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Identification of *Meloidogyne chitwoodi, M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits

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Abstract

This study describes the development of species-specific pairs of PCR primers for the root-knot nematodes *Meloidogyne chitwoodi, M. fallax* and *M. hapla* that amplify species-specific RAPD fragments. After sequencing the fragments, longer primers were designed to complement the terminal sequences of the polymorphic DNA fragments. The resulting pairs of primers were used to generate the sequence-characterized amplified regions (SCARs). Using the developed pairs of SCAR primers, SCAR fragments of *M. chitwoodi, M. fallax* or *M. hapla* were easily amplified from DNA extracts from juveniles, egg masses, females of the particular nematode species investigated, either present alone, in a mixture with other nematode species or in infested plant material. A specially designed multiplex assay using three pairs of SCAR primers enabled the identification of multiple species in a mixture in a single PCR step. Single juveniles were easily identified by applying this multiplex assay followed by a subsequent multiplex PCR using three pairs of nested primers. The SCAR-PCR-based assays described have potential to be optimized for routine practical diagnostic tests. The usefulness of converting RAPD markers into SCAR markers is discussed.

Abbreviations: SCAR – sequence-characterized amplified region.

Introduction

In the past few years, the need for practical reliable techniques for the identification of species of root-knot nematodes has increased. Such methods are needed for plant resistance management and research, design for crop rotation systems and for extension services that test harvested crops or soil samples for the presence of harmful nematodes. This is especially important when it concerns quarantine organisms. PCR based methods are relatively rapid, are independent of the nematode's life cycle stage, and are, as most DNA-based methods, very reliable. In the western part of Europe an increase of the root-knot nematodes *Meloidogyne chitwoodi, M. fallax,* both quarantine organisms, and *M. hapla* has been observed (Mulder and Roosjen, 1990; Molendijk and Mulder, 1996). For this reason considerable effort has been placed on designing PCR-based identification methods for these species. Such methods have been based on the amplification of mitochondrial DNA (Harris et al., 1990; Powers and Harris, 1993; Stanton et al., 1997), satellite DNA (Castagnone-Sereno et al., 1995), ribosomal DNA (Petersen and Vrain, 1996; Petersen et al., 1997; Zijlstra et al., 1995; 1997; Zijlstra, 1997), and randomly amplified polymorphic DNA fragments (RAPDs) (Baum et al., 1994; Blok et al., 1997; Cenis, 1993; Williamson et al., 1997). Initially many of the amplification steps had to be followed by a digestion step resulting in discriminating RFLP patterns (Hugall et al., 1994;

Powers and Harris, 1993; Zijlstra et al., 1995). A different approach, where restriction enzyme digestion is combined with PCR, resulting in amplified fragment length polymorphisms (AFLPs) also enabled the differentiation of the three species (Van der Beek et al., 1998). However, more recent PCR-based identification methods have focussed on designing specific PCR primers that can be used in a PCR assay without the need of a (subsequent) digestion step (Castagnone-Sereno et al., 1995; Petersen and Vrain, 1996; Petersen et al., 1997; 1999; Stanton et al., 1997; Williamson et al., 1997; Zijlstra, 1997).

Of all these PCR-based methods, RAPD and AFLP analyses offer the best means to study genetic variation at species and sub-species level. The other methods have basically only shown polymorphisms at species level. The big disadvantage of RAPD analysis is that the reproducibility between experiments is relatively low. A prerequisite for obtaining reliable and reproducible RAPD patterns is that equal amounts of template DNA are used between experiments and that all other reaction conditions should be identical as well. However, for the specific amplification of a RAPD fragment of interest the procedure can be optimized. After sequencing the particular fragment, longer primers can be designed that complement the terminal sequences. The resulting pairs of primers are used to generate the 'sequence-characterized amplified regions' (SCARs) (Paran and Michelmore, 1993).

The objective of this study was to show the usefulness, the sensitivity, the reliability and several applications of this approach by developing sets of SCAR primers that specifically amplify DNA of the root-knot nematode species *M. chitwoodi*, *M. fallax* or *M. hapla*. The potential of this technique is discussed.

