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A fast PCR assay to identify *Meloidogyne hapla*, *M. chitwoodi*, and *M. fallax*, and to sensitively differentiate them from each other and from *M. incognita* in mixtures

Carolien ZIJLSTRA

DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands.

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Summary – Previously amplified rDNA-ITS fragments of *Meloidogyne chitwoodi*, *M. fallax*, *M. hapla*, and *M. incognita* were cloned and sequenced. Alignment of sequences showed that there was little variation in the coding parts and some variation in the ITS1 region. The largest variation was found in the ITS2 region. The sequences were used to design forward primers H-18S, I-ITS, and CF-ITS that, in combination with reverse primer HCFI-28S, were used for the specific amplification of *M. hapla*, *M. incognita*, *M. chitwoodi*, and *M. fallax*, respectively. This resulted in fragment size polymorphisms that distinguished the four species. When all four primers were used in a single PCR reaction, single juveniles or isolates of *M. chitwoodi*, *M. fallax*, *M. hapla*, and *M. incognita* were identified in a single step. This multiplex PCR approach makes it possible to detect species present in mixtures in proportions as low as 2 to 5%.

Résumé – ***Technique rapide PCR permettant l'identification de* Meloidogyne hapla, M. chitwoodi *et* M. fallax *et la différenciation spécifique de mélanges de ces espèces, ainsi que de* M. incognita** - Des fragments préalablement amplifiés de rDNA-ITS de *Meloidogyne chitwoodi*, *M. fallax*, *M. hapla* et *M. incognita* ont été clonés et séquencés. L'alignement des séquences montre une variabilité faible dans les parties codantes et quelque variabilité dans la région ITS1. La plus importante variabilité est observée dans la région ITS2. Les séquences ont été utilisées pour établir les amorces "sens" H-18S, I-ITS et CF-ITS, lesquelles en combinaison avec l'amorce "anti-sens" HCFI28S ont été utilisées pour l'amplification spécifique de *M. hapla*, *M. incognita*, *M. chitwoodi* et *M. fallax*, respectivement. Il en résulte un polymorphisme de taille des fragments distinguant les quatre espèces. Lorsque les quatre amorces sont utilisées pour une unique réaction PCR, des juvéniles isolés ou des isolats de *M. chitwoodi*, *M. fallax*, *M. hapla* et *M. incognita* peuvent être identifiés en une seule étape. Cette méthode de PCR multiplexe permet la détection des espèces présentes dans un mélange, à des taux aussi faibles que 2 à 5%.

Key-words: diagnostics, ITS, *Meloidogyne*, multiplex PCR, ribosomal DNA, root-knot nematodes.

In Dutch agriculture, an increase of populations of the root-knot nematodes *Meloidogyne hapla*, *M. chitwoodi*, and the recently described *M. fallax* has been observed in recent years. The most affected crops are potato, carrot, beet, and scorzonera. The seriousness of the situation is seen from the fact that *M. chitwoodi* has been designated as quarantine organism by EPPO. *M. fallax* probably will follow. The control of these nematodes is not easy. The Multi-Year Crop Protection Plan of the Dutch government stipulates a drastic reduction in the use of chemical soil pesticides, control by crop rotation is hardly possible because of the wide host range of these nematodes, and there are no resistant cultivars available for the major crops. Accurate and reliable identification of these nematodes is fundamental to many aspects of their effective control and management and to research for the development of control measures.

Resistance used in crop (rotation) systems is the most promising and environmentally safe solution. Proper resistance and virulence research must be conducted on well characterized monospecific populations of root-knot nematodes. In general, the populations of *M. chitwoodi*, *M. fallax*, and *M. hapla* used for research are grown in glasshouses. Even when proper precautions are taken, there is a risk of cross-contamination among these species, or of contamination by *M. incognita*, a species often present in glasshouses. Therefore, methods are required that can sensitively detect mixtures of species. Many molecular techniques have been shown to be valuable tools for the identification of root-knot nematodes, such as RAPD-PCR (Cenis, 1993; Baum *et al.*, 1994), restriction fragment length polymorphisms (RFLPs) of amplified sequences of mitochondrial DNA (Harris *et al.*, 1990; Powers & Harris, 1993; Hugall *et al.*, 1994), and sequence differences of rDNA (Zijlstra *et al.*, 1995; Petersen & Vrain, 1996) or satellite DNA (Piotte *et al.*, 1994, 1995).

