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Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.)

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A molecular protocol is presented for distinguishing seven of the most common and economically important *Meloidogyne* spp. DNA was extracted from individual second-stage juvenile (J2) nematodes of *Meloidogyne* spp. and amplified by PCR (polymerase chain reaction). Fifteen PCRs including amplification of rDNA, specific SCAR (sequence characterized amplified region) and RAPD (random amplified polymorphic DNA) fragments were possible from the extracted DNA. This enabled a molecular diagnostic key for *M. incognita*, *M. javanica*, *M. arenaria*, *M. mayaguensis*, *M. hapla*, *M. chitwoodi* and *M. fallax* to be designed. The key unifies published methods into a single logical schematic using primer combinations that were previously validated and shown to work reliably and specifically. The protocol can be used with single juvenile or adult nematodes and the schematic can readily be expanded to accommodate more species. The use of RAPD amplification to assist with identification of samples which do not yield diagnostic amplification products after the first three steps of the molecular key is also described.

Keywords: diagnostics, identification, *Meloidogyne*, root-knot nematode

Introduction

The genus *Meloidogyne* is comprised of more than 80 species (Karssen, 2002) and on a worldwide basis includes the plant parasitic nematodes most economically damaging to crop production. *Meloidogyne incognita*, *M. javanica*, *M. arenaria*, *M. chitwoodi*, *M. fallax* and *M. hapla* account for more than 95% of the occurrences of this genus and are the most widely distributed species. The impact of these species is enhanced by their wide host ranges; the most common species are estimated to be able to infect more than 5500 plant species (Trudgill & Blok, 2001). *Meloidogyne incognita*, *M. javanica* and *M. arenaria* are distributed mainly in tropical regions, while *M. hapla*, *M. chitwoodi* and *M. fallax* occur in regions with cooler, temperate climates. These species are morphologically similar, making identification difficult for the non-specialist, but distinguishing them is important for utilizing appropriate crop rotations, managing resistance effectively and for plant quarantine requirements.

Recently, the use of isozyme analysis and molecular methods for the identification of root-knot nematode (RKN) species has increased as these methods are fast and can be more accurate than the use of morphological characters such as perineal pattern. However, isozyme analysis is only performed with single females, not single second-stage juveniles (J2s), males or eggs (Esbenshade & Triantaphyllou 1990). Since the female stage is often unavailable in soil samples, the isozyme method requires the time and space to establish and maintain populations in culture from single egg masses or single J2s in order to obtain this stage. The second-stage juvenile is more readily available from soil samples and can be obtained by hatching eggs or releasing juveniles from the eggs by physical pressure. Identification methods using the juvenile stage are therefore more useful for making crop management decisions (Powers & Harris, 1993) because they are quicker and more reliable. Identification of RKN species is important for appropriate deployment of cultivars because of differences in reproduction on different cultivars resulting from host status and resistance specificities, and for monitoring population movement, particularly of quarantine species. Consequently, developing methods to identify *Meloidogyne* species using individual single juveniles has been the objective of various studies. Molecular identification methods for juvenile nematodes may also have applications for studies concerning intraspecific variation between individuals. Several molecular methods which detect DNA polymorphisms between species have been used with DNA extracted from bulked nematode samples:

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for example, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) and specific sequence-characterized-amplified-region (SCAR) primers have been successfully used to identify the most common RKN species. However, only the latter two techniques can be performed with the small amounts of DNA present in a single juvenile.