Materials and methods

Nematodes

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

DNA extraction

Nematode DNA of samples Ab and Ae was kindly provided by M. Fargette (IRD, Montpellier, France). All other isolates DNA were extracted as described by Zijlstra et al. (1997). DNA was extracted from second stage juveniles, females, egg masses and galls of infested tomato roots. Crude extracts from single juveniles of *M. hapla*, *M. chitwoodi* and *M. fallax* were obtained as described by Harris et al. (1990). DNA extraction from galls of infested roots was done as described for DNA extraction from nematodes (Zijlstra et al., 1997) with the exception that a final concentration of 1% PVP was added to the extraction buffer.

PCR-RAPD-analysis

Amplification reactions were done 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) and 3 ng of total DNA. The RAPD primers used were synthesized by Operon Technologies (Alameda, CA). 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 (OPI-01, -02, -03, -04, -06, -07, -08, -09, -10, -11, -12, -13, -14, -15, -17) were first screened on four DNA samples consisting of pooled DNA of isolates of *M. chitwoodi*, *M. fallax*, *M. hapla* or *M. incognita* to determine which yielded strong amplifications for the first three species. The pooled DNA samples were made by mixing equal amounts of DNA of the isolates of *M. chitwoodi*, *M. fallax*, *M. hapla* or *M. incognita* that are listed in Table 1. Subsequently the primers, which produced strong amplification products, were screened on the isolates listed in Table 1.

Cloning of RAPD fragments, sequencing and primer design for SCARs

RAPD amplification products from 3 primers (OPI-01: 5′ ACCTGGACAC, OPI-09: 5′ TGGAGAGCAG, OPI-14: 5′ TGACGGCGGT) were purified from low-melting point agarose gel as described in Zijlstra (1997) and cloned into the pGEM-T vector (Promega) as described in Zijlstra (1997). 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 Qiagen plasmid kit. Sequencing of the inserts was performed in both directions using the Big Dye Terminator (Perkin Elmer) sequencing kit. Specific primer sequences (see Table 2) of 18–24 bases were chosen, beginning on the RAPD primer sequence or within 100 bp of the RAPD primer sequence. Nested primers were located further away from the terminal sequences. The primers were preferentially chosen to have stable 5′ ends but somewhat unstable 3′ ends since these perform best in PCR (Rychlik, 1993).

SCAR amplification and analysis

Amplification reactions for SCARs 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), 0.3 µM of each primer, 3 ng of total DNA or the crude extract of a single juvenile. For SCAR amplifications the thermocycler was programmed for 2 min at 94 °C followed by 30 cycles of

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

Code	Location	Isolate	Source
M. chitwoodi			
Ca	Netherlands	C3022	PD[a]
Cj	Netherlands	C5273f	PD
Ck	Netherlands	C5273g	PD
Cl	Netherlands	C2960	PD
Co	Netherlands	Horst	PAV[b]
Cp	Netherlands		PAV
Cx	Netherlands	C6503	PD
Cat	Washington, USA	WAMC17	WSU[c]
Cba	Oregon, USA	ORMC12, race 1	WSU
Cbd	Washington, USA	WAMC16, race 2	WSU
Cbf	Oregon, USA	ORMC8, race 2	WSU
Cbh	California, USA	CAMC2, race 3	WSU
Cbs	Netherlands	IHO9	PAV
Cbu	Netherlands	KBD4	PAV
Ccg	Portugal		INRA[d]
Cch	Argentina	CArg	INRA
M. fallax			
Fa	Netherlands	CHB	PAV
Fb	Netherlands	C4571	PD
Fc	Netherlands	C6501	PD
Fd	Netherlands	C6466	PD
Fe	Netherlands	W834.769	BLGG[e]
Fg	Netherlands		CPRO-DLO[f]
Fh	France	CHK	INRA
M. hapla			
Hi	Netherlands	Sm.Fei92	PAV
Hj	Netherlands	Sm.Fer92	PAV
Hh	Netherlands		PAV
Hk	Netherlands	Sl.92	PAV
Hak	Netherlands		HLB[g]
Ham	Queensland, Australia	Q114	IRD[h]
Han	South Korea	C2346	PD
Has	Washington, USA	WAMH2	WSU
Hau	Utah, USA	UTMH1	WSU
Haw	France	AN2	INRA, Antibes, France
Hbq	Hungary	C6611	PD
Hbr	Netherlands	C4900-H-1A	PD
Hbt	Netherlands	W834.347	BLGG
Hby	Netherlands	C3029	PD
Hcb	Netherlands	HBW	CPRO-DLO