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Recently, we showed that the ITS-RFLP technique is a reliable and precise method for differentiating species of root-knot nematodes in mixtures (Zijlstra *et al.*, 1997). *Dra*I, *Eco*RI, and *Rsa*I restriction patterns of ITS PCR products from mixtures of *M. hapla*, *M. chitwoodi*, *M. fallax*, and *M. incognita* can detect these species when they are present in proportions of 5% or more of the specimens in a mixture. The ratio of the intensities of the bands of each species-specific restriction pattern observed corresponded with the ratio of the species in the mixture, which indicates that all the individuals of the populations of the different species tested contain the same number of ribosomal cistrons. In the present study, this method has been improved to identify the root-knot nematodes species by the size of their amplified fragments in a single PCR reaction step without the need for subsequent digestion. This was achieved by cloning and sequencing ITS regions of *M. chitwoodi*, *M. fallax*, *M. hapla*, and *M. incognita*, and by designing species-specific forward PCR primers. The method we present is based on a multiplex PCR technique using three forward primers and one common reverse primer. It is used for direct identification of the four species based on length polymorphisms.

Materials and methods

Nematodes

The isolates used are listed in Table 1. Second stage juveniles of *M. chitwoodi* isolate Co, *M. fallax* isolate Fa, *M. hapla* isolate Hi, and *M. incognita* isolate Ia were used for the preparation of mixtures as described in Zijlstra *et al.* (1997). DNA was extracted as described in Zijlstra *et al.* (1997).

Cloning of ITS-fragments and species-specific primer design

ITS regions of *M. hapla*, *M. chitwoodi*, *M. fallax*, and *M. incognita* were amplified from homogenates from single juveniles of Dutch isolates Hk, Ca, Fa, and Ia, respectively. The reaction mixture contained 10 mM Tris pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatine, 0.1% Triton X-100, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.6 µM of each of the ITS-specific primers described by Vrain *et al.* (1992), referred to as 5367 and 5368, 0.6 units of Taq DNA polymerase (Sphaero Q, Leiden, The Netherlands), the crude DNA extract from a single juvenile, and deionized water to a volume of 50 µl. PCR-amplification conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, repeated for 35 cycles.

The generated ITS-fragments of *M. hapla*, *M. chitwoodi*, *M. fallax*, and *M. incognita* were monitored by electrophoresis in 1% low-melting point agarose and subsequently excised from the gel and transferred to Eppendorf reaction tubes. The tubes were kept at 65°C for 10 min; 1/10 of a volume of 3M NaCl was added. The tubes were kept at 65°C for another 20 min. One volume of phenol equilibrated with 0.5 M NaCl was added, followed by another 5 min incubation period at 65°C. The tubes were centrifuged in a bench top table centrifuge for 20 min at full speed. Ten µg of tRNA was added to the supernatant. The supernatant was extracted with a 1:1 mixture of phenol equilibrated with 0.5 M NaCl:chloroform and subsequently with chloroform. The DNA was precipitated by adding 2.5 volumes of ethanol 96%. Tubes were centrifuged for 20 min at full speed. Finally the DNA pellets were resuspended in 20 µl TE.

The ITS-fragments were cloned into the pGEM-T vector (Promega). The cloned inserts were tested by amplification of miniprep plasmid DNA with the ITS-specific primers 5367 and 5368. The amplification products were digested with *Dra*I, *Eco*RI and *Rsa*I in order to obtain the typical species specific ITS-RFLPs described in Zijlstra *et al.* (1995).

Plasmid DNA from the correct insert-containing clones was extracted using a Qiagen plasmid kit. Sequencing of the inserts was performed in both directions at the sequencing facility of the department of molecular biology of the agricultural university in Wageningen.

An alignment of the rDNA sequences of the four ITS fragments was performed using the computer program GAP of the Genetics Computer Group (Devereaux *et al.*, 1984) to select the species-specific forward PCR primers H-18S, CF-ITS and I-ITS (see Fig. 1).