The first method for RKN identification based on the polymerase chain reaction (PCR) was reported by Harris *et al.* (1990), who successfully amplified mitochondrial DNA from a single J2 crushed in a drop of sterile water and added into the PCR reaction. This method was developed further by Powers & Harris (1993), who designed primers to amplify the intervening region between the mitochondrial gene coding for cytochrome oxidase subunit II and the 16S rRNA primers and used them to identify five *Meloidogyne* species: *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. chitwoodi*. However, this method requires restriction enzyme digestion of the amplified product and also only one PCR reaction was obtained from each J2. Cenis (1993) amplified RAPD products from a single J2 in two separate reactions with successful amplification producing species-diagnostic bands with a few minor bands; however, half of the reactions failed to amplify any bands. Williamson *et al.* (1997) successfully identified *M. hapla* and *M. chitwoodi* using specific SCAR primers to amplify DNA extracted from single J2s using a method incorporating proteinase K digestion. They reported that RAPD patterns obtained from a single J2 prepared by this method were not sufficiently consistent for routine identification, presumably because of the variable amount of DNA extracted. Other developments to identify RKN species using a single J2 were reported by Zijlstra (2000), who used nested PCR to identify *M. hapla*, *M. chitwoodi* and *M. fallax* with SCAR primers, while Randig *et al.* (2001) achieved four PCR reactions from the extract of an individual female RKN. More recently Meng *et al.* (2004) designed specific SCAR primers to identify *M. incognita*, *M. javanica* and *M. arenaria*, which allowed identification of these species from single J2s, and three PCR reactions were obtained from extracts of single J2s of these species.

The aim of this study was to produce an efficient and systematic molecular key to aid in the identification of seven of the most common species of *Meloidogyne*: *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. chitwoodi*, *M. fallax* and *M. mayaguensis*, whereby a minimum number of PCR reactions are required to produce diagnostic amplification products for each of the seven species and sufficient DNA extract is produced to allow multiple PCR reactions to be performed, if necessary, to confirm identifications.

Materials and methods

Extraction of DNA from individual nematodes and specific PCR

DNA was extracted from individual J2s from the populations listed in Table 1 using worm lysis buffer [WLB; 50 mM KCl, 10 mM Tris pH 8·2, 2·5 mM $MgCl_2$ 60 $\mu g\ mL^{-1}$ proteinase K (Roche), 0·45% NP40 (Fisher Scientific), 0·45% Tween 20 (Sigma) and 0·01% gelatine] (Castagnone-Sereno *et al.*, 1995) as follows. Individual newly hatched J2s and males were picked up using a small needle and placed in 15 μL of WLB on a glass microscope slide and cut into two pieces using the same instrument under a stereomicroscope (Nikon). The cut nematode, in 10 μL WLB, was then transferred by pipette into a 0·5-mL centrifuge tube containing another 10 μL of WLB. The same procedure was applied to females, but they were picked up with tweezers and squashed with a pipette tip. The tubes were centrifuged at 13 500 r.p.m. for 2 min, then placed at –80°C for 15 min. Mineral oil (7 μL) was added to each tube and incubated at 60°C for 1 h, followed by 90°C for 10 min. The mineral oil was removed by pipette after the aqueous sample was frozen at –20°C.

PCR amplifications using rDNA or specific SCAR primers were carried out in 25 μL reactions and used 0·5 μL of DNA extract, 0·5 μL of each 10-μM primer (Table 2) when using PuReTaq Ready-to-Go™ PCR beads (Amersham) or *Taq* polymerase (Promega). The reactions using *Taq* polymerase also included 2·5 μL 10× buffer, 1·5 μL of 50 mM $MgCl_2$ and 2·5 μL 200-mM of each dNTP and 2 units of enzyme. The PCR amplification conditions used for each primer set are described in Table 3. All amplification tests included a no-template control and a bulk DNA extract when required. All primers were obtained from MWG Biotech.

Table 1 Single egg mass lines used in identification of *Meloidogyne* species

Population code	Origin	Species
J2	Libya[a]	*M. javanica*
L1	Crete[b]	*M. javanica*
L6/3	Crete[b]	*M. javanica*
L4/1	Crete[b]	*M. javanica*
A4	Libya[a]	*M. incognita*
G11	Libya[a]	*M. incognita*
L5	Crete[b]	*M. incognita*
C33	Crete[b]	*M. incognita*
L26	French West Indies[c]	*M. arenaria*
L28	French West Indies[c]	*M. arenaria*
L29	French West Indies[c]	*M. arenaria*
L33	The Netherlands[e]	*M. hapla*
V5	The Netherlands[e]	*M. chitwoodi*
P	France[d]	*M. fallax*
L13	Puerto Rico[c]	*M. mayaguensis*

Sources of populations: [a]M. Adam, Plant Protection Department, Faculty of Agriculture, Omar Al Mukhtar University P.O. Box: 919 El-Beida, Libya; [b]Dr E. Tzortzakakis, National Agricultural Research Foundation, PO Box 2228, 71003, Heraklion, Crete, Greece; [c]M. Fargette, UMR 1062 – CBGP (Centre de Biologie et de Gestion des Populations), Campus International de Baillarguet CS 30 016 34988 Montferrier sur Lez, France; [d]Dr D. Mugniery, INRA, BP 35327, 35653 Le Rheu Cedex, France; [e]Dr C. Zijlstra, L. Poleij and Dr H. van der Beek, Plant Research International, 6700 AA Wageningen, Netherlands.

