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Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays

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Summary – Three randomly amplified polymorphic DNA (RAPD) markers, OPA-12_{420}, OPB-06_{1200} and OPA-01_{700}, species specific to the root-knot nematode species *Meloidogyne arenaria*, *M. incognita* and *M. javanica* respectively, were identified. After sequencing these RAPD-PCR products, longer primers of 18 to 23 nucleotides were designed to complement the terminal DNA sequences of the DNA fragments. This resulted in three pairs of species specific primers that were used to amplify the sequence characterised amplified regions (SCARs). The developed sets of SCAR primers were successfully used in straightforward, fast and reliable PCR assays to identify *M. incognita*, *M. javanica* and *M. arenaria*. The length variant SCAR markers can be amplified from DNA from egg masses, second stage juveniles and females. This species identification technique is therefore independent of the nematode's life cycle stage. Moreover the SCAR-PCR assay was successfully applied using DNA extracts from infested plant material. The method has potential to be optimised for routine practical diagnostic tests facilitating the control of these economically important pest organisms.

Résumé – ***Identification de* Meloigyne incognita, M. javanica *et* M. arenaria *au moyen de l'amplification de régions de séquences caractéristiques (SCAR) par une technique PCR*** – Trois marqueurs d'ADN polymorphique amplifiée au hasard (RAPD) OPA-12_{420}, OPB-$O6_{1200}$ et OPA-OI_{700}, respectivement spécifiques des espèces de nématodes *Meloidogyne arenaria*, *M. incognita* et *M. javanica*, ont été identifiés. Après le séquençage de ces produits RAPD-PCR, les amorces les plus longues de 18 à 23 nucléotides ont été choisies pour compléter les séquences terminales d'ADN des fragments d'ADN. Cela a conduit à trois paires d'amorces spécifiques de l'espèce, utilisées pour amplifier les régions des séquences caractéristiques (SCAR). Les lots d'amorces SCAR mis au point ont été utilisés avec succès lors d'essais directs, rapides et surs pour identifier *M. incognita*, *M. javanica* et *M. arenia*. Les marqueurs peuvent être amplifiés à partir de l'ADN des masses d'œufs, des juvéniles de deuxième stade ou des femelles. Cette technique d'identification spécifique est donc indépendante des différents états de développement du nématode. De plus la technique SCAR-PCR a été appliquée avec succès à l'ADN extrait du matériel végétal infesté. Cette méthode présente des potentialités d'amélioration permettant d'envisager des tests pratiques d'identification de routine, facilitant ainsi le contrôle de ces parasites économiquement importants.

Keywords – diagnostics, RAPD, root-knot nematodes.

Root-knot nematodes (*Meloidogyne* species) are major agricultural pests of a wide range of crops. The genus comprises more than 60 described species that are distributed worldwide. *Meloidogyne incognita*, *M. javanica* and *M. arenaria*, which occur in tropical and subtropical regions as well as in temperate mild regions and in glasshouses, account for the majority of crop losses caused by the root-knot nematodes. Control of plant parasitic nematodes by chemical means has become less desirable because of increased environmental awareness. Therefore the use of resistant plant cultivars and crop rotation have become more attractive alternatives for control measures. Methods that enable the timely detection and identification of these economically important species are necessary for proper research into virulence and resistance and to facilitate resistance management and the design of crop rotation systems.

Available methods of identification based on morphological characters (Jepson, 1987) require a lot of skill and are often inconclusive for individuals because they often vary considerably within a population. Isozyme analysis is a relatively fast way to identify species of *Meloidogyne*

