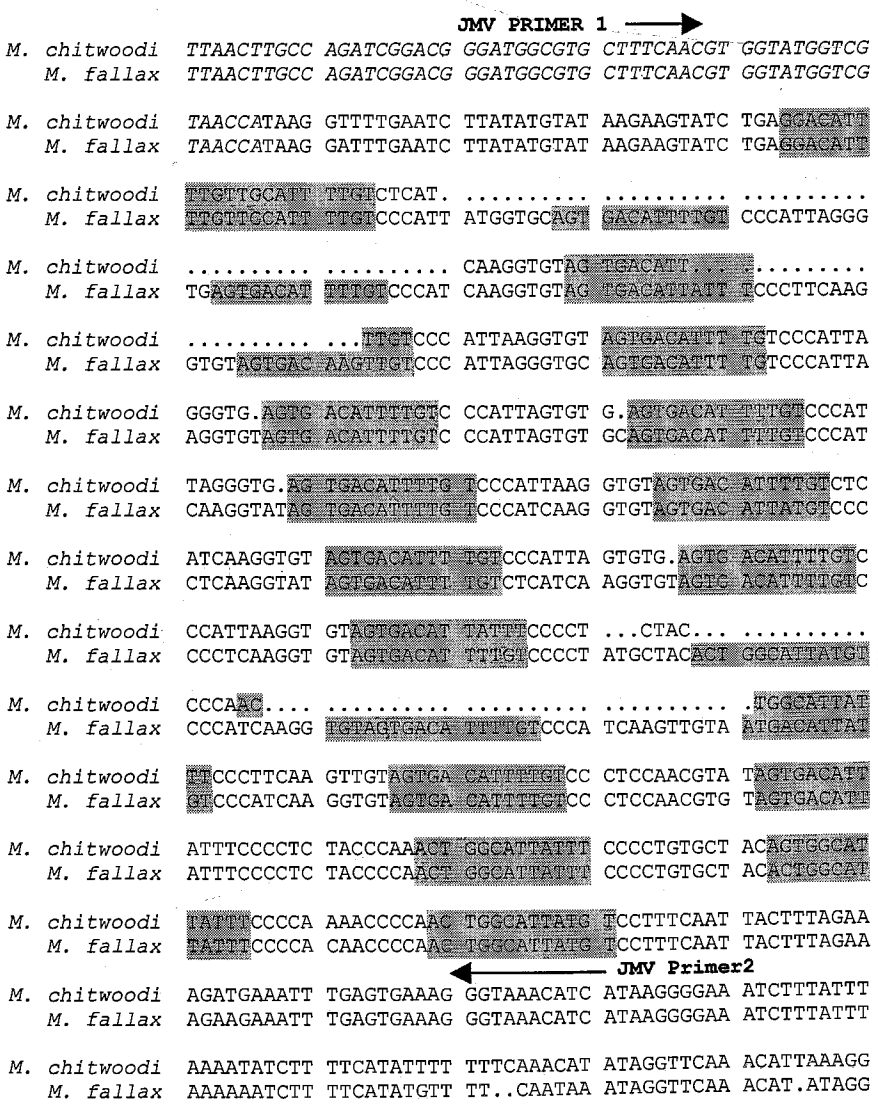

Fig. 6. Alignment of end of the 5S gene (italics) and the first 694 bp of the intergenic spacer 2 (IGS-2) for *Meloidogyne chitwoodi* and *M. fallax*. The species-specific primer sites are indicated. Gray boxes show the short repeats found in both sequences.

obtained from the tropical species was difficult to separate from the *M. chitwoodi* (539 bp) product in standard electrophoresis conditions (data not shown).

When these amplification products were used as probes, dot blots (Fig. 8A) and root blots strongly hybridized with the probes (Fig. 8C) and could clearly differentiate *M. hapla* from *M. chitwoodi* and *M. fallax* but could not differentiate between the closely related *M. chitwoodi* and *M. fallax*.