Species-specific (multiplex) PCR

Fig. 1 shows the location of each primer used for amplifying segments of the ITS regions. The reverse primer HCFI-28S is the backward primer as described by Ferris *et al.* (1993). The reaction mixture contained 10 mM Tris pH 9.0, 3.5 mM $MgCl_2$, 50 mM KCl, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.4 µM each of the forward primers H-18S, CF-ITS, and I-ITS, and 0.4 µM of the reverse primer HCFI-28S, 1 unit of Taq DNA polymerase (Pharmacia), 3 ng of total DNA or the crude DNA extract from a single juvenile, and deionized water to a volume of 50 µl. A touch down cycling profile was used: denaturation at 94°C for 4 min, followed by five cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min with a decrease of 1°C per cycle for the annealing temperature followed by 25 cycles at 94°C for 30 s, 50°C for

Table 1. *Isolates and sources of the species of* Meloidogyne *used in this study.*

Code	Location	Isolate	Source
M. chitwoodi			
Ca	The Netherlands	C3022	PD[a]
Ck	The Netherlands	C5273g	PD
Cl	The Netherlands	C2960	PD
Co	The Netherlands	Horst	PAV[b]
Cba	Oregon, USA	ORMC12, race 1	Mojtahedi/Santo
Cbd	Washington, USA	WAMC16, race 2	Mojtahedi/Santo
Cbf	Oregon, USA	ORMC8, race 2	Mojtahedi/Santo
Cbh	California, USA	CAMC2, race 3	Mojtahedi/Santo
Cbs	The Netherlands	IHO9	PAV
Cbu	The Netherlands	KBD4	PAV
Ccg	Portugal		Mugniéry
Cch	Argentina	CArg	Mugniéry
M.fallax			
Fa	The Netherlands	CHB	PAV
Fb	The Netherlands	C4571	PD
Fc	The Netherlands	C6501	PD
Fd	The Netherlands	C6466	PD
Fe	The Netherlands	W834.769	BLGG[c]
Ff	Belgium		Moens
Fg	The Netherlands		CPRO-DLO[d]
Fh	France	CHK	Mugniéry
M. hapla			
Hi	The Netherlands	Sm.Fei92	PAV
Hj	The Netherlands	Sm.Fer92	PAV
Hk	The Netherlands	Sl.92	PAV
Ham	Queensland, Australia	Q114	Fargette
Han	South Korea	C2346	PD
Has	Washington, USA	WAMH2	Mojtahedi/Santo
Hau	Utah, USA	UTMH1	Mojtahedi/Santo
Hbj	Canada	Canada	MacGuidwin
Hbq	Hungary	C6611	PD
Hbr	The Netherlands	C4900-H-1A	PD
Hbt	The Netherlands	W834.347	BLGG
Hby	The Netherlands	C3029	PD
Hcb	The Netherlands	HBW	CPRO-DLO
M. incognita			
Ia	The Netherlands		IPO-DLO[e]
Ib	The Netherlands	Inc568-93	PD
If	The Netherlands	KnolE	De Ruiter[f]
Ih	The Netherlands	KnolG	De Ruiter
Ij	The Netherlands	KnolH	De Ruiter

a Plant Protection Service, Wageningen, The Netherlands.
b Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands.
c Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.
d Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands.
e DLO-Research Institute for Plant Protection, Wageningen, The Netherlands.
f De Ruiter Seeds, Bleiswijk, The Netherlands.

