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Specific probes efficiently distinguish root-knot nematode species using signature sequences in the ribosomal intergenic spacer

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Summary – Molecular probes were designed to identify *Meloidogyne* species by hybridizing to unique signature sequences located within variable regions of the ribosomal intergenic spacer (IGS). IGS nucleotide sequences were obtained by sequencing the corresponding PCR-amplified DNA. Sequence alignments of the IGS from *M. chitwoodi* and *M. fallax* revealed several areas of localized dissimilarity to which species-specific PCR primers were synthesized. When used in combination with non-specific (conserved) primers, these primer pairs produced species-specific PCR amplification products as revealed by agarose gel electrophoresis. Size separation of amplified products from a single PCR reaction utilizing a combination of five specific and non-specific primers was demonstrated to provide an accurate, single-test assay, without the need for restriction digests. Multiplex-PCR amplification of DNA from single juveniles or a small number of eggs efficiently distinguished *M. chitwoodi* and *M. fallax* from *M. hapla, M. incognita, M. javanica, M. arenaria,* and *M. mayaguensis.*

Résumé – ***Sondes spécifiques permettant de distinguer les espèces de* Meloidogyne *en utilisant des signatures séquentielles de l'espaceur intergénique ribosomal*** – Ont été établies des sondes moléculaires -destinées à identifier les espèces de *Meloidogyne*- grâce à des différences spécifiques dans l'espaceur intergénique (IGS) de l'ADN ribosomal. Les séquences de nucléotides de l'IGS ont été obtenues en séquençant l'ADN amplifié par PCR. L'alignement des séquences de l'IGS de *M. chitwoodi* et *M. fallax* a révélé plusieurs régions contenant des différences localisées. Des amorces PCR ont été synthétisées qui ont donné des produits d'amplification spécifiques lorsqu'utilisées avec des amorces non spécifiques (conservées). Les produits d'amplification d'une seule réaction PCR utilisant cinq amorces, spécifiques et non spécifiques, ont pu être séparés par leur taille dans un gel d'agarose, procurant ainsi un test fiable et précis ne nécessitant pas de restriction enzymatique. L'amplification de l'ADN d'un nématode juvénile ou d'un oeuf par PCR multiplex a permis d'identifier *M. chitwoodi* et *M. fallax* et de les séparer de *M. hapla, M. incognita, M. javanica, M. arenaria* et *M. mayaguensis.*

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

Meloidogyne chitwoodi is an important pathogen of potato and other crops in the USA (Nyczepir *et al.*, 1982) and in Europe, and considerable efforts are being directed toward discovering the source of genetic resistance to this species in wild Solanaceae (Brown *et al.*, 1996; Janssen *et al.*, 1996). Until its formal description as a new species (Golden *et al.*, 1980), this nematode was known in the potato producing states of the northwestern USA as a particularly virulent race of *M. hapla*. *M. fallax* was previously classified as *M. chitwoodi* until its distinction as a new pathogenic species of potato in the Netherlands (Karssen, 1996). Due to the significance of *M. chitwoodi* as a pathogen, many countries including Canada are implementing quarantine restrictions. Simple, rapid and reliable means of identification of *Meloidogyne* spp. are therefore essential, not only for population management in the field, but also for quarantine purposes.

A number of molecular techniques have been developed for the routine identification of *Meloidogyne* species. Curran *et al.* (1986) and Powers *et al.* (1986) pioneered the use of RFLP's in genomic and mitochondrial DNA to identify nematode species. Subsequently, Harris *et al.* (1990) and Powers *et al.* (1993) separated single juveniles of the five major species of *Meloidogyne* using size variations in PCR amplified mitochondrial DNA.