Table 2 Primer codes used for identification of *Meloidogyne* species, their sequences and sources

Code	Primer sequence 5′–3′	Specificity and source
194	TTAACTTGCCAGATCGGACG	5S-18S ribosome region
195	TCTAATGAGCCGTACGC	Blok *et al.* (1997)
Far	TCGGCGATAGAGGTAAATGAC	*M. arenaria*-specific SCAR
Rar	TCGGCGATAGACACTACAAACT	Zijlstra *et al.* (2000)
Fjav	GGTGCGCGATTGAACTGAGC	*M. javanica*-specific SCAR
Rjav	CAGGCCCTTCAGTGGAACTATAC	Zijlstra *et al.* (2000)
Finc	CTCTGCCCAATGAGCTGTCC	*M. incognita* specific SCAR
Rinc	CTCTGCCCTCACATTAGG	Zijlstra *et al.* (2000)
MI-F	GTGAGGATTCAGCTCCCCAG	*M. incognita*-specific SCAR
MI-R	ACGAGGAACATACTTCTCCGTCC	Meng *et al.* (2004)
F	TAGGCAGTAGGTTGTCGGG	*M. incognita*-specific SCAR
R	CAGATATCTCTGCATTGGTGC	Dong *et al.* (2001)
Inc-K14-F	GGGATGTGTAAATGCTCCTG	*M. incognita*-specific SCAR
Inc-K14-R	CCCGCTACACCCTCAACTTC	Randig *et al.* (2002)
JMV1	GGATGGCGTGCTTTCAAC	*M. hapla*-, *M. chitwoodi*- and
JMV2	TTTCCCCTTATGATGTTTACCC	*M. fallax*-specific IGS-SCAR
JMV hapla	AAAAATCCCCTCGAAAAATCCACC	Wishart *et al.* (2002)
SC 10–30	CCGAAGCCCT	Zijlstra *et al.* (1997)
OPG –13	CTCTCCGCCA	Operon Technologies
OPG –19	GTCAGGGCAA	Operon Technologies

All primers synthesized by MWG Biotech.

Table 3 PCR amplification profiles used with different primers for identification of *Meloidogyne* species

		45 cycles			
		50°C (194/195)			
		61°C (Far/Rar)			
		64°C (Fjav/Rjav)			
		62°C (MI-F/MI-R)			
		50°C (F/R)			
		64°C (Inc-K14-F/Inc-K14-R)			
		54°C (Finc/Rinc)			
94°C	94°C	50°C (JMV primers)			
2 min	30 secs		72°C	72°C	
			90 secs (194/195)	7 min	
		30 secs	90 secs (JMV primers)		4°C
			1 min for remaining primers		∞

DNA extraction from bulk nematode samples

DNA was extracted from a 50-mg sample of eggs using the DNeasy Tissue kit (Qiagen).

RAPD-PCR amplification from individuals

Amplification reactions were prepared with 1 μL of 10-μM RAPD primer and 23 μL sterile distilled water, which were added to the PuReTaq Ready-to-Go™ PCR beads. Reactions with Promega *Taq* were as described for specific PCR, but used 3 U of *Taq* polymerase. Amplification reactions were exposed to UV light using either an XL-1500 UV cross-linker, (Spectronics Corporation) for 5 min or a light box (Vilber Lourmat) for 4 min to cross-link non-template DNA from the amplification reactions. Then 1 μL of DNA extracted from individual nematodes was added to each reaction and the tubes centrifuged briefly to ensure that the reaction contents were mixed. Amplification was carried out using cycling conditions of 94°C for 2 min, followed by 45 cycles of 94°C for 1 min, 38°C for 1 min and 72°C for 2 min, and one final cycle of 72°C for 10 min. Ramp speeds of 50% from 94°C to 38°C and 40% from 38°C to 72°C were used with an Applied Biosystems thermocycler 9700. Amplification products were separated by electrophoresis in a 2% Tris borate (TBE) buffer agarose gel at 50 V, followed by UV illumination after staining with ethidium bromide (Sambrook *et al.*, 1989).