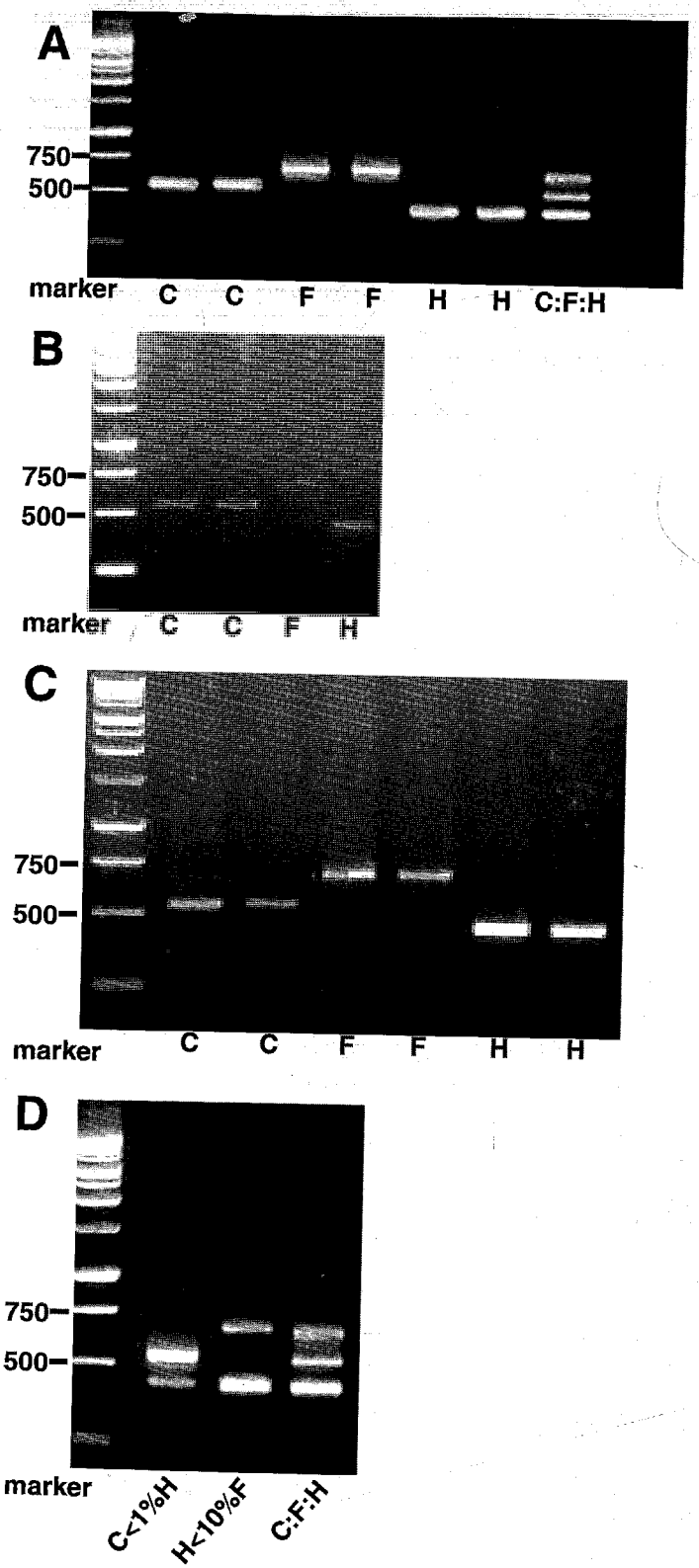

Fig. 7. One percent ethidium bromide-stained agarose gel showing the species-specific polymerase chain reaction products obtained after amplification from *Meloidogyne chitwoodi* (C), *M. fallax* (F), and *M. hapla* (H) using the JMV primers. **A,** Bulk DNA extracted from eggs including a 1:1:1 mixture of the three species (C/F/H); **B,** single juveniles; **C,** single females; and **D,** mixed DNA samples.