Recent emphasis has been placed on more rapid techniques which, while as reliable as previous methods to separate *Meloidogyne* species, do not require digestion with restriction enzymes. Zijlstra *et al.* (1995), using nested PCR primers from Vrain *et al.* (1992) and Ferris *et al.* (1993) to amplify the internal transcribed spacer of rDNA (ITS), found enough variability in that region to separate *M. hapla* from *M. chitwoodi*. Powers *et al.* (1998) found identical sequences in the ITS region of *M. arenaria*, *M. javanica* and *M.*

incognita, pointing to the need to search other variable regions of the genome to distinguish these species. While Blok *et al.* (1997) also found no size differences between the ITS-1 PCR products of *M. incognita*, *M. arenaria* and *M. javanica*, the PCR products of the less related *M. hapla* and *M. mayaguensis* were distinguishable from the other three species.

The intergenic spacer (IGS) has become another focal point in molecular diagnostics since this region of rDNA more easily yields polymorphisms between *Meloidogyne* species. Petersen and Vrain (1996) reversed the ITS primers of Vrain *et al.* (1992) to amplify a 5.8 kb fragment including the IGS of *M. chitwoodi*. They mapped, obtained partial nucleotide sequences, and designed new PCR primers to the 3' end of the 28S rRNA gene and to the 5' end of the 18S rRNA gene. These primers amplified the IGS region of *M. chitwoodi*, *M. hapla* and *M. fallax*, revealing distinct polymorphisms between these three species, and providing molecular evidence to confirm the status of the newly described *M. fallax* (Karssen, 1996).

A further advance in molecular identification of nematode populations is to synthesize oligonucleotide probes which specifically hybridize to a single species, but this requires species-specific DNA targets to be identified. Williamson *et al.* (1997) synthesized PCR primers derived from the sequence of cloned RAPD bands that distinguished single juveniles of *M. chitwoodi* from *M. hapla*. Zijlstra (1997) cloned and sequenced the ITS region of *M. chitwoodi*, *M. fallax*, *M. hapla*, and *M. incognita*, and identified a great deal more variability in the ITS2 region than in the ITS1. PCR primers were designed to the variable regions to separate these four species. The amplification product from *M. chitwoodi* was easily separated from *M. hapla* and *M. incognita*, but the size of the PCR product of *M. fallax* differed from that of *M. chitwoodi* by only 8 bp, making it difficult to distinguish between the two using standard gel electrophoresis.

Since selection pressure decreases mutation rates within protein coding DNA sequences, a higher degree of genetic variability is often found in non-coding regions. Indeed, diagnostic probes capable of distinguishing *Pratylenchus penetrans* and *P. scribneri* were designed from intron sequences in the major sperm protein (*msp*) gene (Setterquist *et al.*, 1996). The non-coding nature of the IGS, combined with size polymorphisms within PCR-amplified IGS fragments from closely related species (Petersen & Vrain, 1996), indicated a high potential for utilizing this region for producing species-specific probes. We have synthesized two probes, each targeting signature sequences within the IGS of either *M. chitwoodi* or *M. fallax*, and utilized them in mutiplex PCR to distinguish and differentiate *Meloidogyne* species via a single-tube assay format.

Materials and methods

Nematodes

The populations of *Meloidogyne* spp. used in this study are listed in Table 1. Genomic DNA was purified from nematode juveniles or eggs essentially as previously described (Vrain *et al.*, 1992; Zijlstra *et al.*, 1995).

Polymerase chain reaction

PCR amplification of the entire IGS region utilized primers previously described (Petersen & Vrain, 1996). PCR reactions typically contained 3-5 ng genomic DNA template, 2 mM each dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase, and 1 X *Taq* buffer (60 mM Tris-SO_4 [pH 9.1], 15 mM $(NH_4)_2SO_4$, Appligene-Oncor, France). Cycling parameters typically consisted of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 72°C extension for 60 s. An initial denaturation step (95°C for 3 min) was performed prior to thirty amplification cycles, and a final extension step (72°C for 5 min) was performed immediately following amplification. PCR reactions utilizing the "pentaprimer" mix described below were conducted using an annealing temperature of 64°C.

