Plant Pathology. (2014) **63**, 476–483 Doi: 10.1111/ppa.12108

Genetic diversity of the root-knot nematode *Meloidogyne ethiopica* and development of a species-specific SCAR marker for its diagnosis

V. R. Correa[ab], V. S. Mattos[a], M. R. A. Almeida[a], M. F. A. Santos[a], M. S. Tigano[a], P. Castagnone-Sereno[c] and R. M. D. G. Carneiro[a]*

[a]*Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF 70770-900, Brazil;* [b]*Departamento de Fitopatologia, Universidade de Brasília, Brasília, DF 70910-900, Brazil; and* [c]*Institut Sophia Agrobiotech, INRA-UMR1355, UNSA, CNRS-UMR7254, 400 route des Chappes, BP167, Sophia Antipolis 06903, France*

Meloidogyne ethiopica is an important nematode pathogen causing serious economic damage to grapevine in Chile. In Brazil, *M. ethiopica* has been detected with low frequency in kiwifruit and other crops. The objectives of this study were to evaluate the intraspecific genetic variability of *M. ethiopica* isolates from Brazil and Chile using AFLP and RAPD markers and to develop a species-specific SCAR-PCR assay for its diagnosis. Fourteen isolates were obtained from different geographic regions or host plants. Three isolates of an undescribed *Meloidogyne* species and one isolate of *M. ethiopica* from Kenya were included in the analysis. The results showed a low level of diversity among the *M. ethiopica* isolates, regardless of their geographical distribution or host plant origin. The three isolates of *Meloidogyne* sp. showed a high homogeneity and clustered separately from *M. ethiopica* (100% bootstrap). RAPD screenings of *M. ethiopica* allowed the identification of a differential DNA fragment that was converted into a SCAR marker. Using genomic DNA from pooled nematodes as a template, PCR amplification with primers designed from this species-specific SCAR produced a fragment of 350 bp in all 14 isolates of *M. ethiopica* tested, in contrast with other species tested. This primer pair also allowed successful amplification of DNA from single nematodes, either juveniles or females and when used in multiplex PCR reactions containing mixtures of other root-knot nematode species, thus showing the sensitivity of the assay. Therefore, the method developed here has potential for application in routine diagnostic procedures.

Keywords: amplified fragment length polymorphisms, diagnostic, intraspecific diversity, multiplex SCAR-PCR, RAPD, root-knot nematodes

Introduction

Meloidogyne ethiopica is a root-knot nematode (RKN) found throughout the tropics (Hunt & Handoo, 2009). Recently, it has been detected in Europe (Širca *et al.*, 2004; Conceição *et al.*, 2012) and has been placed in the 2011 EPPO alert list. Additional information was recently added to the taxonomic status of this nematode based on its detection in Brazil and Chile, parasitizing kiwifruit (*Actinidia deliciosa*) and grapevine crops (*Vitis vinifera*), respectively (Carneiro *et al.*, 2003, 2004, 2007). The characteristics of those isolates were compared with the type isolate of *M. ethiopica* and an isolate originating from Kenya: all isolates showed the characteristics typical for the species (Carneiro *et al.*, 2004).

The identification of *M. ethiopica* requires a detailed study of a suite of distinctive morphological features of the female, male and second-stage juveniles (J2). However, the perineal patterns of the female are highly variable and are similar to *M. arenaria* or to *M. incognita*. Isozyme phenotyping is currently the most useful character for routine differentiation of *M. ethiopica* from other RKN species as the esterase E3 is species-specific (Carneiro *et al.*, 2004). However, the technique requires fully developed female nematodes in good condition, which are sometimes difficult to obtain from field samples.

In Brazil, *M. ethiopica* has been reported causing severe infection of root systems of kiwifruit plants, which show defective growth, are distorted by small and large multiple galls, and are devoid of fine roots (Carneiro *et al.*, 2003). It is hypothesized that this nematode was introduced into Brazil in 1989 on kiwifruit seedlings imported from Chile (Carneiro *et al.*, 2003). Furthermore, given the large number of host species, *M. ethiopica* is considered a difficult nematode to eradicate (Carneiro *et al.*, 2003, 2004). Therefore, it is important to protect uninfected sites by planting certified nematode-free stocks and avoiding other pathways by which this RKN species might be introduced.

The wide distribution of *M. ethiopica* throughout the various Chilean regions is probably because of the use of

*E-mail: regina.carneiro@embrapa.br

Published online 31 July 2013

Plant Pathology

contaminated grapevine seedlings. In Chile, the prohibition of producing grapevine seedlings in areas with RKN and on the movement of infested seedlings into new areas seems to be ineffective, as all nurseries surveyed were severely infested with *M. ethiopica* and commercialization of uncertified plant materials is allowed (Carneiro *et al.*, 2007).

PCR-based analysis of neutral molecular markers, e.g. amplified fragment length polymorphisms (AFLP) and random amplified polymorphic DNA (RAPD), has been very useful to detect genetic variability within RKN species and such methods are sensitive, rapid and relatively simple, showing several loci scattered throughout the genome without requiring prior knowledge of the genetic information of the target species (Welsh & McClelland, 1990; Williams *et al.*, 1990; Cenis, 1993; Castagnone-Sereno *et al.*, 1994; Vos *et al.*, 1995; Carneiro *et al.*, 1998, 2008a; Randig *et al.*, 2002; Fargette *et al.*, 2005; Santos *et al.*, 2012). In addition, differential DNA fragments resulting from these markers have been used to design longer, species-specific PCR primers. Such PCR-based detection tools used for RKN species are attractive because they are simple, fast and can be used routinely on a large number of samples, particularly for quarantine purposes, without requiring prior multiplication of the target nematode or living J2 or females (Zijlstra, 2000; Zijlstra *et al.*, 2000; Randig *et al.*, 2002; Powers, 2004; Blok, 2005; Blok & Powers, 2009; Tigano *et al.*, 2010).