Table 1. Continued

Code	Location	Isolate	Source
M. incognita			
Ia	Netherlands		IPO-DLO[i]
Ib	Netherlands	Inc568-93	PD
Ik	Egypt	Mynia	AGERI[j]
Il	Egypt	Giza	AGERI
M. javanica			
Jb		C3059	PD
Jc	South Africa	Mooirivier	GCI[k]
Jd	North Carolina	JNC	AGERI
M. arenaria			
Aa		C4393	PD
Ab	Ivory Coast		IRD
Ae	Portugal	ARE-POR	IRD

[a]Plant Protection Service, Wageningen, The Netherlands.
[b]Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands.
[c]Washington State University, Prosser, USA.
[d]INRA, Le Rheu, France.
[e]Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.
[f]Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands.
[g]Hillebrands Laboratorium, Assen, The Netherlands.
[h]IRD (ex-ORSTOM), Montpellier, France.
[i]DLO-Research Institute for Plant Protection, Wageningen, The Netherlands.
[j]Agricultural Genetic Engineering Institute, Agricultural Research Centre (ARC), Cairo, Egypt.
[k]Grain Crops Institute, Potchefstroom, South Africa.

Table 2. Nucleotide sequence of primer used for each SCAR derived from the RAPD markers

RAPD marker	Name	SCAR primers Sequence	Size of SCAR (bp)
OPI-01_{550}	Ff	CCAAACTATCGTAATGCATTATT	515
	Rf	ggacacAGTAATTCATGAGCTAG	
	Ff2	**CCATTTCTGCTAAATGCCAAACTA**	**530**
	Rf	**ggacacAGTAATTCATGAGCTAG**	
	FfN	*GCACCGAAACGTCCTGTAAA*	*280*
	RfN	*CTGCGTTGAACCGACCTATACT*	
OPI-14_{610}	Fh	tgacggcggtGAGTGCGA	610
	Rh	tgacggcggtACCTCATAG	
	Fh	**tgacggcggtGAGTGCGA**	**610**
	Rh	**tgacggcggtACCTCATAG**	
	FhN	*GCCTTCTTTGGATTCCTCTCA*	*420*
	RhN	*GGCTCATCCTTGCTGTAAAT*	
OPI-09_{900}	Fc	TGGAGAGCAGCAGGAGAAAGA	800
	Rc	GGTCTGAGTGAGGACAAGAGTA	
	Fc2	**GGCATTGACGTGCTCCGAGAGT**	**755**
	Rc	**GGTCTGAGTGAGGACAAGAGTA**	
	FcN	*CGCTGATAATCAGAGCAAAC*	*600*
	RcN	*GCCAATTCATAAGTGTTGTCTAG*	

Pairs are shown that are used in normal PCR reactions using two primers to specifically detect a single species. In bold pairs are shown that are used in multiplex PCR reactions used to detect multiple species in mixtures. In italics pairs are shown that can be used in a subsequent nested PCR. Lower case letters represent (part of) the sequence of the progenitor RAPD primer.

30 s at 94 °C, 30 s at the annealing temperature and 1 min at 72 °C. Annealing temperatures were 58 °C for SCAR reactions using the primers Ff/Rf and for the multiplex PCR using the primers Ff2/Rf/Fh/Rh/Fc2/Rc and 60 °C using primers Fh/Rh and Fc/Rc. To amplify from single juveniles, the same conditions were used, except that the number of cycles was increased from 30 to 40 and the reaction volume was 50 µl.

Identification of single juveniles using nested PCR

In order to identify the species of a single juvenile, the DNA extract of a single juvenile was first submitted to a multiplex PCR using the primers Ff2/Rf/Fh/Rh/Fc2/Rc. Subsequently 1 µl of this PCR product was used in a nested PCR using the primers FfN/RfN/FhN/RhN/FcN/RcN (Table 2). Nested PCR reactions 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), 1 µl PCR product as template DNA and 0.24 µM each of nested primers. For the nested multiplex PCR the thermocycler was programmed for 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 59 °C and 1 min at 72 °C. When the nested PCR was done with only one pair of nested primers the annealing temperatures were 64, 60 and 59 °C when primers FfN/RfN, FhN/RhN or FcN/RcN were used respectively.