DNA sequencing

Amplified fragments were separated from the genomic DNA template and primers by electophoresis using 2% (w/v) low-melting point agarose (Gibco-BRL, Burlington, ON, Canada) gels, containing 0.25 µg/ml ethidium bromide (EtBr), in Tris-acetate EDTA buffer at 90 V for 1 h. Visualization of EtBr-stained DNA was accomplished using a UV transilluminator, and the size of each fragment calculated using the 100 bp marker (BRL) as a size standard. DNA bands were excised from gels with a sterile scalpel and purified from the agarose using Qiaquick spin columns (Qiagen Inc, Chatsworth, CA, USA), eluted in 10 mM Tris-HCl, pH 8.0, and stored frozen until further use. Sequencing was accomplished using the FSTaq Ready Reaction Kit (Perkin Elmer, Mississaugua, ON, Canada) and analyzed by ABI310 capillary electrophoresis system according to the manufacturers directions. The sequencing data obtained for the IGS regions were aligned using Sequence Navigator so that additional primers could be designed to specifically target DNA from individual nematode species.

Oligonucleotide synthesis

Cloned DNA fragments containing portions of the rDNA IGS (Petersen & Vrain, 1996) were sequenced and used as the basis for primer constructions. The DNA sequences of oligonucleotide primers used in

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

Code	Location	Isolate	Source	Pentaprimer bands[a]
M. chitwoodi				
Ca	The Netherlands	C3022	Pd[b]	C, I
Ck	The Netherlands	C5273g	PD	C, I
Cl	The Netherlands	C2960	PD	C, I
Co	The Netherlands	Horst	PAV[c]	C, I
Cba	Oregon, USA	ORMC12, race 1	USDA[d]	C, I
Cbd	Washington, USA	WAMC16, race 2	USDA	C, I
Cbf	Oregon, USA	ORMC8, race 2	USDA	C, I
Cbh	California, USA	CAMC2, race 3	USDA	C, I
Cbs	The Netherlands	IHO9	PAV	C, I
Cbu	The Netherlands	KBD4	PAV	C, I
Ccg	Portugal		INRA[e]	C, I
Cch	Argentina	CArg	INRA	C, I
M.fallax				
Fa	The Netherlands	CHB	PAV	F, I
Fb	The Netherlands	C4571	PD	F, I
Fc	The Netherlands	C6501	PD	F, I
Fd	The Netherlands	C6466	PD	F, I
Fe	The Netherlands	W834.769	BLG[f]	F, I
Ff	Belgium		Moens[g]	F, I
Fg	The Netherlands		CPRO-DLO[h]	F, I
Fh	France	CHK	INRA	F, I
M. hapla				
Hc	The Netherlands	C3064	PD	I
He	The Netherlands	Dr.91	PAV	I
Hh	The Netherlands	Wa.90 (raceB)	PAV	I
Hi	The Netherlands	Sm.Fer92	PAV	I
Hj	The Netherlands	Sm.Fer92	PAV	I
Hk	The Netherlands	Sl.92	PAV	I
Ham	Queensland, Australia	Q114	ORSTOM[i]	I
Han	South Korea	C2346	PD	I
Has	Washington, USA	WAMH2	USDA	I
Hau	Utah, USA	UTMH1	USDA	I
Hbj	Canada	Canada	Uw[j]	I
Hbq	Hungary	C6611	PD	I
Hbr	The Netherlands	C4900-H-1A	PD	I
Hbs	The Netherlands	C7388	PD	I
Hbt	The Netherlands	W834.347	BLGG	I
Hby	The Netherlands	C3029	PD	I
Hcb	The Netherlands	HBW	CPRO-DLO	I
L33	The Netherlands		SCRI[k]	I

End of Table 1 next page.