In this study phylogenetic analyses based on AFLP and RAPD markers were used to assess the genetic diversity within *M. ethiopica* isolates obtained from different hosts and regions of Brazil, Chile and Kenya. In addition, a sequence characterized amplified region (SCAR) marker was developed that can be used individually or in multiplex PCR reactions containing mixtures of species for fast and routine diagnosis of this important nematode species from field samples.

Materials and methods

Nematode isolates

Fourteen populations of *M. ethiopica* originating from Brazil and Chile, one *M. ethiopica* population from Kenya and three other *Meloidogyne* isolates found on kiwifruit plants (Table 1) were identified and purified using isozyme analyses (esterase and malate dehydrogenase) according to Carneiro *et al.* (1996) and Carneiro & Almeida (2001). Ten other RKN species, each represented by one single isolate, were also included in the analysis (Table 1). Purified nematode isolates were multiplied on tomato plants (*Solanum lycopersicum* cv. Santa Clara) under greenhouse conditions.

Table 1 *Meloidogyne* spp. isolates used in this study

Species	Code	Geographic region[a]	Original host plant	Isozyme phenotypes (EST/MDH)[b]
M. ethiopica	meth1	Kenya	Tomato	E3/N1
	meth2	Brasília, DF, Brazil	Tomato	E3/N1
	meth3	Farroupilha, RS, Brazil	Kiwifruit	E3/N1
	meth4	Itapetininga, SP, Brazil	Soybean	E3/N1
	meth5	Braganey, PR, Brazil	Tobacco	E3/N1
	meth6	Requínoa, Cachapoal, Chile	Grapevine	E3/N1
	meth7	Casablanca, Santa Rita, Chile	Grapevine	E3/N1
	meth8	Codigua, Melipilla, Chile	Grapevine	E3/N1
	meth9	Rengo, Cachapoal, Chile	Kiwifruit	E3/N1
	meth10	Fundo El Quadro, Casablanca, Chile	Grapevine	E3/N1
	meth11	San Clemente, Talca, Chile	Grapevine	E3/N1
	meth12	Curicó, Chile	Grapevine	E3/N1
	meth13	Los Robles, Santa Emiliana, Chile	Grapevine	E3/N1
	meth14	Sagrada Família, Monte Frutal, Chile	Kiwifruit	E3/N1
	meth15	Casa Blanca, La Rotunda, Chile	Grapevine	E3/N1
Meloidogyne sp.	Sp1	Caxias do Sul, RS, Brazil	Lavender	L3/N1
	Sp2	Brasília, DF, Brazil	Cucumber	L3/N1
	Sp3	Iran	Rose	L3/N1
M. arenaria	are	El Salvador	Coffee	A2/N1
M. morocciensis	mor	Londrina, PR, Brazil	Soybean	A3/N1
M. javanica	jav	Londrina, PR, Brazil	Coffee	J3/N1
M. hispanica	hisp	Sevilla, Spain	Nectarine	H3/N1
M. hapla	hap	Farroupilha, RS, Brazil	Kiwifruit	H1/H1
M. incognita	inc	Londrina, PR, Brazil	Coffee	I2/N1
M. enterolobii	ent	Petrolina, PE, Brazil	Guava	M2/N1a
M. exigua	exi	Costa Rica	Coffee	E1/N1
M. paranaensis	par	Londrina, PR, Brazil	Coffee	P1/N1
M. inornata	ino	São Paulo, SP, Brazil	Yakon	I3/N1

[a]Brazilian states: DF, Distrito Federal; RS, Rio Grande do Sul; SP, São Paulo; PR, Paraná; PE, Pernambuco.
[b]Esterase and malate dehydrogenase phenotyping.

DNA extraction

Nematode eggs were extracted from infected roots of tomato plants according to McClure *et al.* (1973) and stored at −80°C. For each nematode isolate, total genomic DNA was extracted and purified from aliquots of 200–300 μL of eggs according to a protocol described by Randig *et al.* (2002). Genomic DNA was also extracted from a single J2 nematode and/or female following a method described by Castagnone-Sereno *et al.* (1995).

AFLP analysis

For each isolate, *c.* 1 μg of total genomic DNA was digested overnight at 37°C with *Eco*RI (15 U μL^{-1}; Invitrogen) and ligated to the specific adapters following the method of Suazo & Hall (1999). A total of 30 random 22-mer primers (Integrated DNA Technologies) were used, consisting of the *Eco*RI adapter core sequences 5′-GACTGCGTACCAATTCAGT-3′ plus the 3′ selective nucleotides (AGT, ACT, AGC, ATT, GGC, CAG, TGG, CCT, ACC, TCG, ATA, AGG, AAT, AAC, GCC, CGA, GGG, CTC, CAT, TTA, TTG, TAC, GAC, GTG, CCG, TCT, GAG, TGC, CGT and CAC). PCR reactions were performed in a 25 μL final volume containing 1 μL (50 ng μL^{-1}) digested DNA, 2·5 μL 10× PCR buffer without magnesium chloride (Invitrogen), 1 μL 50 mM $MgCl_2$, 0·5 μL 10 mM dNTPs, 1 μL 10 μM primer and 0·3 μL *Taq* DNA polymerase (5 U μL^{-1}; Invitrogen). DNA was amplified using the PTC-100 thermocycler (MJ Research) under the following cycling parameters: 1 min at 95°C; 37 cycles of 1 min at 94°C, 1 min at 56°C, 2·5 min at 72°C; and a final extension of 10 min at 72°C (Suazo & Hall, 1999). PCR products were separated by electrophoresis in a 1·5% (w/v) agarose–synergel (0·7% agarose, 0·4% synergel; Diversified Biotech), stained with ethidium bromide (0·3 μg mL^{-1}) and photographed under UV light. The analysis was repeated three times.