Results

RAPD-PCR of species

All primers produced good amplification products and were tested on the isolates listed in Table 1. An example is shown in Figure 1 (primer OPI-14). PCR with primers OPI-01, -02, -06, -07, -08, -09, -10, -11, -12, -14 and -17 produced species-specific patterns for all four species, taking even though into account that *M. chitwoodi* and *M. fallax* patterns always shared one or more common bands. PCR with primers OPI-03 and -13 produced identical patterns for isolates of *M. chitwoodi* and *M. fallax*. OPI-04 RAPD patterns of isolates of *M. chitwoodi* and *M. fallax* contained species-specific bands but showed a lot of intraspecific variation. OPI-04 RAPD patterns of the *M. hapla* populations tested were all different, not reproducible and did not share common bands. Primer OPI-15 produced

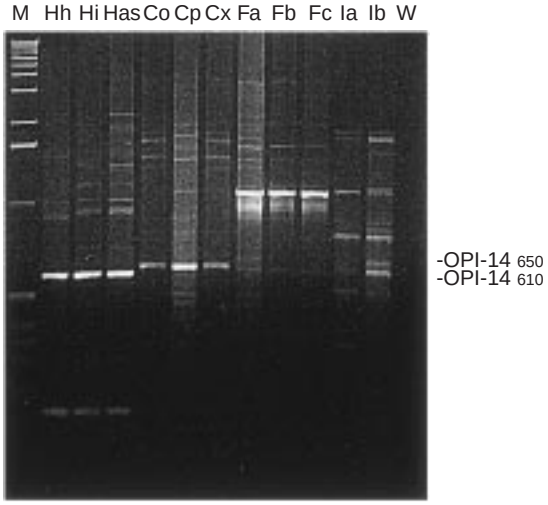

Figure 1. RAPD patterns using RAPD primer OPI-14. The *M. hapla* specific OPI-14_{610} RAPD fragment (the subscripts refer to the approximate fragment size in base pairs) was successfully used for *M. hapla*-specific SCAR primers design.

species-specific patterns for *M. hapla* but the RAPD patterns of isolates of *M. chitwoodi* and *M. fallax* were all different with no common bands.

Development of species-specific SCAR primers

First the *M. fallax*-specific OPI-01_{550} fragment (the subscripts refer to the approximate fragment size in base pairs), was isolated from the gel, cloned and sequenced. A *M. fallax*-specific SCAR primer set was designed resulting in primers Ff and Rf (Table 2). Using this primer set a 530 bp fragment was amplified when *M. fallax* was used as template (Figure 2, lane F). No amplification could be observed when DNA of *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. arenaria* or *M. javanica* were used as templates.

Similarly the SCAR primer set Fh/Rh (Table 2) was designed to terminal sequences of the *M. hapla*-specific OPI-14_{610} fragment (Figure 1). Using the Fh/Rh primer set a 610 bp fragment was amplified when *M. hapla* was used as template (Figure 2, lane H). No amplification could be observed when *M. chitwoodi*, *M. fallax*, *M. incognita*, *M. javanica* or *M. arenaria* DNA were used as templates. The SCAR primer set Fc/Rc was designed to terminal sequences of the *M. chitwoodi* OPI-09_{900}. Using the Fc/Rc primer set a 800 bp fragment was amplified when *M. chitwoodi* DNA was used as template (Figure 2, lane C). No amplification

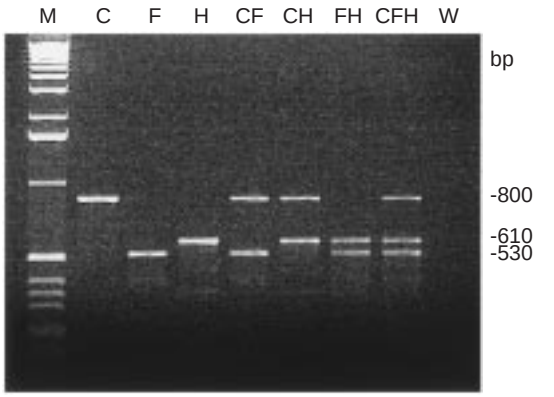

Figure 2. Typical amplification products of PCR reactions using primers Fc2/Rc (lane C) or Fh/Rh (lane H) or Ff/Rf (lane F) and of multiplex PCR reactions using primers Ff2/Rf/Fh/Rh/Fc2/Rc (all lanes) using 3 ng of template DNA of *M. chitwoodi* (C), *M. fallax* (F), *M. hapla* (H), 1 : 1 mixtures of *M. chitwoodi* and *M. fallax* (CF), *M. chitwoodi* and *M. hapla* (CH), *M. fallax* and *M. hapla* (FH), a 1 : 1 : 1 mixture of *M. chitwoodi*, *M. fallax* and *M. hapla* (CFH). M: 1 kb size marker DNA. W: no template DNA control.

could be observed when *M. hapla*, *M. fallax*, *M. incognita*, *M. arenaria* or *M. javanica* DNA were used as templates.