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(Esbenshade & Triantaphyllou, 1990). However, for clear, reliable results, isozyme analysis can only be done with females of a specific developmental stage, whereas soil samples only contain juveniles or eggs. DNA-based diagnostics provide attractive solutions, because they do not rely on the expressed products of the genome, they are independent of environmental influence and of the stage of the nematode life cycle, and are potentially extremely discriminating. Accordingly, several studies have been performed that have looked at polymorphisms of DNA banding patterns between and within the species *M. incognita*, *M. javanica* and *M. arenaria*. Early DNA analyses predominantly assayed restriction fragment length polymorphisms that enabled the differentiation of the species or could differentiate populations within species (Curran *et al.*, 1985, 1986; Powers *et al.*, 1986; Castagnone-Sereno *et al.*, 1991, 1993; Garate *et al.*, 1991; Cenis *et al.*, 1992; Piotte *et al.*, 1992; Xue *et al.*, 1992; Fargette *et al.*, 1996). However, since the RFLP technique requires relatively large amounts of DNA, more recent studies have used PCR-based methods that require much less DNA. In particular, the exploration of PCR-based methods that use multicopy DNA sequences as target DNA, such as ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA), initially became popular. Unfortunately, the rDNA sequences of *M. incognita*, *M. javanica* and *M. arenaria* studied turned out to be so homologous that rDNA based differentiation is not possible (Zijlstra *et al.*, 1995; Powers *et al.*, 1997; Blok *et al.*, 1997a). Harris *et al.* (1990) and Powers and Harris (1993) managed to separate the three *Meloidogyne* species by mtDNA sequence analysis. A disadvantage of the technique is that the PCR reaction has to be followed by a restriction enzyme digestion step. More rapid techniques are desirable for practical purposes.

Random amplified polymorphic DNA (RAPD) fingerprinting is a rapid technique and was successfully used to distinguish the species *M. incognita*, *M. javanica* and *M. arenaria* (Cenis, 1993; Baum *et al.*, 1994; Blok *et al.*, 1997b).

Similar to previous studies regarding species identification of plant parasitic nematodes using species specific primers derived from RAPD fragments (Williamson *et al.*, 1997; Fullaondo *et al.*, 1999), the objective of this study was to select species specific RAPD-fragments for *M. incognita*, *M. javanica* and *M. arenaria*, to determine their DNA sequences and to design longer species specific pairs of primers. These primers would be used to amplify the sequence characterised amplified regions (SCARs), enabling the straightforward, fast and reliable identification of species *M. incognita*, *M. javanica* and *M. arenaria*.

Materials and methods

Nematodes

The isolates used are listed in Table 1. DNA of samples Io, Ip, Ir, Jh, Ji, Jj, Ab, Ag, Me and Mf was extracted from second stage juveniles as described in Fargette *et al.* (1996). DNA of the other samples was extracted from second stage juveniles, females, egg masses and galls of infested tomato roots as described in Zijlstra *et al.* (1997). DNA extraction from galls of infested roots was done as described for DNA extraction from nematodes (Zijlstra *et al.*, 1997) with the exception that PVP was added to the extraction buffer at a final concentration of 1%.

PCR-RAPD-analysis

Amplification reactions were carried out in volumes of 50 μl containing 10 mM Tris pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.3 μM primer, 1 unit of Taq DNA polymerase (Pharmacia, Peapack, NJ, USA) and 3 ng of total DNA. The RAPD primers used were synthesised by Operon Technologies (Alameda, CA, USA). For RAPD amplifications, the thermocycler was programmed for 45 cycles of 1 min at 94°C, 2 min at 38°C and 3 min at 72°C, with a temperature ramp of 1°C per 5 s for the 38-72°C transition. Control reactions without template DNA were included to avoid misinterpretations of the RAPD patterns due to artifacts.

RAPD primers were first screened on pooled DNA of isolates of a species to determine which yielded strong amplifications. The strongly amplifying primers of interest were screened on the isolates listed in Table 1.

Cloning of RAPD fragments, sequencing and primer design for SCARs

RAPD amplification products from three primers (OPA-01: 5′CAGGCCCTTC, OPA-12: 5′TCGGCGATAG, OPB-06: 5′TGCTCTGCCC) were purified from gel and cloned into the pGEM-T vector (Promega, Madison, WI, USA) as described in Zijlstra (1997) or into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen BV, Groningen, The Netherlands). The cloned inserts were tested by amplification of miniprep plasmid DNA with the original RAPD primers. Plasmid DNA from the correct insert-containing clones was extracted using a Qia-