RAPD analysis

PCR reactions were performed in a 13 μL final volume containing 1·3 μL 10× PCR reaction buffer (Phoneutria Biotecnologia & Serviços), 0·4 μL 10 μM primer (Operon Technologies), 2 μL 1·25 mM dNTPs (Invitrogen), 0·2 μL 5 U μL^{-1} *Taq* DNA polymerase (Phoneutria Biotecnologia & Serviços) and 3 μL total genomic DNA (3 ng μL^{-1}) of the *M. ethiopica* isolates, *Meloidogyne* sp. and *M. enterolobii*, a species distantly related to *M. ethiopica* (Table 1). The following 33 random 10-mer oligonucleotide primers (Operon Technologies) were used for assessing the genetic diversity of *M. ethiopica*: OPA-02, OPA-03, OPA-04, OPA-05, OPA-07, OPA-13, OPA-15, OPA-18, OPB-13, OPC-09, OPC-11, OPC-16, OPC-18, OPD-08, OPD-18, OPD-20, OPE-02, OPE-12, OPE-16, OPE-18, OPG-02, OPG-03, OPG-06, OPJ-10, OPJ-19, OPJ-20, OPK-01, OPK-05, OPN-08, OPP-01, OPP-02, OPR-07 and OPR-08. Another set of 57 RAPD primers was used for screening the *M. ethiopica* genome to find DNA markers present only in this species as compared to the other RKN species listed in Table 1. These primers comprised: OPA-01, OPA-04, OPA-07, OPA-12, OPA-13, OPA-14, OPA-18, OPAB-02, OPAB-03, OPAB-04, OPAB-06, OPAS-08, OPB-04, OPB-05, OPB-06, OPB-07, OPB-09, OPB-11, OPB-12, OPC-02, OPC-07, OPC-09, OPC-20, OPD-05, OPD-16, OPE-05, OPE-07, OPE-08, OPF-06, OPG-02, OPG-03, OPG-05, OPG-06, OPG-13, OPH-01, OPJ-10, OPJ-20, OPK-01, OPK-07, OPK-09, OPK14, OPK-16, OPK-19, OPK-20, OPL-08, OPL-19, OPM-10, OPM-20, OPN-10, OPO-01, OPO-02, OPO-05, OPQ-12, OPR-03, OPR-04, OPR-07 and OPR-08. The amplification was performed on a PTC-100 thermocycler, using the following settings: 5 min at 94°C; 40 cycles of 30 s at 94°C, 45 s at 36°C, 2 min at 70°C; and a final extension of 10 min at 70°C (Randig *et al.*, 2002). PCR products were separated by electrophoresis in a 1·5% (w/v) agarose gel, stained with ethidium bromide (0·3 μg mL^{-1}) and visualized under UV light. All RAPD analyses were repeated three times.

Phylogenetic analyses

AFLP and RAPD markers were used to determine the intraspecific genetic diversity among 15 isolates of *M. ethiopica* and three isolates of *Meloidogyne* sp., using *M. enterolobii* as an out-group (Table 1). The experiments were run three times and only DNA fragments consistently present between repeats were recorded as present or absent directly from the gels. DNA fingerprints were converted into a 0–1 binary matrix and phylogenetic reconstruction was performed using the neighbour-joining (NJ) algorithm (Saitou & Nei, 1987) in PAUP* v. 4b10 (Swofford, 2002), considering the data as unordered with no weighting.

To test the node support of the generated trees, the analysis was performed on 1000 bootstrap replicates and only values above 50% were considered. As the AFLP and RAPD markers could be considered to be independent characters, the two data sets were combined into a global NJ analysis, using the total evidence approach proposed by Huelsenbeck *et al.* (1996). The same settings used for the individual analysis were used for this combined data set.

Development of species-specific SCAR-PCR

A DNA fragment present only in the *M. ethiopica* genome was extracted from the RAPD gel using the Wizard SV Gel/PCR Clean Up system (Promega) and cloned into the pGEM-T Easy vector (Promega), following the manufacturer's instructions. Sequencing of the insert was carried out on two independent clones by Macrogen. A pair of species-specific primers, with longer sequences, higher annealing temperature (Tm) and high G+C content compared to the RAPD primer, was designed based on the consensus sequences using PRIMER3 v. 4.0 (Rozen & Skaletsky, 2000) and synthesized by Invitrogen. Specific SCAR primers were designed spanning the RAPD primer sequences. Further analysis using a primer pair designed from OPE-07, which differentially amplified a DNA fragment in *M. ethiopica*, was performed to test its specificity to this species. The sequences of this fragment were BLAST searched against the NCBI databases (http://blast.ncbi.nlm.nih.gov). SCAR-PCR was performed in a 25 μL final volume, containing 1× reaction buffer (Phoneutria Biotecnologia & Serviços), 200 μM of each dNTP (dATP, dTTP, dGTP and dCTP; Invitrogen), 10 μM each primer (meth-F 5′-ATGCAGCCGCAGGGAACGTAGTTG-3′ and meth-R 5′-TGTTGTTTCATGTGCTTCGGCATC-3′), 1 U *Taq* DNA polymerase (Phoneutria Biotecnologia & Serviços) and 6 ng total genomic DNA from *M. ethiopica* and other RKN species tested (Table 1). Amplifications were performed using a PTC-100 thermocycler with the following cycling conditions: 5 min at 94°C; followed by 33 cycles of 30 s at 94°C, 45 s at 66°C, 1 min at 70°C; and a final extension of 8 min at 70°C. Amplification conditions of SCAR-PCR using a single J2 or female were the same as described above, except that after the denaturation step a total of 39 amplification cycles were used. PCR products were