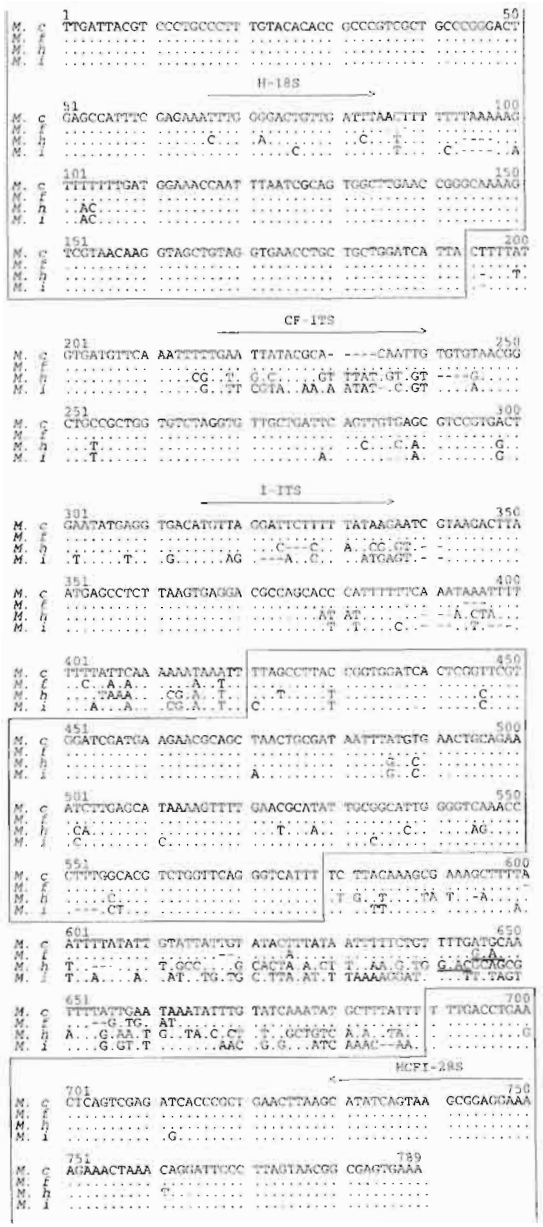

Fig. 1. *Alignment of rDNA sequences of* Meloidogyne chitwoodi *(M. c),* M. fallax *(M. f),* M. hapla *(M. h), and* M. incognita *(M. i) obtained by PCR using the ITS-specific primers described by Vrain* et al. *(1992). The 3' end of the 18S coding region, the 5.8S coding region, and the 5' end of the 28S coding region are outlined. Arrows mark the locations of primers H-18S, CF-ITS, I-ITS, and HCFI-28S. The restriction sites of RsaI are underlined.*

30 s, and 72°C for 1 min. To amplify from single juveniles, the same conditions were used, except that the number of cycles was increased from 25 to 30.

Results

Sequencing data revealed that the sizes of the cloned amplified ITS regions of *M. chitwoodi*, *M. fallax*, *M. hapla,* and *M. incognita* were 784 bp, 775 bp, 768 bp, and 767 bp, respectively. The sequence alignments of the amplified ITS regions are shown in Fig. 1.

Three species-specific forward primers were designed. Their positions on the sequences are shown in Fig. 1: H-18S: 5'-CTTGGAGACTGTTGATC-3'; CF-ITS: 5'-GAATTATACGCACAATT-3'; I-ITS: 5'-TGTAGGACTCTTTAATG-3'.

First, PCR reactions were performed with HCFI-28S and one of the three forward primers. The sizes of the fragments obtained after amplification could be estimated from the sequences and were as expected. The combination of primers H-18S and HCFI-28S resulted in an amplified fragment of 660 bp when *M. hapla* DNA was used as a template. No amplification could be observed when *M. chitwoodi*, *M. fallax*, or *M. incognita* was used as template DNA. PCR with primers CF-ITS and HCFI-28S resulted in a fragment of 525 bp when *M. chitwoodi* DNA was used as template and in a smaller fragment of 517 bp when *M. fallax* was used as template DNA. No amplification could be observed when DNA of *M. hapla* and *M. incognita* was used as template DNA. PCR with primers I-ITS and HCFI-28S resulted in amplification of a 415 bp fragment when *M. incognita* was used as template DNA. No amplification could be observed when the other species served as template DNA. All isolates listed in Table 1 were included in every experiment. The amplified fragments in the first four lanes of Fig. 2 represent 10 µl of the reaction products of the PCRs described above when using 3 ng of template DNA. Similar signals were obtained when the crude extract of a single juvenile was used as template DNA and when 35 PCR cycles were used.