Results

Extraction method and PCR reactions

The extraction method used, which took 2 h to complete, yielded DNA from individuals (J2s and females) that was sufficient for more than 15 PCR reactions. Moreover, PCR amplification products were obtained from 95% of the extracts. This facilitated the design of a molecular key for the identification of common species of RKN (*M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. chitwoodi*, *M. fallax* and *M. mayaguensis*) (Table 1).

Evaluation of primers for use in the molecular diagnostic key

The non-transcribed spacer region between the 5S and 18S rDNA genes was routinely amplified from single juveniles of all species with the 194/195 primers (Blok *et al.*, 1997) (Fig. 1, panel 1). The specific SCAR primers Fjav/Rjav (*M. javanica*) and Far/Rar (*M. arenaria*) (Zijlstra *et al.*, 2000) gave very consistent results and the products were readily amplified from single individual juvenile nematodes (Fig. 1, panels 2·1, 2·2). Four pairs of *M. incognita*-specific SCAR primers were tested with extracts from single individuals. The Finc/Rinc primers (Zijlstra *et al.*, 2000) gave a product, but inconsistently, whereas the *M. incognita* F/R primers (Dong *et al.*, 2001) and Inc-14k F/R (Randig *et al.*, 2002) gave no amplification products from single individual *M. incognita* juveniles, although an appropriate product was obtained when bulk DNA was used (data not shown). Only the MI-F/MI-R *M. incognita*-specific SCAR primers (Meng *et al.*, 2004) amplified products consistently from DNA extracted from single individual juveniles of *M. incognita* and produced a 999-bp fragment (Fig. 1, panel 2·3). All the PCR results shown in Fig. 1 were obtained using the PuReTaq Ready-to-Go™ PCR beads. Equivalent results were obtained with Promega *Taq*, but are not shown. Examples of the use of JMV and Fjav/Rjav primers with template from single females can be found in Wishart *et al.* (2002) and Tzortzakakis *et al.* (2005), respectively.

RKN molecular diagnostic key

A molecular key for the identification of seven *Meloidogyne* species is shown in Fig. 1. At the first stage, the seven species are divided into five groups according to the size of the PCR product obtained following amplification of the intergenic spacer region between the 5S and 18S ribosomal genes as follows: (a) *M. incognita*, *M. javanica*, *M. arenaria* (720 bp), (b) *M. mayaguensis* (780 bp), (c) *M. hapla* (700 bp), (d) *M. fallax* (1600 bp) and *M. chitwoodi* (1700 bp) and (e) others. To distinguish the species in group a, the second step of the key uses *M. incognita*-, *M. javanica*- and *M. arenaria*-specific SCAR primers. Only *M. incognita* should produce a 999-bp product with the MI-F/R primers, *M. javanica* amplifies a 670-bp fragment with the Fjav/Rjav primers, whereas *M. arenaria* produces a 420-bp fragment with the Far/Rar primers. Whilst the first stage of the key enables differentiation of *M. chitwoodi* and *M. fallax* by means of a small product size difference, it is possible to confirm the identification of *M. chitwoodi*, *M. fallax* and *M. hapla* by use of the third step in the key. This involves a multiplex PCR assay including three JMV primers (Table 2) (Wishart *et al.*, 2002) and results in amplification products of 540, 670 and 440 bp from these three species, respectively (Fig. 1, panel 3).