resolved in a 1·2% agarose gel, stained with ethidium bromide (0·3 μg mL^{-1}) and photographed under UV light. PCR products were also resequenced to check whether the same original sequences were obtained.

Because some of the RKN species listed in Table 1 are found together in the field, combinations of multiplex PCR were carried out. Multiplex PCR reactions of *M. ethiopica* + *M. incognita* or *M. ethiopica* + *M. arenaria* were performed with 5 μM of each primer pair: (i) methF/R (*M. ethiopica*) plus inc14-F 5′-GGGATGTGTAAATGCTCCTG-3′ and inc14-R 5′-CCCGCTACACCCTCAACTTC-3′ (*M. incognita*) (Randig *et al.*, 2002); and (ii) methF/R plus ar-F 5′-TCGGCGATAGAGGTAAATGAC-3′ and ar-R 5′- TCGGCGATAGACACTACAACT-3′ (*M. arenaria*) (Zijlstra *et al.*, 2000). Similarly, multiplex PCR reactions of *M. ethiopica* + *M. hapla* or *M. ethiopica* + *M. javanica* were performed with: (iii) 10 μM methF/R plus 5 μM h-F 5′-TGACGGCGGTGAGTGCGA-3′ and h-R 5′-TGACGGCGGTACCTCATAG-3′ (*M. hapla*) (Zijlstra *et al.*, 2000); and (iv) methF/R plus 5 μM jav-F 5′-GGTGCGCGATTGAACTGAGC-3′ and jav-R 5′-CAGGCCCTTCAGTGGAACTATAC-3′ (*M. javanica*) (Zijlstra *et al.*, 2000). All multiplex PCR reactions were performed using 6 ng pooled nematode DNA from eggs or DNA extract from a single individual. PCR conditions were the same as described previously for individual SCAR analysis. All experiments were run three times.

Results

Isozyme phenotyping

Results for the isozyme analysis are shown in Table 1. All isolates of *M. ethiopica* showed the E3 esterase phenotype (EST) typical for this species (Carneiro *et al.*, 2003, 2004). For the three undescribed *Meloidogyne* isolates an L3 EST phenotype was obtained. Additional information, such as phylogenetic analysis and morphology, indicated that these latter isolates very probably represent an undescribed species of RKN (authors' unpublished results). All other species showed typical EST phenotypes as previously described: *M. arenaria* (A2), *M. morocciensis* (A3), *M. javanica* (J3), *M. hispanica* (H3), *M. hapla* (H1), *M. incognita* (I2), *M. enterolobii* (M2), *M. exigua* (E1), *M. paranaensis* (P1) and *M. inornata* (I3). All nematode species listed in Table 1 showed the N1 malate dehydrogenase phenotype, except for *M. hapla* which showed the H1 phenotype.

Genetic diversity within *M. ethiopica* isolates

The genetic variability within 15 *M. ethiopica* isolates was analysed using a total of 30 AFLP and 33 RAPD random primers (Fig. 1). The sizes of amplified fragments ranged from 150 to 2500 base pairs (Fig. 1) and the number of reproducible amplified fragments observed with each marker ranged from 207 to 384 (Table 2). Overall, there was *c.* 22·5% polymorphism among *M. ethiopica* isolates and *c.* 28% polymorphism between *M. ethiopica* and *Meloidogyne* sp. (Table 2). Reproducible amplified fragments resulting from these markers were recorded to build a 0–1 matrix from which phylogenetic analysis was performed. The NJ trees obtained for each of the two types of markers were similar. After combining data together into one single analysis, the same tree topology was observed, with all isolates of *M. ethiopica* belong to a single cluster (82% bootstrap value) and without any supported subspecific clustering of the isolates (Fig. 2). The similar topologies indicate that the tree provides an accurate estimate of the rela-