Multiplex PCRs were performed while using all four primers H-18S, CF-ITS, I-ITS, and HCFI-28S in a single PCR reaction. The following templates were used: *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. incognita*, 1:1 mixtures of *M. chitwoodi* and *M. fallax*, *M. chitwoodi* and *M. hapla*, and *M. fallax* and *M. hapla*, a 1:1:1 mixture of *M. chitwoodi*, *M. fallax*, and *M. hapla*, and a 1:1:1:1 mixture of *M. chitwoodi*, *M. fallax*, *M. hapla*, and *M. incognita*. Results presented in Fig. 2 show that this multiplex PCR easily differentiates the species *M. hapla* and *M. incognita* from each other and from *M. chitwoodi* and *M. fallax*, even in mixtures. To facilitate the differentiation between *M. fallax* and *M. chitwoodi*, the amplification products were digested with *Rsa*I. As could be deduced from observing the sequences, this resulted in restricted fragments of 416 and 101 bp for *M. fallax* and restricted fragments of 554 and 106 bp for *M. hapla*.

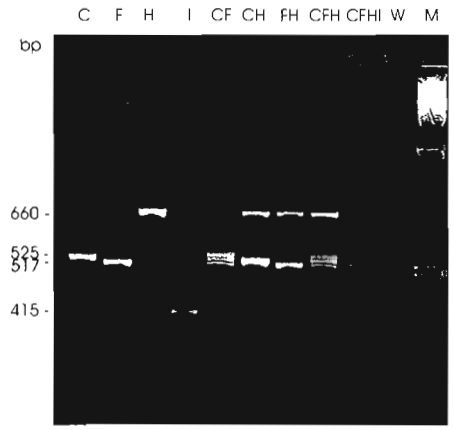

Fig. 2. *Typical amplification products of multiplex PCR reactions using forward primers H-18S, CF-ITS, I-ITS and reverse primer HCFI-28S in a single PCR reaction with 3 ng of template DNA of* Meloidogyne chitwoodi *(C),* M. fallax *(F),* M. hapla *(H),* M. incognita *(I), 1:1 mixtures of* M. chitwoodi *and* M. fallax *(CF),* M. chitwoodi *and* M. hapla *(CH), and* M. fallax *and* M. hapla *(FH), a 1:1:1 mixture of* M. chitwoodi, M. fallax *and* M. hapla *(CFH), and a 1:1:1:1 mixture of* M. chitwoodi, M. fallax, M. hapla *and* M. incognita *(CFHI). M: 1 kb size marker DNA. W: no template DNA control.*

The amplified fragments of *M. chitwoodi* and *M. incognita* were not restricted by *Rsa*I. Results are shown in Fig. 3.

Fig. 4 shows that a proportion as low as 2 to 5% of *M. chitwoodi* or *M. hapla* in mixtures can be detected by this multiplex PCR technique. The technique presents the same sensitivity for the detection of *M. incognita* and *M. fallax* in mixtures (data not shown).

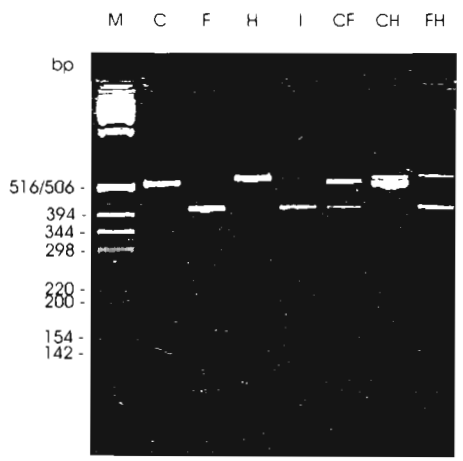

Fig. 3. *RsaI restriction products of the PCR-products shown in Fig. 2. Capital letters: see Fig. 2.*