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

Species	Code	Location	Isolate	Source
M. incognita	Ia	The Netherlands		IPO-DLO[a]
	Ib	The Netherlands	Inc568-93	PD[b]
	Ik	Egypt	Mynia	AGERI[c]
	Il	Egypt	Giza	AGERI
	Io	Senegal	line 16	IRD[d]
	Ip	Burkina Fasso	line 17	IRD
	Ir	Chad	line 18	IRD
M. javanica	Ja	Unknown		IPO-DLO
	Jb	"	C3059	PD
	Jc	South Africa	Mooirivier	GCI[e]
	Jd	USA (NC)	JNC	AGERI
	Jh	Burkina Fasso	line 22	IRD
	Ji	Burkina Fasso	line 23	IRD
	Jj	Spain	line 24	IRD
M. arenaria	Aa		C4393	PD
	Ab	Ivory Coast		IRD
	Ag	French W.I.	line 31	IRD
	Ah	The Netherlands	Xa	PD
M. mayaguensis	Me	Ivory Coast	line 1	IRD
	Mf	Ivory Coast	line 5	IRD
M. chitwoodi	Co	The Netherlands	Horst	PAV[f]
	Cba	USA (OR)	ORMC12, race 1	WSU[g]
	Cbd	USA (WA)	WAMC16, race 2	WSU
	Cbh	USA (CA)	CAMC2, race 3	WSU
	Ccg	Portugal		INRA[h]
M. fallax	Fa	The Netherlands	CHB	PAV
	Fe	The Netherlands		BLGG[i]
	Fh	France	CHK	INRA
M. hapla	Hh	The Netherlands		PAV
	Hk	The Netherlands	Sl.92	PAV
	Han	South Korea	C2346	PD
	Has	USA (WA)	WAMH2	WSU
	Hbq	Hungary	C6611	PD

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Table 2. *Nucleotide sequence of primer used for each SCAR derived from the RAPD markers. Pairs shown were used in PCR reactions using two primers to specifically detect a single species. Lower case letters represent (part of) the sequence of the progenitor RAPD primer.*

RAPD marker	Name of SCAR primer	Sequence of SCAR primer	Size of SCAR
OPA-12_{420}	Far	TcggcgatagAGGTAAATGAC	420
	Rar	tcggcgatagACACTACAACT	
OPB-06_{1200}	Finc	ctctgcccAATGAGCTGTCC	1200
	Rinc	ctctgcccTCACATTAAG	
OPA-01_{700}	Fjav	GGTGCGCGATTGAACTGAGC	670
	Rjav	caggcccttcAGTGGAACTATAC	

gen plasmid kit. Sequencing of the inserts was performed in both directions. Specific SCAR primer sequences (Table 2) of 18-23 bases were designed, beginning on the RAPD primer sequence or within 100 bp of the primer sequence.

SCAR AMPLIFICATION AND ANALYSIS

Amplification reactions for SCAR were performed in 25 μl reaction volumes containing 10 mM Tris pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.5 unit of Taq DNA polymerase (Pharmacia) and 1 to 10 ng of total DNA. For PCR reactions using the primers Far/Rar (Table 2), they were used at a final concentration of 0.3 μM. For PCR reactions using Finc/Rinc or Fjav/Rjav (Table 2) a primer concentration of 0.24 μM was used. For SCAR amplifications, the thermocycler was programmed for 2 min at 94°C followed by 35 cycles of 30 s at at 94°C, 30 s at the annealing temperature and 1 min at 72°C. Annealing temperatures were 54°C using the primers FincRinc, 61°C using primers Far/Rar and 64°C using primers Fjav/Rjav.

Results

Initially, 12-base primers (OPA-01, -09, -11, -12, OPB-06, -07, -08, -09, -11, -12, -15, -17) were tested on three DNA samples consisting of pooled DNA of isolates of the species *M. incognita*, *M. javanica* and *M. arenaria*. All primers produced good amplification products and different patterns for the three species tested. With each of these primers the RAPD patterns of the species shared one or two bands of the same size.

Primers OPA-01, -12 and OPB-06 that produced RAPD patterns with clear bands that clearly distinguished one species from the others were subsequently tested on the isolates listed in Table 1. This showed that the bands of interest, OPA-12_{420}, OPA-01_{700} and OPB-06_{1200}, where the subscripts refer to the approximate fragment size in base pairs, appeared indeed to be species specific for *M. arenaria*, *M. javanica* and *M. incognita*, respectively. An example of RAPD patterns is shown in Fig. 1.