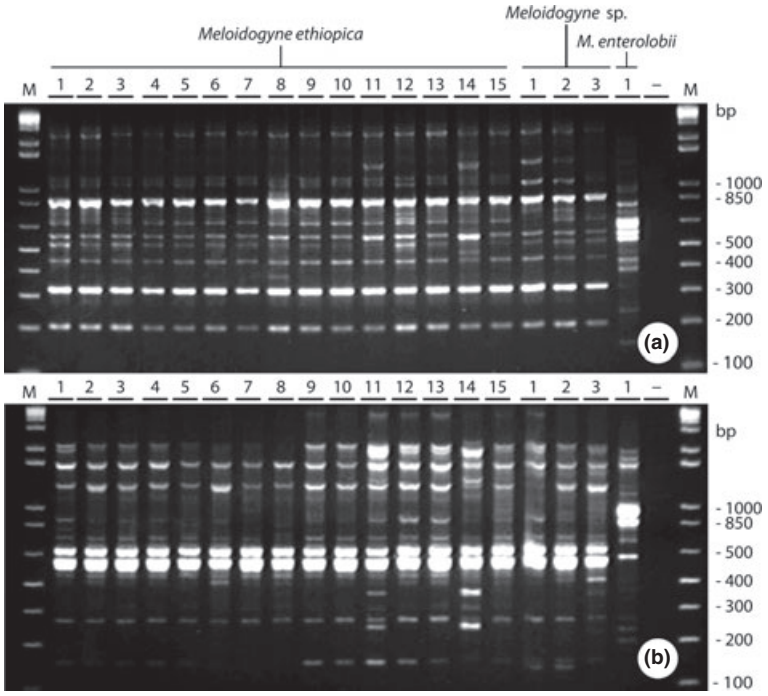

Figure 1 Genetic diversity of *Meloidogyne ethiopica* and *Meloidogyne* spp. as inferred from (a) RAPD and (b) AFLP markers. Numbers represent isolate codes listed in Table 1. RAPD and AFLP patterns shown were generated using primers OPR-07 (5′-ACTGGCCTGA-3′) and aflp-29 (5′- GACTGCGTACCAATTCAGTCGT-3′), respectively. M, 1 kb DNA Plus ladder.

Table 2 Polymorphisms observed within *Meloidogyne ethiopica* isolates and related species

	Marker characteristic						
	AFLP		RAPD		Combined AFLP + RAPD		
Species combination	Amp[a]	Poly[b]	Amp	Poly	Amp	Poly	%
M. ethiopica vs *Meloidogyne* sp.	294	80	210	62	504	142	28·2
M. ethiopica vs *M. enterolobii*	384	297	272	236	656	533	81·3
M. ethiopica (Brazil) vs (Chile) vs (Kenya)	272	57	207	51	479	108	22·5

[a]Amp, number of amplified bands.
[b]Poly, number of polymorphic bands.

tionships of the isolates studied. The three isolates of *Meloidogyne* sp., which showed the L3 esterase profile, clustered together.

Development of a PCR-based detection tool for *M. ethiopica*

To look for a species-specific DNA marker for *M. ethiopica* that can be used as a PCR diagnostic tool, *M. ethiopica* and the other RKN species were screened using a total of 57 10-mer RAPD primers. Amplification with primer OPE-07 resulted in a fragment of 350 bp that was present only in *M. ethiopica* isolates (Fig. 3). This DNA fragment was cloned, sequenced and converted into a SCAR marker. A BLAST search against the NCBI databases using this sequence resulted in no hits to any sequences deposited in the database (results not shown).

SCAR-PCR reactions with primer pair methF/R produced a single amplicon of 350 bp for *M. ethiopica*, in comparison to no amplicons produced from other *Meloidogyne* spp. tested (Fig. 4). DNA sequencing of this PCR product resulted in the same original sequence as the cloned fragment. The specificity and reliability of this primer pair was also tested against 14 isolates of *M. ethiopica*, and results revealed amplification of a DNA product of the expected size (Fig. 5). This primer pair was also successful in amplifying DNA from single J2 and individual females from *M. ethiopica* isolates (Fig. 6) or when used in multiplex PCR reactions containing mixtures of other major RKN species (Fig. 7).

Discussion

This study demonstrated that *M. ethiopica* isolates from Brazil and Chile had an overall low genetic diversity of *c.* 22·5%. In addition, a primer pair that can be used for specific diagnosis of *M. ethiopica* was developed. The specificity of the primer pair was validated by analysing other RKN species and 14 isolates of *M. ethiopica*. The sensitivity of the reaction allowed the identification of single individual nematodes of the target species when used in mixtures with other major RKN nematode species associated with grapevine and kiwifruit plants.

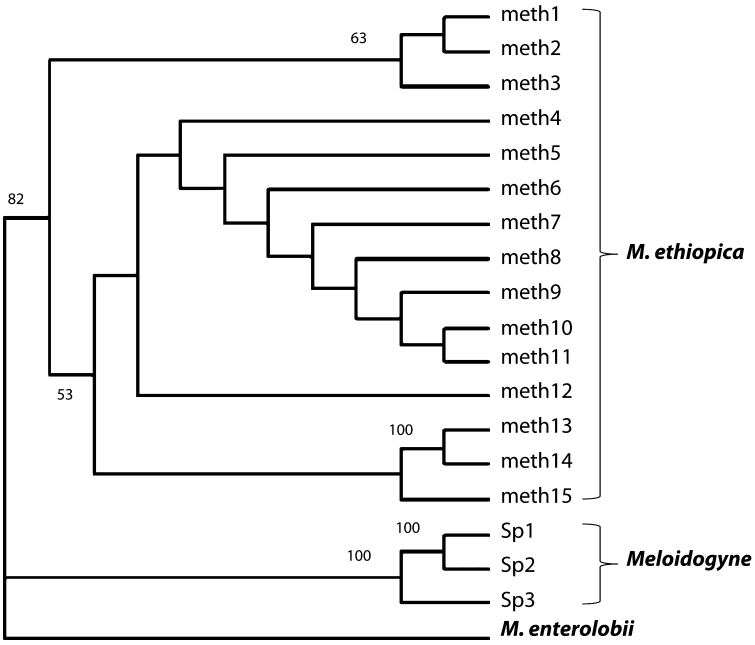

Figure 2 Neighbour-joining tree showing the phylogenetic relationships of *Meloidogyne ethiopica* and *Meloidogyne* sp. based on the combined AFLP and RAPD data set. Numbers to the left of the branches are bootstrap values for 1000 replicates. *Meloidogyne enterolobii* served as an out-group. Isolate codes are given in Table 1.