Table 1. *(cont.)*

Code	Location	Isolate	Source	Pentaprimer bands[a]
Pak1	Pakistan		SCRI	I
Cyp1	Cyprus		SCRI	I
M. incognita				
Ia	The Netherlands		IPO-DLO[l]	I
Ib	The Netherlands	Inc568-93	PD	I
If	The Netherlands	KnolE	De Ruiter[m]	I
Ih	The Netherlands	KnolG	De Ruiter	I
Ij	The Netherlands	KnolH	De Ruiter	I
I3			SCRI	I
L11	Louisiana		SCRI	I
L15	Thailand		SCRI	I
L17	Burkina Faso		ORSTOM	I
L19	French West Indies		ORSTOM	I
M. arenaria				
L10	Ivory Coast		ORSTOM	I
L26	Portugal		SCRI	I
L32	French West Indies		ORSTOM	I
M. mayaguensis				
L30	Burkina Faso		ORSTOM	I
L3	Ivory Coast		ORSTOM	I
M. javanica				
Ja	The Netherlands	C3059	PD	I
L25	Portugal		SCRI	I
L23	Burkina Faso		ORSTOM	I

[a] Amplified fragment sizes resulting from PCR using Pentaprimers are indicated as follows: 900 bp band = C (*chitwoodi*-specific), 1100 bp band = F (*fallax*-specific), 750 bp band = I (ITS-specific).

[b] Plant Protection Service, Wageningen, The Netherlands.

[c] Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands.

[d] United States Department of Agriculture, Prosser, Washington, USA.

[e] Institut National de Recherche Agronomique, Le Rheu, France.

[f] Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.

[g] Centrum Landbouwkundige Onderzoek, Merelbeke, Belgium.

[h] Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands.

[i] Office de la Recherche Scientifique et Technique Outre-Mer, Montpellier, France.

[j] Department of Plant Pathology, University of Wisconsin, Madison, WI, USA.

[k] Scottish Crop Research Institute, Invergowrie, Dundee, United Kingdom.

[l] DLO-Research Institute for Plant Protection, Wageningen, The Netherlands.

[m] De Ruiter Seeds, Bleiswijk, The Netherlands.

this study are listed in Table 2. Conserved primers included those designed to amplify the ITS (18S and 28S) and a conserved sequence primer which binds within the IGS (1839). These three primers were mixed with primers specific for *M. chitwoodi* (C64) and *M. fallax* (F64) to produce the "pentaprimer"

Table 2. *Oligonucleotide primer sequences.*

Primer	Sequence	Origin
18S	5'-TCATTACGTCCCTGCCCTTTG-3'	Vrain *et al.*, 1992
28S	5'-TTTCACTCGCCGTTACTAAGG-3'	Vrain *et al.*, 1992
1839	5'-AGCCAAAACAGCGACCGTCTAC-3'	This study
C64	5'-GATCTATGGCAGATGGTATGGA-3'	This study
F64	5'-TGGGTAGTGGTCCCACTCTG-3'	This study

mix. All oligonucleotides were synthesized by phosphoramidite chemistry using the Oligo1000M (Beckman, Mississaugua, ON, Canada).

Results

Amplified IGS fragments were initially obtained from races 2 and 3 of *M. chitwoodi* (Cbd and Cbn, respectively). DNA sequencing via primer walking resulted in a series of primers to both strands of the IGS. These primers successfully amplified the *M. fallax* IGS and were subsequently used to sequence genomic DNA from two populations of *M. fallax* (Cg and Cae). The nucleotide sequences of *M. chitwoodi* and *M. fallax* were compared with other nematode or related rDNA sequences present within the Genbank database. These alignments revealed several variable regions to which primers were designed for species-specific amplification of the IGS (Table 2). Specificity of primer annealing was confirmed by pairwise PCR analyses using DNA from each species (data not shown).