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 the first three lanes of Figure 2 equal 8 µl amounts 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 40 PCR cycles were used.

Multiplex PCR

Multiplex PCRs were performed using three sets of SCAR primers in a single PCR reaction. In order to optimize the multiplex PCR results in such a way that the intensities of the three SCAR bands were similar when using a 1 : 1 : 1 mixture of *M. chitwoodi*, *M. fallax* and *M. hapla*, different forward primers for the *M. fallax* and *M. chitwoodi* were selected. The PCR annealing temperature was 58 °C. Results presented in Figure 2 show that by using this multiplex PCR *M. hapla*, *M. chitwoodi* and *M. fallax* can easily be differentiated from each other, even in mixtures. Figure 3 illustrates that this multiplex PCR technique enables the detection of a proportion as low as 1–2% of

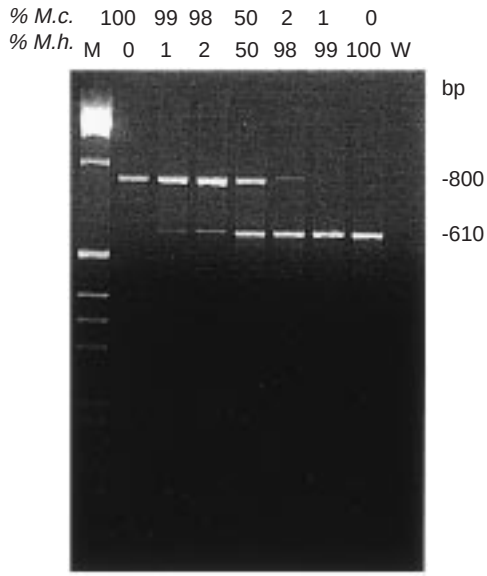

Figure 3. Typical amplification products of multiplex PCR reactions using primers Ff2/Rf/Fh/Rh/Fc2/Rc in a single PCR reaction using 3 ng of template DNA of mixtures of *M. chitwoodi* (M.c) and *M. hapla* (M.h). The sizes in base pairs of the fragments are indicated on the right. M: 1 kb size marker DNA.

M. chitwoodi or *M. hapla* in mixtures. The same was found for detection of *M. fallax* in mixtures (data not shown).

Identification of single juveniles

The DNA extract of a single juvenile was sufficient to amplify the species-specific SCAR fragment when using primer set Fc/Rc, Ff/Rf or Fh/Rh (data not shown). However, the multiplex PCR using the primers Ff2, Rf, Fh, Rh, Fc2, Rc never resulted in a visible amplified fragment when the extract of a single juvenile was used. Therefore this multiplex PCR product was used in a subsequent multiplex PCR using the nested primers FfN, RfN, FhN, RhN, FcN and RcN. This nested multiplex PCR always resulted in species-specific fragments of 280 bp for *M. fallax*, 420 bp for *M. hapla* and 600 bp for *M. chitwoodi*. Results are shown in Figure 4.

Discussion

To find species-specific RAPD-fragments, RAPD-PCRs were performed with DNA of *M. chitwoodi*,