First, the *M. arenaria* specific OPA-12_{420} fragment was isolated from the gel, cloned and sequenced. A *M. arenaria* specific SCAR primer set was designed resulting in primers Far and Rar (Table 2). Using this primer set, a 420 bp fragment was amplified when *M. arenaria* was used as template. No amplification could be observed when *M. incognita*, *M. javanica*, *M. chitwoodi*, *M. fallax*, *M. mayaguensis* or *M. hapla* were used as template DNA (Fig. 2).

Similarly, the SCAR primer set Fjav/Rjav (Table 2) was designed to DNA sequence of terminal ends of *M. javanica* specific OPA-01_{700}. Using the Fjav/Rjav primer set, a 670 bp fragment was amplified when *M. javanica* was used as template. No amplification could be observed when *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. incognita*, *M. mayaguensis* or *M. arenaria* were used as template DNA (Fig. 2).

Similarly, the SCAR primer set Finc/Rinc (Table 2) was designed to DNA sequence of terminal ends of *M. incognita* specific OPB-06_{1200}. Using the Finc/Rinc primer set, a 1200 bp fragment was amplified when *M. incognita* was used as template. No amplification could be observed when *M. chitwoodi*, *M. hapla*, *M. fallax*, *M. javanica*, *M. mayaguensis* or *M. arenaria* were used as template DNA (Fig. 2).

In every experiment all isolates listed in Table 1 were included and DNA extracts from juveniles, egg masses, females and galls of infested tomato roots were tested. The amplified fragments in Fig. 1 equal 8 μl amounts (out of 25 μl of total reaction) of the reaction products of the PCRs described above when using 3 ng of template DNA.

Discussion

RAPD-PCR distinguishes the species *M. incognita*, *M. javanica* and *M. arenaria* as shown in this study as well as in previous studies (Cenis, 1993; Baum *et al.*, 1994; Blok *et al.*, 1997b). However, reproducible patterns can only be obtained when identical conditions are used between experiments. Differences in the template concentration and the source and purity of the DNA may result in different fingerprints. This makes RAPD-PCR not a very suitable method for routine identification purposes. Moreover, the use of short, non specific primers will allow amplification of any 'contaminant' (including microbial 'contaminants') which may be present in extracts. It is likely that the RAPD-PCR technique will be sensitive enough to produce banding patterns from 'contaminant' template DNA, which will confuse the nematode pattern.

In order to develop PCR-assays that allow the identification of these three economically important root-knot nematode species under a wide range of conditions, species specific pairs of primers that amplify species specific RAPD fragments were developed in this study. After sequencing the fragments, longer primers were designed to complement the terminal sequences of the polymorphic DNA fragments. These specific primers were used to generate SCARs. SCARs that are well optimized tend to be less sensitive than RAPDs to varying reaction conditions.

The newly designed primers for the identification of *M. arenaria* contain the whole sequence of the original RAPD primer OPA-12 plus additional nucleotides. Those for the identification of *M. incognita* contain the last eight nucleotides of the original RAPD primer OPB-06. When these new sets of SCAR primers are used in a PCR, amplification products are obtained that have the same sizes as those of the original selected RAPD fragments. The newly selected reverse primer for the identification of *M. javanica* contains the whole sequence of the original RAPD primer OPA-01, whereas the newly designed forward primer for the identification of *M. javanica* does not contain the original RAPD primer sequence but is located 30 bp downstream of the sequence of the selected RAPD fragment. This is why the size of the amplified fragment upon PCR with the *M. javanica* specific SCAR primers is 30 bp shorter than that of the size of the selected RAPD-fragment. The reason for selecting this forward primer downstream of the end of the RAPD fragment is that a much stronger signal was obtained than when the forward primer contained the sequence of the RAPD primer. The reason for this could be preferential annealing due to a better secondary or tertiary structure of the template DNA at the downstream located primer binding site. The resulting sets of species specific SCAR primers proved to be powerful tools for the straightforward, fast and reliable identification of *M. incognita*, *M. javanica* and *M. arenaria* in PCR assays.