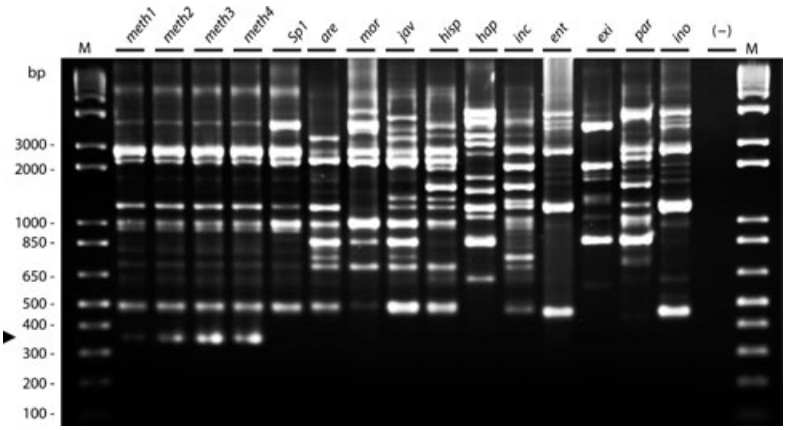

Figure 3 RAPD patterns for *Meloidogyne ethiopica* and other *Meloidogyne* spp. obtained with primer OPE-07. The *M. ethiopica*-specific 350 bp fragment is shown with an arrowhead. Isolate codes correspond to those listed in Table 1. M, 1 kb DNA Plus ladder.

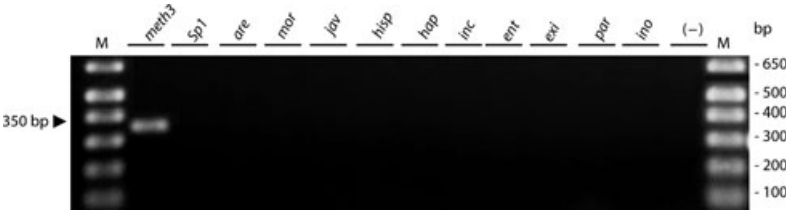

Figure 4 SCAR-PCR amplification pattern for *Meloidogyne ethiopica* and other root-knot nematode species generated with primer pair methF/R. Arrowhead indicates the specific fragment detected only in *M. ethiopica* (meth3). M, 1 kb DNA ladder. PCR amplifications were performed using purified DNA from nematode eggs. Isolate codes are listed in Table 1.

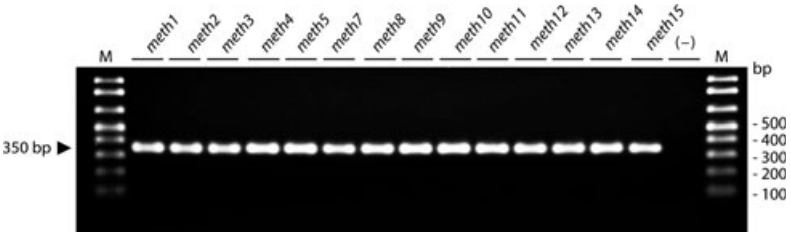

Figure 5 SCAR-PCR amplification pattern for *Meloidogyne ethiopica* isolates using the species-specific primer pair methF/R. Arrowhead indicates the 350 bp specific fragment identified in this species. M, 1 kb DNA ladder. PCR amplifications were done using purified DNA from nematode eggs.

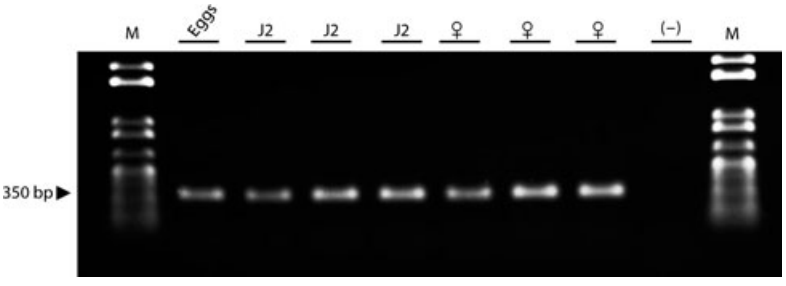

Figure 6 PCR amplification pattern for *Meloidogyne ethiopica* (meth3) single individuals using the SCAR primer pair methF/R. Only the 350 bp specific fragment is amplified. M, 1 kb DNA ladder. PCR reactions were done in triplicate using DNA extracted from single J2 or female individuals.

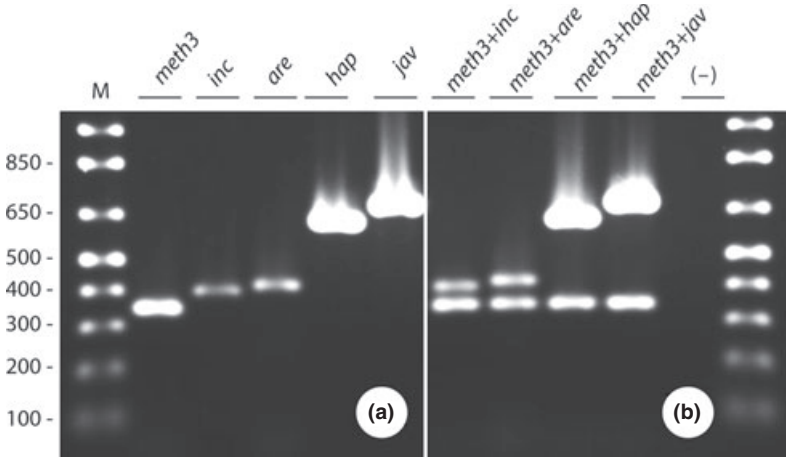

Figure 7 Single-species (a) and multiplex (b) SCAR-PCR amplification patterns of *Meloidogyne ethiopica* and other root-knot nematode species associated with grapevine and kiwifruit crops. Isolate codes are listed in Table 1. (–) DNA-negative control; M, 1 kb Plus DNA ladder. Single-species PCR was done using a single primer pair and purified DNA from eggs for each species listed. Multiplex PCR was done using a combination of two primer pairs and mixtures of purified DNA from eggs.