A mixture of five PCR primers (pentaprimers) was prepared using equal molar concentrations of: *i*) a conserved forward primer (18S); *ii*) a conserved reverse primer (28S), for non-specific amplification of the ITS; *iii*) a conserved reverse primer (1839), binding within the IGS 3' region; *iv*) a *M. chitwoodi*-specific forward primer (C64) binding within the IGS 5' region, and *v*) a *M. fallax*-specific forward primer (F64) binding within the IGS 5' region (Fig. 1). Use of the pentaprimer mixture in PCR reactions employing DNA from individual lines of *M. chitwoodi* and *M. fallax* species demonstrated binding of either the C64 or F64 primers, resulting in specific multiplex amplification products of 900 and 1100 bp, respectively (Fig. 2, lanes 1, 3). A non-specific fragment of approximately 750 bp, corresponding to binding of the conserved ITS primers, served as a positive internal control.

Primer specificity was further tested by amplification of unknown *M. chitwoodi*, *M. fallax*, or *M. hapla* samples using the pentaprimers, and the reaction products compared with band sizes amplified from known controls. Correct identification of the unknown sample was deduced in all cases, including correct species identification from DNA extracted from each half of a single *M. chitwoodi* J2, and from as few as six eggs (Fig. 2). These latter experiments also demonstrated the sensitivity of our PCR reactions.

Pentaprimer specificity was further investigated by testing four additional *Meloidogyne* species. The universality of primer binding was established by amplifying DNA from 58 different *Meloidogyne* populations originating from twelve countries (Table 1). Specific banding patterns corresponding to the presence of

Fig. 1. *Genetic map of the* Meloidogyne chitwoodi *and* M. fallax *rDNA including the IGS region between rDNA cistrons. The annealing locations of oligonucleotide primers, and their 3' end orientations are indicated by arrows. The size of amplification products generated by PCR primer pairs (dashed lines) are also shown.*

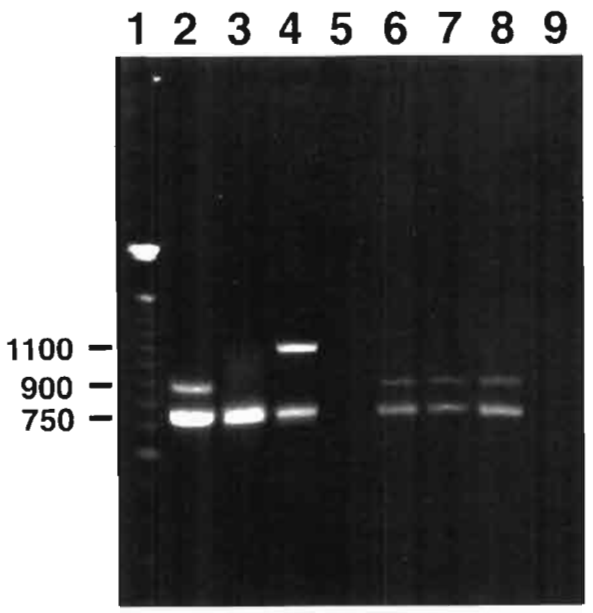

Fig. 2. *Agarose gel separation of species-specific amplicons. Multiplex PCR was conducted using the pentaprimer mix as described in Materials and Methods. Templates added to each reaction were either control DNAs (3 ng) from* Meloidogyne chitwoodi *(lane 2),* M. hapla *(lane3), and* M. fallax *(lane 4), no DNA template (negative control, lane 5), or DNA extracted from each half of a sliced single* M. chitwoodi *juvenile (lanes 6, 7), or six* M. chitwoodi *eggs (lane 8) or a single* M. chitwoodi egg *(lane 9). Size confirmations were made by comparison with the 100 bp ladder (Gibco, BRL) in lane 1.*

DNA from *M. chitwoodi* or *M. fallax* were accurately identified in all cases.