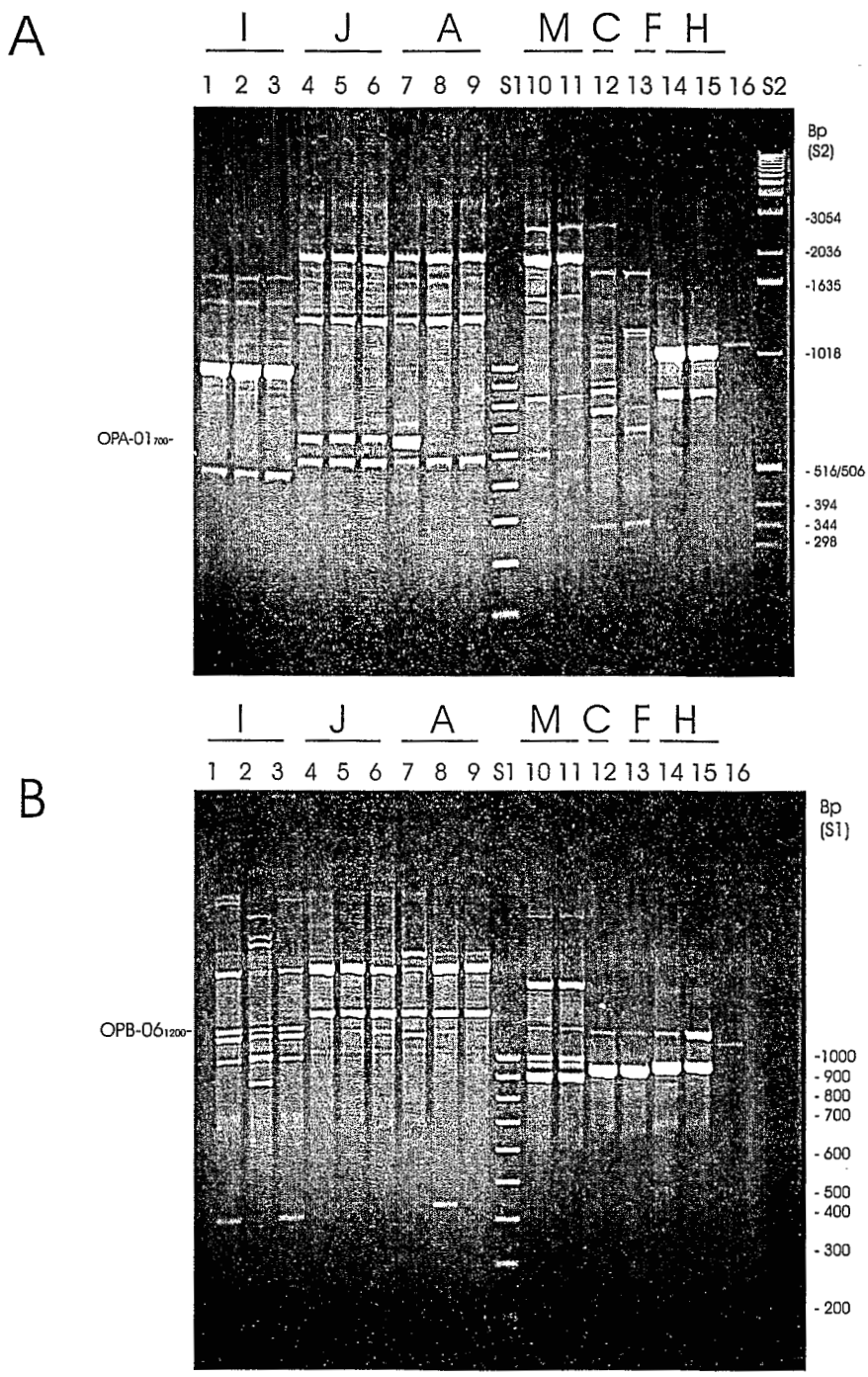

Fig. 1. *RAPD patterns using RAPD primers OPA-01 (A) and OPB-06 (B). OPA-01*$_{700}$ *and OPB-06*$_{1200}$ *are species specific RAPD markers that were successfully used for* Meloidogyne javanica *and* M. incognita *specific SCAR primers design, respectively. I:* M. incognita*; J:* M. javanica*; A:* M. arenaria*; M:* M. mayaguensis*; C:* M. chitwoodi*; F:* M. fallax*; H:* M. hapla. *Lanes 1-10: Isolates Io, Ip, Ir, Jh, Ji, Jj, Ag, Ab, Ah and 100 bp size marker, respectively; Lanes 11-17: Isolates Me, Mf, Co, Fa, Hh, Hk and no template DNA control, respectively.*