Thus, these species-specific PCR primers can be used in the diagnosis of this important plant-parasitic nematode.

Meloidogyne ethiopica is a damaging and extremely polyphagous species, having the potential to spread globally and to cause a significant impact on important crops (Carneiro *et al.*, 2003, 2007; Lima *et al.*, 2009; Strajnar *et al.*, 2011; Conceição *et al.*, 2012). Although this nematode is not yet considered to be a quarantine pest in Brazil, it has been placed on the 2011 European alert list as a potential treat to agriculture, following its recent detection on tomato plants in Slovenia (Širca *et al.*, 2004) and on kiwifruit and maize (*Zea mays*) in Greece (Conceição *et al.*, 2012). Furthermore, the finding that *M. ethiopica* is able to survive at subzero temperatures in open fields (Strajnar *et al.*, 2011) makes establishment of this pathogen a possibility and hence it could pose a significant threat not only to tropical and subtropical regions but also to places such as Europe. To the authors' knowledge, esterase phenotyping was the only routine reliable method to identify this nematode when the present study was initiated. Unfortunately, few laboratories are able to use this technique. Hence, the PCR-based detection tool described here will contribute to faster and simpler detection of this important nematode and help prevent its further spread worldwide.

The low genetic variability observed among *M. ethiopica* isolates might be related to the mode of reproduction of *M. ethiopica* via mitotic parthenogenesis, as this results in clonal progenies (Triantaphyllou, 1985). This finding is similar to other studies, e.g. Randig *et al.* (2002) reported a diversity of 19·5% and 30·1% for *M. javanica* and *M. incognita*, respectively, two species with the same mode of reproduction as *M. ethiopica*. Conversely, the low genetic diversity observed within *M. ethiopica* isolates might be because the isolates were mainly from Brazil and Chile, with *M. ethiopica* probably having been introduced into Brazil from Chile with imported grapevine and kiwifruit seedlings (Carneiro *et al.*, 2003). In this case, it would be necessary to increase the number of isolates from other countries in order to verify the data on the genetic variability of *M. ethiopica*. Nonetheless, the discovery of this low genetic diversity observed among *M. ethiopica* isolates could be important for future breeding programmes that ultimately lead to improved control management practices against this pathogen in Brazil and Chile.

The phylogenetic analysis indicated that there was no specific clustering related to original host plant or geographic origin of the isolates tested. Correlations between the geographic location for each isolate of *M. ethiopica* and the genetic distance between them were inconsistent. This result indicates a nonsignificant geographic–genetic correlation among this group of accessions.

Phylogenetic analysis further supported the grouping of the three isolates that had the same, L3, esterase phenotype. This cluster was successfully separated on the basis of AFLP and RAPD data, suggesting that these three isolates may belong to the same, as yet undescribed, species. The same unidentified RKN have been compared to several of the most common species of *Meloidogyne* in a phylogenetic study based on mitochondrial DNA sequences, and were shown to be closely related (*c.* 85% bootstrap value) to isolates that have since been confirmed as *M. inornata* (Carneiro *et al.*, 2008b) and *M. ethiopica* (Tigano *et al.*, 2005). However, the SCAR marker developed here for *M. ethiopica* did not result in any PCR amplification for this *Meloidogyne* sp. In addition, esterase profiling and morphology (authors' unpublished data) give further support to the classification of isolates from this cluster as belonging to an undescribed RKN species. Altogether, the results presented here suggest the need for further studies using a more comprehensive set of individuals of *M. ethiopica* to validate the evaluation of the intraspecific variability of this species.

Acknowledgements

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and EMBRAPA—Recursos Genéticos e Biotecnologia for their financial support. VRC thanks CAPES for his scholarship.

References

Blok VC, 2005. Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Canadian Journal of Plant Pathology* **27**, 176–85.

Blok VC, Powers O, 2009. Biochemical and molecular identification. In: Perry RN, Moens M, Starr JL, eds. *Root Knot Nematodes*. Wallingford, UK: CABI International, 98–118.

Carneiro RMDG, Almeida MRA, 2001. Técnica de eletroforese usada no estudo de enzimas dos nematóides de galhas para identificação de espécies. *Nematologia Brasileira* **25**, 35–44.

Carneiro RMOG, Almeida MRA, Carneiro RG, 1996. Enzyme phenotypes of Brazilian populations of *Meloidogyne* spp. *Fundamental and Applied Nematology* **19**, 555–60.

Carneiro RMDG, Castagnone-Sereno P, Dickson DW, 1998. Variability among four populations of *Meloidogyne javanica* from Brazil. *Fundamental and Applied Nematology* **21**, 319–26.

Carneiro RMDG, Gomes CB, Almeida MRA, Gomes ACMM, Martins I, 2003. Primeiro registro de *Meloidogyne ethiopica* Whitehead, 1968, em plantas de quivi no Brasil e reação em diferentes plantas cultivadas. *Nematologia Brasileira* **27**, 151–8.