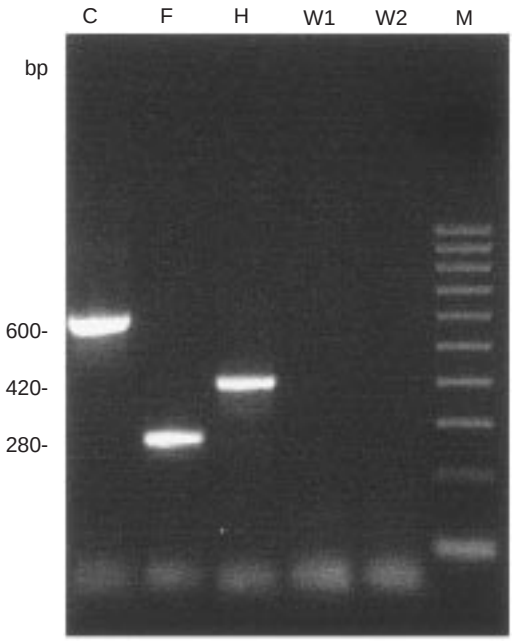

Figure 4. Typical amplification products of nested PCR reactions using primers FfN/RfN/FhN/RhN/FcN/RcN and 1 µl PCR product of a multiplex PCR using primers Ff2/Rf/Fh/Rh/Fc2/Rc performed on DNA extracts from single juveniles of *M. chitwoodi* (lane C), *M. fallax* (lane F) and *M. hapla* (lane H). W1: no DNA control of the nested PCR; W2: nested PCR product of the no template DNA control product of the first multiplex PCR. M: 100 bp size marker.

M. fallax and *M. hapla*. As was shown before, it was not difficult to find species-specific RAPD banding patterns for *M. chitwoodi*, *M. hapla* and *M. incognita* (Baum et al., 1994; Blok et al., 1997; Cenis, 1993; Williamson et al., 1997). However, this is the first report of RAPD studies on *M. fallax*. Some RAPD primers did not distinguish *M. chitwoodi* from *M. fallax* and most of the RAPD patterns showed many common bands between the two species indicating that both species share a lot of homology in their DNA sequences. Previous studies had shown that *M. chitwoodi* and *M. fallax* have considerable homology in their satellite DNA sequences (Castagnone-Sereno et al., 1999) as well as in their ribosomal ITS-DNA sequences (Zijlstra, 1997). Together with the data of the present study this indicates that both species are closely related.

In 1997, Williamson et al. reported a set of SCAR primers, based on RAPDs, that specifically amplified *M. chitwoodi*. However, when these primers were tested in our laboratory they also appeared to amplify *M. fallax*, a species that had not been described at the time of their studies. However, it again illustrates the relatedness of the two species. It also shows that new species-specific primers should be tested on as many relevant species as possible.

The PCR-assays using one pair of species-specific SCAR primers yielded large amounts of the desired amplification product, regardless of the source of template DNA. The SCAR fragments of *M. chitwoodi*, *M. fallax* or *M. hapla* were easily amplified from DNA extracts of juveniles, egg masses or females of the particular nematode species investigated, either present alone, in a mixture with other nematode species or in infested plant material. The procedure was sensitive enough to amplify the SCAR fragment from the crude DNA extract of a single juvenile.

Unfortunately, these three SCAR primer sets were not suitable for a multiplex PCR assay. Often SCARs of one or two of the species were not amplified or were hardly visible. By developing new forward SCAR primers for *M. chitwoodi* and *M. fallax* these problems were solved. Nevertheless this optimized multiplex PCR did not produce enough PCR product to be visualized when it was performed on DNA extracts from single juveniles. However, it was sufficient to serve as template DNA for the subsequent nested multiplex PCR, enabling the reliable identification of single juveniles. The fact that use of the nested primers in a subsequent PCR reaction enhances the sensitivity even more, offers possibilities to design extremely sensitive identification assays in which tiny amounts of nematode DNA could be detected and identified.

The SCAR-PCR methods described in this study will have potential for the application of routine diagnostic purposes using DNA extracts from single juveniles, soil samples or infected plant material. Mixtures can also easily be identified.

This report shows the usefulness of converting RAPD markers into reliable SCAR markers. One can imagine that RAPDs can provide a large number of markers for numerous traits. Unfortunately, using RAPD-PCR, these markers are not always reproducible since this technique is very sensitive to varying reaction conditions (Ellsworth et al., 1993; Munthali et al., 1992). SCAR primers direct 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 makes the desired SCAR fragments more readily obtained in PCR reactions and less dependent of the amount

and source of template DNA that is offered. Although SCAR-PCR can be very sensitive, the design of nested primers can increase the sensitivity even more. By combining multiple pairs of SCAR primers in one PCR reaction, mixtures can be identified. In this study SCAR primers were designed to identify species, but the method offers great possibilities to create markers at the sub-species level, serving a much wider range of identification levels and facilitating genetic research.

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