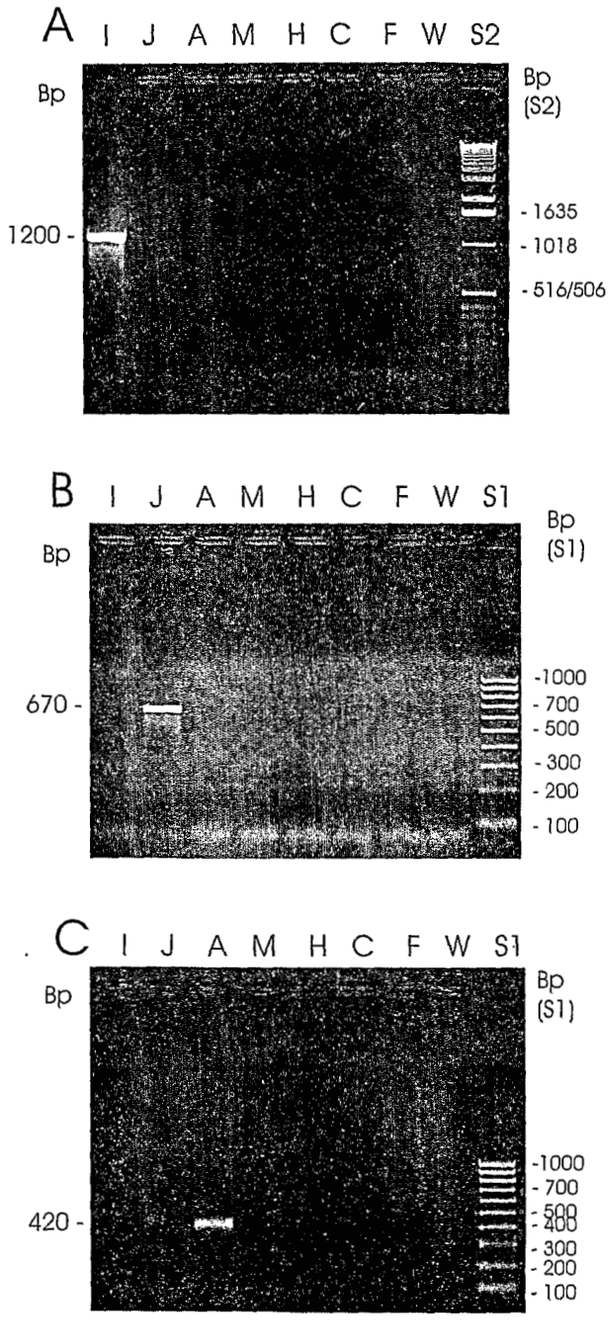

Fig. 2. *Typical amplification products of PCR reactions using primers Finc/Rinc (A), Far/Rar (B) or Fjav/Rjav (C) using 3 ng template DNA of I:* Meloidogyne incognita*; J:* M. javanica*; A:* M. arenaria*; M:* M. mayaguensis*; H:* M. hapla*; C:* M. chitwoodi*; F:* M. fallax*; W: No template DNA control; S: Size marker.

A set of SCAR primers directs the amplification of a single fragment with a specific size from the target DNA. Since SCAR primers are longer than RAPD primers, higher annealing temperatures can be used. This means that the desired SCAR fragments are easily obtained in PCR reactions, independent of the amount and source of template DNA that is offered. Template amounts of 1 to 10 ng, isolated from egg masses, females, second stage juveniles or galls of infested tomato roots allowed proper amplification of the species specific, length variant, SCAR markers.

The method will have potential for the application of routine diagnostic purposes using DNA extracts from soil samples or infected plant material. Mixtures can also easily be identified using this method. The reliable and rapid identification of these economically important pest organisms is an increasingly important component of plant protection. *M. incognita*, *M. javanica* and *M. arenaria* have the same geographical distribution and wide, but somewhat different, host ranges. This enables them to be controlled by crop rotation and resistance management, which requires accurate identification. Moreover, the approach described adds an identification tool to the already existing tools for identification of other *Meloidogyne* species. *M. chitwoodi*, for instance, can like *M. incognita*, *M. javanica* and *M. arenaria* be found in temperate mild regions. Its host range, however, is completely different. Now the chance of positively identifying all the possible *Meloidogyne* species present in a sample is increased and subsequently the possibilities of controlling them as well.

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