Carneiro RMDG, Randig O, Almeida MRA, Gomes ACMM, 2004. Additional information on *Meloidogyne ethiopica* Whitehead, 1968 (Tylenchida: Meloidogynidae), a root-knot nematode parasitising kiwi fruit and grape-vine from Brazil and Chile. *Nematology* **6**, 109–23.

Carneiro RMDG, Almeida MRA, Cofcewicz ET, Magunacelaya JC, Aballay E, 2007. *Meloidogyne ethiopica*, a major root-knot nematode parasitizing *Vitis vinifera* and other crops in Chile. *Nematology* **9**, 635–41.

Carneiro RMDG, Santos MFA, Almeida MRA, Mota FC, Gomes ACMM, Tigano MS, 2008a. Diversity of *Meloidogyne arenaria* using morphological, cytological and molecular approaches. *Nematology* **10**, 819–34.

Carneiro RMDG, Mendes ML, Almeida MRA, Santos MFA, Gomes ACMM, Karssen G, 2008b. Additional information on *Meloidogyne inornata* Lordello, 1956 (Tylenchida: Meloidogynidae) and its characterisation as a valid species. *Nematology* **10**, 123–36.

Castagnone-Sereno P, Vanlerberghe-Masutti F, Leroy F, 1994. Genetic polymorphism between and within *Meloidogyne* species detected with RAPD markers. *Genome* **37**, 904–9.

Castagnone-Sereno P, Esparrago G, Abad P, Leroy F, Bongiovanni M, 1995. Satellite DNA as a target for PCR-specific detection of the plant-parasitic nematode *Meloidogyne hapla*. *Current Genetics* **28**, 566–70.

Cenis JL, 1993. Identification of four major *Meloidogyne* spp. by random amplified polymorphic DNA (RAPD-PCR). *Phytopathology* **83**, 76–80.

Conceição IL, Tzortzakakis EA, Gomes P, Abrantes I, da Cunha MJ, 2012. Detection of the root-knot nematode *Meloidogyne ethiopica* in Greece. *European Journal of Plant Pathology* **134**, 451–7.

Fargette M, Lollier V, Phillips M, Blok V, Frutos R, 2005. AFLP analysis of the genetic diversity of *Meloidogyne chitwoodi* and *M. fallax*, major agricultural pests. *Comptes Rendus Biologies* **328**, 455–62.

Huelsenbeck JP, Bull JJ, Cunningham CW, 1996. Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11**, 152–8.

Hunt DJ, Handoo ZA, 2009. Root knot nematodes: taxonomy, identification and principal species. In: Perry RN, Moens M, Starr JL, eds. *Root Knot Nematodes*. Wallingford, UK: CABI International, 55–97.

Lima EA, Mattos JK, Moita AW, Carneiro RG, Carneiro RMDG, 2009. Host status of different crops for *Meloidogyne ethiopica* control. *Tropical Plant Pathology* **34**, 152–7.

McClure MA, Kruk TH, Migashi I, 1973. A method for obtaining quantities of clean *Meloidogyne* eggs. *Journal of Nematology* **5**, 230.

Powers T, 2004. Nematode molecular diagnostics: from bands to barcodes. *Annual Review of Phytopathology* **42**, 367–83.

Randig O, Bongiovanni M, Carneiro RMDG, Castagnone-Sereno P, 2002. Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome*, **45**, 862–70.

Rozen S, Skaletsky HJ, 2000. PRIMER3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, NJ, USA: Humana Press, 365–86.

Saitou N, Nei M, 1987. The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–25.

Santos MFA, Furlanetto C, Almeida MRA *et al.*, 2012. Biometrical, biological, biochemical and molecular characteristics of *Meloidogyne incognita* isolates and related species. *European Journal of Plant Pathology* **134**, 671–84.

Širca S, Urek G, Karssen G, 2004. First report of the root-knot nematode *Meloidogyne ethiopica* on tomato in Slovenia. *Plant Disease* **88**, 680.

Strajnar P, Širca S, Knapič M, Urek G, 2011. Effect of Slovenian climatic conditions on the development and survival of the root-knot nematode *Meloidogyne ethiopica*. *European Journal of Plant Pathology* **129**, 81–8.

Suazo A, Hall HG, 1999. Modification of the AFLP protocol applied to honey bee (*Apis mellifera* L.) DNA. *BioTechniques* **26**, 704–9.

Swofford DL, 2002. PAUP*: *Phylogenetic Analysis Using Parsimony (*and Other Methods)* version 4b10. Sunderland, USA: Sinauer Associates.

Tigano MS, Carneiro RMDG, Jeyaprakash A, Dickson DW, Adams BJ, 2005. Phylogeny of Meloidogyne spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. *Nematology* **7**, 851–62.

Tigano M, De Siqueira K, Castagnone-Sereno P *et al.*, 2010. Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development for SCAR marker for this guava-damaging species. *Plant Pathology* **59**, 1054–61.

Triantaphyllou AC, 1985. Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes. In: Sasser JN, Carter CC, eds. *An Advanced Treatise on* Meloidogyne, *Vol. I. Biology and Control*. Raleigh, NC, USA: North Carolina State University Graphics, 113–26.

Vos P, Hogers R, Bleeker M *et al.*, 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407–14.

Welsh J, McClelland M, 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213–8.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV, 1990. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Research* **18**, 6531–5.

Zijlstra C, 2000. 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. *European Journal of Plant Pathology* **106**, 283–90.

Zijlstra C, Donkers-Venne DTHM, Fargette M, 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology* **2**, 847–53.