Testing the RKN molecular diagnostic key

DNA obtained from individual single-stage J2s of 15 single egg mass lines belonging to seven species (*M. javanica* lines J2, L1, L6/3 and L4/1, *M. incognita* lines G11, C33, A4 and L5, *M. arenaria* lines L26, L28 and L29 and one line of each of *M. hapla* L33, *M. chitwoodi* V5, *M. fallax* P and *M. mayaguensis* L13) were used to demonstrate the performance of the molecular diagnostic key. PCR amplification products from single J2s of these lines using 194/195 primers produced amplification products of 720 bp from the tropical species populations *M. javanica* (J2, L1, L6/3, L4/1), *M. incognita* (G11, C33, A4, L5) and *M. arenaria* (L26, L28, L28), 700 bp from *M. hapla* (L33), 780 bp from *M. mayaguensis* L13 and 1600, and 1700 bp from *M. chitwoodi* and *M. fallax*, respectively (Fig. 1, panel 1). The specificity of these primers with 22 other isolates of *M. javanica*, *M. arenaria*, *M. incognita* and *M. mayaguensis* was reported in Blok *et al.* (1997).

For the tropical species of RKN, PCR with *M. javanica*-specific SCAR primers (Fjav/Rjav) resulted in a 720-bp SCAR fragment only from individuals from *M. javanica* populations (J2, L1, L6/3 and L4/1) (Fig. 1, panel 2·1). *Meloidogyne incognita* populations (G11, C33, A4 and L5) produced 999-bp products when MI-F/R *M. incognita*-specific SCAR primers were used (Fig. 1, panel 2·2) and *M. arenaria* populations (L26, L28 and L29) produced a 420-bp fragment with *M. arenaria* SCAR primers Far/Rar (Fig. 1, panel 2·3). The specificity of the Fjav/Rjav and Far/Rar primers was previously been reported for 33 isolates of seven species of RKNs by Zijlstra *et al.* (2000) and 42 isolates of five species of RKN by Meng *et al.* (2004).

The identification of the three species in group b, which included *M. chitwoodi*, *M. fallax* and *M. hapla*, was confirmed using the JMV multiplex PCR assay. Amplification products of 540, 670 and 440 bp were produced from these three species, respectively (Fig. 1, panel 3). Forty-two isolates from six species of RKN were previously tested for species specificity of the JMV primers by Wishart *et al.* (2002).

RAPD results

Using the DNA extraction technique described above, successful RAPD amplifications were obtained from individuals of *Meloidogyne* species using 1 μL (1/20 of the extract) of the individual nematode lysate with 1 μL of 10-μM RAPD primer and PureTaq Ready-To-Go PCR beads or with Promega *Taq* polymerase. Pretreatment of PCR