DISCUSSION

The three species of *Meloidogyne* compared in this study are diploid, facultative meiotic parthenogenetic species that reproduce either sexually or, in the absence of male gametes, parthenogenetically, except for *M. hapla* race B, which is an obligate mitotic parthenogenetic species and is usually triploid (34). *M. chitwoodi* and *M. fallax* are closely related as seen in two-dimensional protein profiles (33,38), satellite DNA (3), ITS sequence (42,44,45),

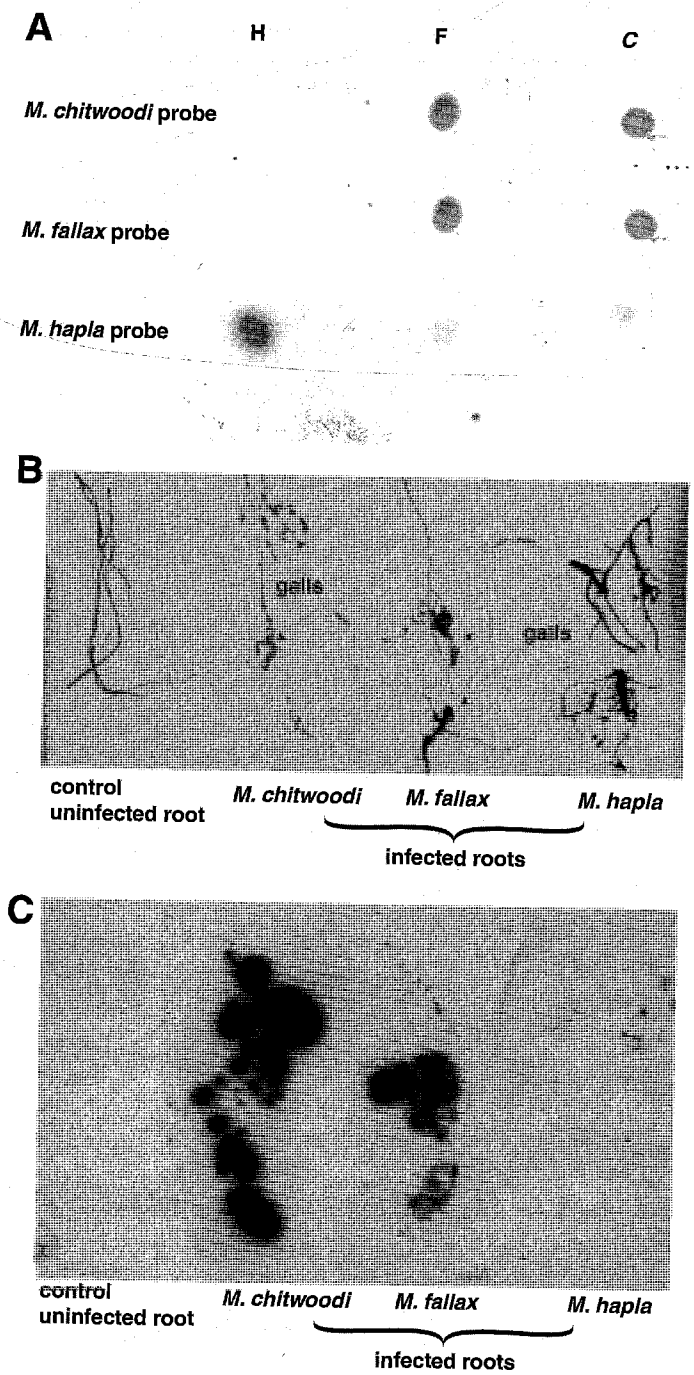

Fig. 8. A, Dot blots of DNA from *Meloidogyne chitwoodi* (C), *M. fallax* (F), and *M. hapla* (H) dotted directly onto nitrocellulose membrane and hybridized with the JMV polymerase chain reaction (PCR) product from each species. **B and C,** Root blots of infected and uninfected roots **B,** blotted and **C,** hybridized with the JMV PCR product from *M. chitwoodi*.

and RAPD analysis (41,43). It is possible to produce hybrid females from *M. chitwoodi* and *M. fallax* but the offspring are not viable (39). All this information suggests a common phylogeny and possible recent speciation. As well as the interspecific similarity between these two species, there is evidence for low intraspecific variability (two-dimensional gel electrophoresis [2-DGE] studies [38], ITS sequencing [14], and ITS RFLPs [44]). It had been shown by using primers that amplified the whole IGS region between the large and small subunit of rDNA that *M. chitwoodi* and *M. fallax* were similar to each other and differed from the tropical *Meloidogyne* spp. and *M. hapla* in the length and sequence of the IGS region (24).