Discussion

The sequences in Fig. 1 were very useful for providing sequences of species-specific primers. It should be noted that the data in Fig. 1 are the sequences of cloned ITS-PCR fragments of isolates Co of *M. chitwoodi*, Fa of *M. fallax*, Hk of *M. hapla*, and Ia of *M. incognita*. These cloned fragments were sequenced in both directions to detect possible sequencing artefacts. However, it cannot be excluded that (slight) sequence differences may exist within one species or even within one individual between the different ribosomal gene cluster repeat units. Zijlstra *et al.* (1995) gave strong evidence that different ITS sequences are present within single individuals of *M. hapla*. Digestion of amplified ITS regions of *M. hapla* with restriction enzymes *Hin*dIII, *Hin*fI, and *Msp*I always resulted in patterns that contained additional minor bands. This was not due to contamination or partial digestion. It was proposed that a major part of the *M. hapla* ITS fragments has a restriction map resulting in the restriction patterns containing strong signals; a minor part of the *M. hapla* ITS fragments has a different restriction map resulting in the minor bands in the restriction patterns (Zijlstra *et al.*, 1995). Consequently, we subjected the *M. hapla* ITS region inserts of the selected clones to a restriction analysis with *Hin*dIII, *Hin*fI, and *Msp*I to select those clones that contained ITS fragments with a restriction map producing the major restriction patterns. As expected, most of the inserts gave major restriction patterns without any evidence of minor bands, whereas a minor part of the inserts gave the minor restriction patterns for one or more of the restriction enzymes *Hin*dIII, *Hin*fI, and *Msp*I without any evidence of the major bands (unpubl.). The sequences in Fig. 1 show restriction sites at locations that agree with the ITS-RFLP data for *M. chitwoodi*, *M. fallax*, and *M. incognita* and with the fragment sizes of the major bands of the *M. hapla* ITS-restriction patterns. This indicates that even if the sequence of a minority of the ITS fragments present within individuals is different from those shown in Fig. 1, which is the case in at least *M. hapla*, most of the sequences of the ITS-fragments present in the species will most likely be as shown in Fig. 1. Moreover, this study has clearly shown that the (intraindividual) ITS heterogeneity does not prevent the identification of *M. chitwoodi*, *M. fallax*, and *M. hapla* and the differentiation of these species from each other and from *M. incognita* in mixtures, when the approach des- cribed in this study is applied.

In previous papers (Zijlstra *et al.*, 1995, 1997), the size of the ITS-PCR products of *M. hapla*, *M. chitwoodi*, *M. fallax*, and *M. incognita* measured by using the ITS-specific primers 5367 and 5368 was approximately 760 bp. The sequence data of the present study clearly show that there are slight size differences

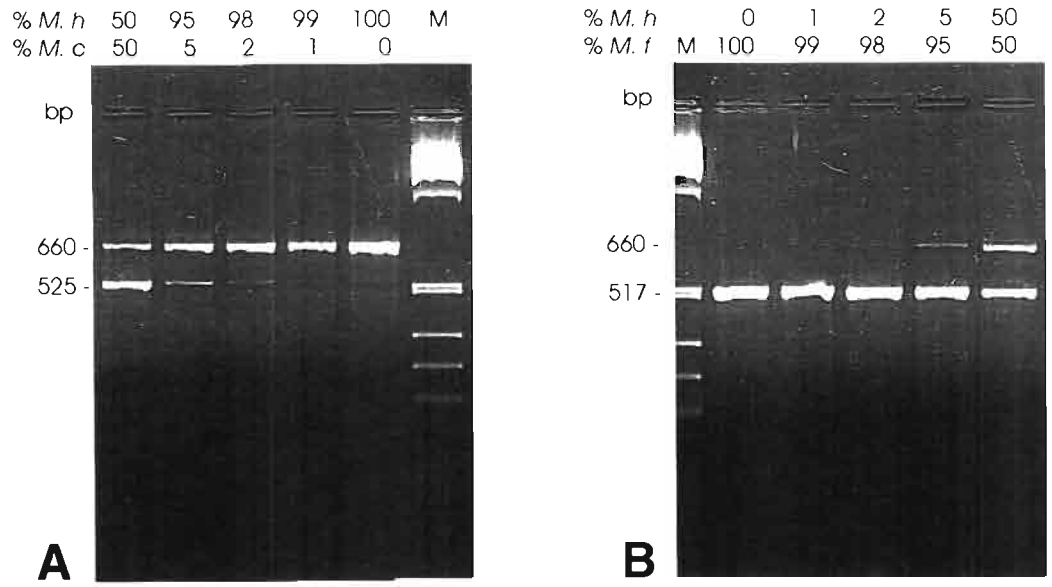

Fig. 4. *Amplification products of multiplex PCR reactions using forward primers H-18S, CF-ITS, I-ITS and reverse primer HCFI-28S in a single PCR reaction with 3 ng of template DNA of mixtures of A:* Meloidogyne hapla *(M. h) and* M. chitwoodi *(M. c); B:* M. hapla *(M. h) and* M. fallax *(M. f). The sizes of the fragments in base pairs are indicated on the left.*