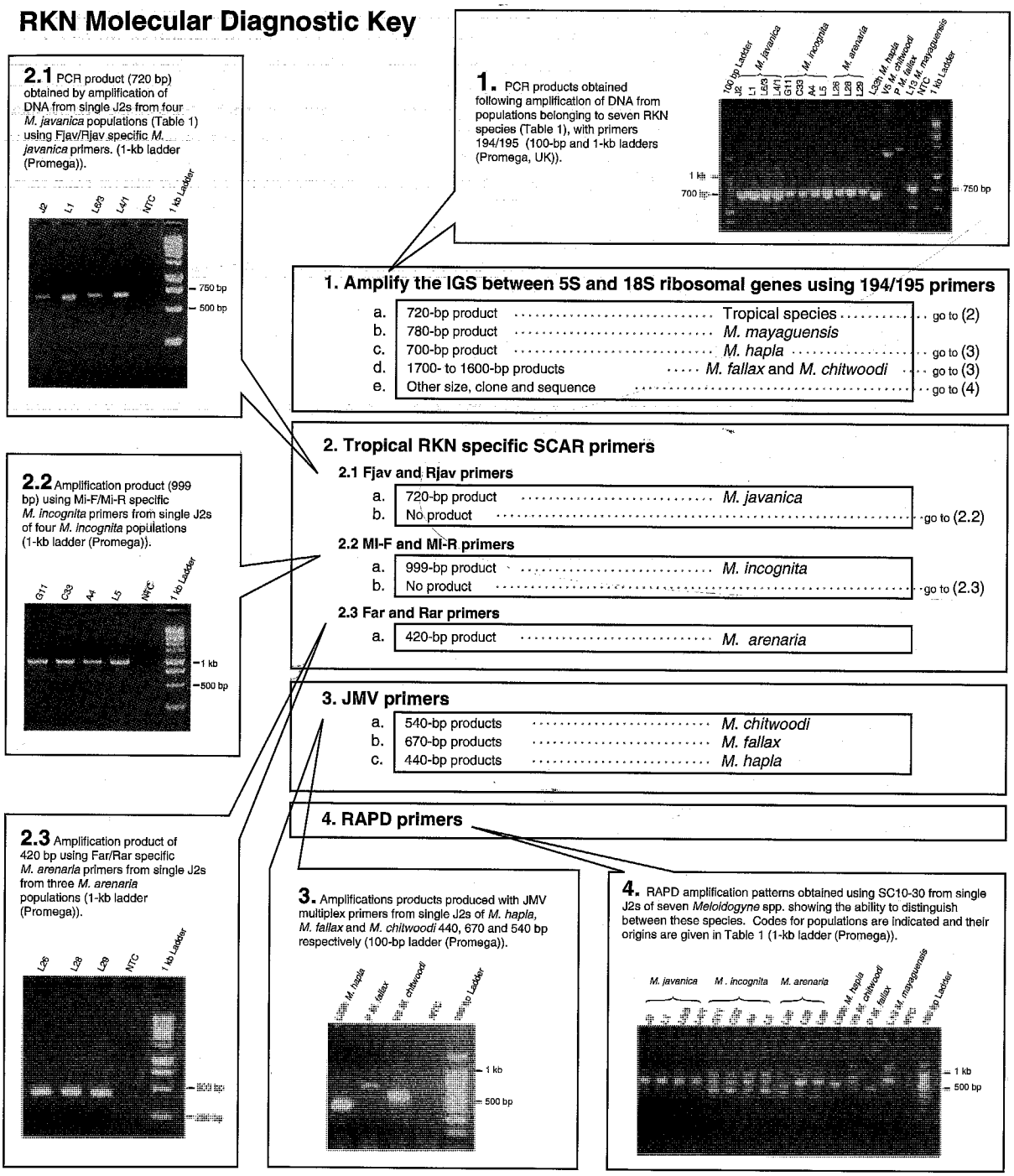

Figure 1 RKN Molecular Diagnostic Key.

reaction mixes with UV did not affect the efficiency of PCR amplification (results not shown). Amplification products were obtained with 10 RAPD primers from the DNA extracts of single J2 nematodes and bulk DNA of *M. javanica*, and were compared. Three primers, SC10-30, OPG-13 and OPG19, gave the same amplification patterns from single J2s and bulk DNA of *M. javanica* (results not shown). An example of the amplification products obtained with the SC10-30 RAPD primer from individuals (J2 males and females) and bulk DNA *M. javanica* is shown in Fig. 2. The amplification products produced from single juveniles from seven species with OPG-13 show the ability of this primer to distinguish *M. arenaria*, *M. javanica* and *M. incognita* from the other

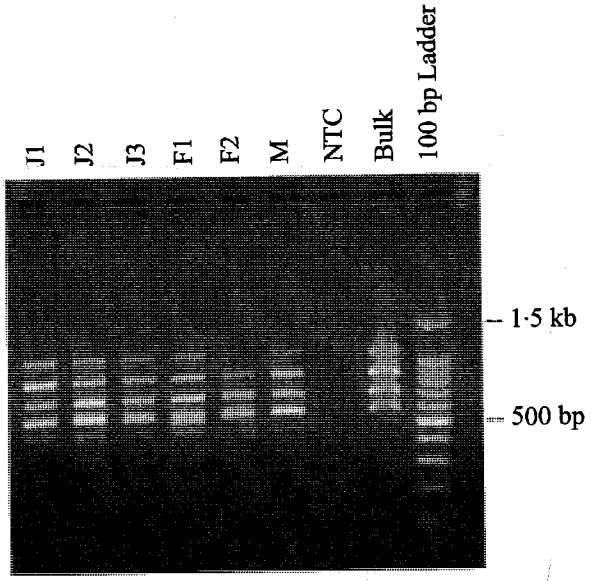

Figure 2 RAPD amplification patterns obtained using primer SC10-30 from different life stages of *Meloidogyne javanica* line J2 from three juveniles (J1, J2, J3), two females (F1, F2) and a male (M) compared with products obtained from a bulk extraction of DNA [100-bp ladder (Promega)].

species by the fact that these three share similar patterns (Fig. 3). The SC10-30 primer showed consistent patterns within the *M. javanica* and *M. incognita* isolates, but revealed variation between the three isolates of *M. arenaria* tested (Fig. 1, panel 4).