Intraspecific molecular variability is evident in *M. hapla* (ITS RFLPs [44], 2-DGE [38], and ITS sequence [14]), and this combined with the existence of two cytological races, with race B isolates being polyploid (34), suggests divergent phylogeny from *M. chitwoodi* and *M. fallax*. 2-DGE and amplified fragment length polymorphism analyses (38) demonstrated that *M. hapla* was more closely related to the tropical species than to *M. chitwoodi* or *M. fallax*. It was expected, therefore, from previously published work on these groups, that *M. chitwoodi* and *M. fallax* would be very similar and that *M. hapla* would differ from these two species, with the race B isolates showing extra variability due to polyploidy.

By using the 5S gene as primer site and amplifying the IGS in two sections, it was possible to identify regions of high variability in *M. hapla*. Genomic blots probed with the IGS-1 PCR product provided evidence for a single rDNA IGS size class in *M. chitwoodi* and *M. fallax* (Fig. 2) and confirmed the variability found in *M. hapla* (especially within the race B isolate) (Fig. 3). Amplification of the IGS-1 separately (primers Blo4/Blo5) enabled a comparison of the two parts of the IGS (upstream and downstream from the 5S gene). The multiple products produced after amplification of IGS-1 from the tropical *Meloidogyne* spp., as well as from certain populations of *M. hapla*, could be a result of variability in IGS cistron length in this region or a result of lack of conservation of the site for the 28S primer. Using one primer located at the 3′ end of the 28S gene and the one primer in the 18S gene, Peterson and Vrain (24) amplified a product of only 600 bp for the complete IGS of *M. hapla*. This is inconsistent with the 680 bp reported for the IGS-2 only (1) and suggests the presence of cistrons containing large deletions. Variability within *M. arenaria* 28S-5S is well documented (2,10,12,35,36), and one of the *M. hapla* clones sequenced in this study shows a deletion corresponding to a loss of one 34- and one 35-bp repeat. It seems likely therefore, that the variability identified within *M. hapla* was due to polymorphisms because of a loss of repeats within the IGS-1. More populations would have to be studied and genomic clones sequenced to fully interpret the variability in this region.

M. chitwoodi and *M. fallax* were the only species in this study that produced a single size of PCR product following amplification of the complete IGS region, suggesting that only in these two species was the ribosomal cistron completely homogenized throughout the rDNA arrays. This was consistent with the Southern blot results. The *M. chitwoodi* and *M. fallax* IGS sequences were highly similar. In the repeat region, possibly associated with transcription termination and initiation sites previously reported in the IGS of other organisms (11), there was evidence of divergence. Length polymorphism in the *M. fallax* sequence, including some of the short repeats and spacer sequence, is likely to be indicative of replication slippage and homogenization of the repeats the result of unequal crossing over (8,19).

The variability in the mitotically reproducing *M. hapla* (race B) population Hh could be a result of polyploidy arising from heterogeneous progenitors and diverse phylogenetic origins may also explain the variation found in the other *M. hapla* populations. It is also possible, because very little is known of the occurrence of sexual reproduction within these groups, that it is more commonly occurring in *M. hapla* populations than in *M. chitwoodi* or *M. fallax*. If this were the case, one could hypothesize that the homogenization processes occurring during meiosis are counterbalanced by the heterogeneity introduced by sexual reproduction. If sexual reproduction was rare, then meiosis without sexual reproduction could lead to homogenization throughout the rDNA arrays.

This study, in revealing the areas of nonvariability, enabled a set of species-specific primers to be designed for the differentiation of *M. chitwoodi*, *M. fallax*, and *M. hapla* with a one-step multiplex PCR. Amplifications were possible from bulk samples of DNA as well as from individual juveniles, males, and females. Previously published molecular diagnostic tools have either required an additional restriction digest (13,26,44), have not differentiated *M. chitwoodi* from *M. fallax* (41), or require a second nested PCR to differentiate between DNA extracted from individuals (43). The primer set described by Peterson et al. (25) does distinguish all three species but identifies *M. hapla* by the absence of a band. The primers reported here enable positive differentiation of all three species.

This multiplex primer set allows the positive detection of low levels of a contaminating species in a mixture and can be used as a probe to detect these species in infected root samples and in dot blotted DNA.

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