between the species, ranging from 766 bp for *M. incognita* to 784 bp for *M. chitwoodi*. According to the alignment, there is little variation in the parts of the coding regions. More variation is present in the ITS1 region. The ITS1 sequences of *M. hapla* and *M. incognita* differ from each other and from *M. chitwoodi* and *M. fallax*. The ITS1 sequences of the last two species are very similar. Highest variation is found in the ITS2 region. The sequences of *M. chitwoodi* and *M. fallax* are almost identical, except a small deletion and some sequence differences at the 3' end of the ITS1 region and a few differences in the ITS2 region. This sequence similarity made it very difficult to find suitable primers that would specifically amplify *M. fallax* or *M. chitwoodi*. The differing sequences in the ITS1 and ITS2 regions are very AT-rich with long stretches of A and T, which make them not suitable for specific primer design. Therefore, the CF-ITS primer was selected. It should allow amplification of *M. chitwoodi* and *M. fallax* resulting in a PCR product of 525 bp for *M. chitwoodi* when reverse primer HCFI-28S was used and a fragment of 517 bp for *M. fallax,* which is shorter due to deletions in its ITS1 and ITS2 regions. The primers H-18S and I-ITS, which allow species-specific amplification of *M. hapla* and *M. incognita,* respectively, were much easier to select. By using all three forward primers with one common reverse primer in a single PCR reaction, the species of the root-knot nematode sample tested could be identified in a single step. Template DNA originating from mixtures containing similar amounts of nematodes of different species revealed the amplified species specific products of all species present in the mixture. These patterns were as expected, except for those mixtures that contained *M. chitwoodi* and *M. fallax*. Besides the expected fragments of 525 and 517 bp, these mixtures always showed a larger third fragment (see Fig. 2). Because of the high sequence similarity between *M. chitwoodi* and *M. fallax,* this third fragment can be assumed to be a heteroduplex. Because of its DNA conformation, it will migrate differently in electrophoretic gels than the homoduplexes (Keen *et al.*, 1991; Ganguly *et al.*, 1993). This phenomenon is ideal as an indication that both species are present in the sample tested. However, when only one fragment of about 520 bp in the PCR pattern is visible and a reference fragment of *M. fallax* or *M. chitwoodi* is missing, it is hard to estimate whether the sample tested contains *M. chitwoodi* or *M. fallax*. This will be shown by digestion with *Rsa*I, as seen in Fig. 3. When a reference fragment of *M. chitwoodi* or *M. fallax* is used, this technique can identify single juveniles or isolates of *M. hapla*, *M. chitwoodi*, *M. fallax,* and *M. incognita* using a single PCR reaction.

In the recently described ITS-RFLP based identification approach (Zijlstra *et al.*, 1997), the relative

intensities of the bands of each species-specific restriction pattern observed corresponded with the proportion of these species in mixtures. In the present study, the ratio of the intensities of the amplified fragments did not correspond with the ratio of the species present in the mixture (Fig. 2: lane CH; Fig. 4: lanes 50%50%). If individual nematodes of the different species tested contain a similar number of cistrons (Zijlstra *et al.*, 1997), this phenomenon could be due to the fact that three different forward primers are used that can differ in their affinity to anneal to their respective annealing sites under the given PCR conditions. Moreover, secondary and tertiary structures surrounding the annealing sites of the different forward primers might influence the success of annealing during the PCR. Nevertheless, compared to the ITS-RFLP approach, the present technique is faster and cheaper since no restriction enzyme incubation is required. Moreover, the two techniques have similar sensitivity and detect species present in mixtures in proportions as low as 2 to 5%. The new technique can become widely used in routine diagnostic tests to identify species and to sensitively differentiate them from each other in field populations.

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