Discussion

The use of individual single-stage nematodes (J2s males or females) of *Meloidogyne* spp. for the identification of RKN species was demonstrated in this study. The identification of RKN species using J2s is of great practical importance because this stage is the most commonly found in field samples and can be readily obtained from eggs. A lysis method was used, following cutting juveniles or squashing of females, which provided sufficient extract for more than 15 PCR reactions. The method described is an improvement on the original method described by Castagnone-Sereno *et al.* (1995) where the juvenile nematode was not cut and the entire extract (2·5 μL) was used in the PCR reaction. Also, with the improved method, more than 95% of the extracts tested from over 100 juveniles gave amplification products with both the Ready-to-Go beads and Promega *Taq* polymerase. Ready-to-Go beads are stable at room temperature and the latter enzyme is a widely available general-use *Taq* polymerase. Another advantage of this method is that it saves time and space, as field samples can be identified immediately without the need for maintaining populations in the glasshouse and waiting for them to reproduce. Moreover, only 2 h is required for DNA extraction.

To date, other methods for identification of *Meloidogyne* spp. from individual nematodes have reported only 1–3 PCR reactions from DNA obtained from single J2s (Powers & Harris, 1993; Williamson *et al.*, 1997; Meng *et al.*, 2004). Similarly, four RAPD-PCR reactions were obtained from single females by Randig *et al.* (2001). Zijlstra (2000) used a two-step nested PCR to identify *M. hapla*, *M. chitwoodi* and *M. fallax* from single J2s. The method described here enables species identification from single J2s, males or females. Because of the large number of PCR reactions which can obtained, this method can be used with several primer sets to confirm identification and to study variation between individuals. Moreover, this DNA extraction method and PCR performance should be applicable to other plant parasitic nematodes (similar results have been obtained with *Globodera pallida*) because of the success with the different stages (juveniles and females) of *Meloidogyne* spp. which differ in size and protein content.

The 194/195 ribosomal primers, Fjav/Rjav *M. javanica*-, Far/Rar *M. arenaria*-, MI-F/R *M. incognita*-specific SCAR primers and JMV ribosomal primers successfully amplified fragments of the expected size from DNA obtained from single individual stages of RKN from 2·5% of the

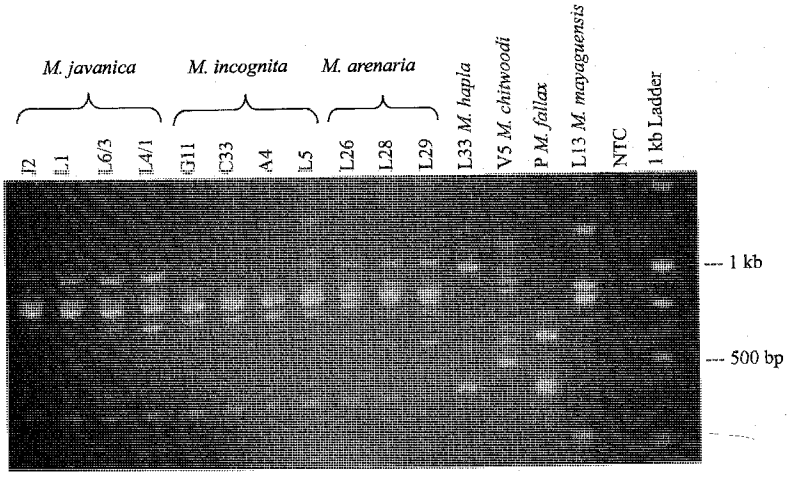

Figure 3 RAPD amplification patterns obtained with primer OPG-13 from single J2s of seven *Meloidogyne* spp. [1-kb ladder (Promega)].