Discussion

Difficulties in molecular diagnostics stem primarily from the lack of reproducible results between, and within, various laboratories. Subjective assessments of band intensities from agarose-separated RAPD or RFLP reaction products contributes further to the limitations of these methods for routine nematode identification purposes. Consequently, many researchers are seeking species-specific probes which yield positive/negative diagnostic results when used in combination with a non-specific primer in the PCR. Diagnostic markers have already been designed to target the *msp* gene of *Pratylenchus* spp (Setterquist *et al.*, 1996). These primers hybridize to variable sequences within an intron located between conserved, flanking coding regions. Primers designed to RAPD markers specific to *M. hapla* or *M. chitwoodi* were also constructed, but only successfully amplified 80-90% of J2s tested (Williamson *et al.*, 1997). DNA sequencing has also assisted construction of primers which differentiate various *Meloidogyne* species using variations in the ITS. Species-specific primers for *M. hapla, M. javanica,* and *M. incognita* have been synthesized and demonstrated to yield amplicon polymorphisms in combination with a conserved reverse primer (Zijlstra, 1997; Powers *et al.*, 1998). However, the extensive homology observed between *M. chitwoodi* and *M. fallax* ITS sequences precluded species-specific DNA probes from being developed.

In contrast to the ITS, the high degree of variability identified within the IGS allowed us to expand the range of root-knot nematode species-specific probes to include *M. chitwoodi* and *M. fallax*. Primer sequences were chosen to have approximately 50% GC content, to lack obvious secondary structure, to lack homology with all other sequences in the Genbank database, and to provide a high annealing temperature (near that of the *Taq* extension cycle) to minimize non-specific amplification. Provided annealing temperatures are designed accordingly, additional primers can be included in future reactions since the multiplex PCR reaction conditions were the same as for the single primer pairs. Preliminary work with other species demonstrates the IGS may be generally applicable for species-specific probe development. We anticipate that such probes will facilitate rapid determination of nematode species identity.

Naturally, the universality of any species-specific primer needs to be proven. To this end, we tested the specificity of our pentaprimers upon nearly 60 different *Meloidogyne* spp. isolates from around the world. These tests were conducted in three laboratories (Canada, UK, and The Netherlands) so that the variations in extraction procedures, reaction techniques and hardware could be assessed simultaneously. The reliability of the method was clearly evidenced by accurate determination of all samples.

In addition to distinguishing *M. chitwoodi* and *M. fallax*, our pentaprimer mix provides several intrinsic features which advance upon previously published methods and which should prove useful for routine assessment of nematode identity: *i*) the species-specific markers included are complementary to the high copy-number rDNA IGS regions; the repetitive nature of these sequences increases the sensitivity of the PCR diagnosis, making possible the identification of a single juvenile or several eggs; *ii*) identification is based upon the presence or absence of species-specific bands so there is no cause for discrepancy arising from laboratory to laboratory variation in band intensity; *iii*) our pentaprimer mixture includes an internal control primer pair to verify the integrity of the detection system. The PCR can be extremely sensitive to poor DNA template quality or the presence of

inhibitors, a major problem when directly amplifying DNA of nematodes extracted from soil. Since the ITS primer pair generates an amplified fragment in all positive reactions, its visualization ensures that the other species-specific data (presence or absence) are valid; whereas if the ITS control band fails to appear then the absence of a particular nematode species DNA will not be assumed without reanalysis.

Having demonstrated the potential of the pentaprimer mixture, we look forward to incorporating additional species-specific probes into a multiplex PCR reaction mix to increase the range of species detected. Although no upper limit to the number of primers used in a multiplex reaction has been reported, limitations may arise due to competition between amplicons, limitations of substrates, and formation of chimaeras when too many primers are present. Reverse dot blot hybridizations (Iida *et al.*, 1993) may circumvent such problems. By immobilizing several species-specific probes in a filter array, amplified labelled-DNA from complex genomic samples such as those obtained from soil extracts or root washes can be hybridized to the filter to assess the species-biodiversity. This technique has been especially useful in detecting bacterial and viral pathogens on food destined for human consumption (Ehrmann *et al.*, 1994; Cromeans *et al.*, 1997), and has recently been modified for high through-put procedures using a microplate format (Soumet *et al.*, 1997). Application of this technique for detection or identification of plant-parasitic nematodes on imported agricultural commodities, or in field soils holds great potential for reducing the spread of quarantined organisms and the losses associated with nematode infestations.

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