DNA extract. With the 194/195 primers the amplification products from the tropical species (*M. incognita*, *M. javanica* and *M. arenaria*) and *M. hapla* are of very similar sizes (720 and 700 bp, respectively) and inclusion of positive controls could be helpful to resolve the different products on an agarose gel. Three pairs of *M. incognita*-specific SCAR primers, Finc/Rinc (Zijlstra *et al.*, 2000), F/R (Dong *et al.*, 2001) and Inc-14k F/R (Randig *et al.*, 2002) failed to routinely amplify their expected products from a single *M. incognita* J2. An explanation could be that there were low copy numbers of the corresponding target regions for these primer sets.

RAPDs are an established technique which has been used to study intraspecific variation amongst RKN species and to develop species-specific diagnostic SCAR primers such as the Fjav/Rjav, Far/Rar and MI-F/R primers used in this study. SCAR primers are based on the isolation of a particular amplification product from a representative sample of isolates from a species. Application of the RAPD technique to individual nematodes requires that reproducible amplification patterns are obtained from different individuals that do not differ from a bulk extraction of DNA or from different life stages, as shown with the SC10-30 primer in Fig. 2. Pretreatment of the PCR reaction mixture with UV light before addition of template DNA was required to eliminate non-specific amplification products, but this did not affect the subsequent PCR amplification. Primers differ in their reliability and the amount of intraspecific variation they reveal in their amplification patterns with particular templates. In this study consistent RAPD patterns were obtained with three of 10 primers evaluated with DNA extracts from single juveniles, with no background amplification in the no-template control. This result differs from what has been reported in other studies (Cenis, 1993; Williamson *et al.*, 1997). This could be a result of the different extraction method, PCR enzyme and primers used in the present study. Variation in reproducibility with different RAPD primers was also reported by Randig *et al.* (2001), who found that only four of 20 primers tested gave reproducible RAPD amplifications with extracts from single females.

The first three stages in the molecular key facilitate identification of seven *Meloidogyne* species. Failure to identify an unknown sample at this stage could be caused by a change at a primer site preventing amplification of a diagnostic product, a change in the intervening region between the diagnostic primer sites, giving a product with an aberrant size, or the sample being from a different species to the seven in the molecular key. The RAPD technique can be used to test for amplification patterns typical to reference samples of a particular species or group of species supporting other identification information, including host range, geographic origin and morphological data. Alternatively, it may be possible to use mitochondrial DNA genetic markers or sequencing of part of the 18s ribosomal gene as reported by Powers *et al.* (2005) to assist with identification of samples that are not resolved with the molecular key.

The molecular key unifies a number of published protocols into a simple, logical one. The steps required for easy, reliable and quick identification of seven of the world's most important and damaging *Meloidogyne* spp., based on PCR with different combinations of primers, are summarized in the key. Only 2–4 PCR reactions are usually required to identify these seven species. This key can be used with bulk extractions of DNA as well as DNA obtained from single juvenile or adult individuals. Multiple PCR reactions can be performed with the extracts from single nematodes, allowing confirmation of identifications. Use of the 194/195, Fjav/Rjav and Finc/Rinc primers to identify single *M. incognita* and *M. javanica* females from RKN isolates from Crete was reported by Tzortzakakis *et al.* (2005) and Fjav/Rjav and Finc/Rinc were used with *M. incognita* and *M. javanica* isolates from Libya (Adam *et al.*, 2005). In the latter study the Finc/Rinc SCAR primers did not reliably amplify a product from a *M. incognita* isolate that was subsequently identified using RAPDs. The present study has improved the DNA extraction protocol for use with single juvenile nematodes and SCAR primer set MI-F/R has been identified as being able to give a reliable amplification product from single juveniles of *M. incognita*.

All of the specific primers used in the molecular key have previously been tested on a wide range of isolates as described in the original publications, although isolates that fail to amplify because of changes in the priming sites may occur. Testing of all of these primers on further isolates will indicate whether there are differences in the conservation of the priming sites for rDNA-based (194/195 and JMV) and SCAR primers which will influence their reliability. The use of the RAPD technique with samples that failed to produce diagnostic amplification products in the first three stages of the molecular key is also described. Alternatively, amplification of a diagnostic region of mt DNA followed by restriction enzyme digestion or amplification of the 18S region combined with sequence analysis as used by Powers *et al.* (2005) could be used with samples not identified by the molecular key. The key could also be readily expanded to include other RKN species in order to produce a logical and broad-based diagnostic protocol.

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