Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species

Onivaldo Randig, Michel Bongiovanni, Regina M.D.G. Carneiro, and Philippe Castagnone-Sereno

Abstract: RAPD markers were used to characterize the genetic diversity and relationships of root-knot nematodes (RKN) (*Meloidogyne* spp.) in Brazil. A high level of infraspecific polymorphism was detected in *Meloidogyne arenaria*, *Meloidogyne exigua*, and *Meloidogyne hapla* compared with the other species tested. Phylogenetic analyses showed that *M. hapla* and *M. exigua* are more closely related to one another than they are to the other species, and illustrated the early divergence of these meiotically reproducing species from the mitotic ones. To develop a PCR-based assay to specifically identify RKN associated with coffee, three RAPD markers were further transformed into sequence-characterized amplified region (SCAR) markers specific for *M. exigua*, *Meloigogyne incognita* and *Meloidogyne paranaensis*, respectively. After PCR using the SCAR primers, the initial polymorphism was retained as the presence or absence of amplification. Moreover, multiplex PCR using the three pairs of SCAR primers in a single reaction enabled the unambiguous identification of each species, even in mixtures. Therefore, it is concluded that the method developed here has potential for application in routine diagnostic procedures.

Key words: diagnostic, multiplex PCR, phylogeny, RAPD, root-knot nematodes.

Résumé : Des marqueurs RAPD ont été utilisés pour caractériser la diversité génétique et les relations entre les nématodes à galles (*Meloidogyne* spp.) du Brésil. Un important polymorphisme infraspécifique a été détecté chez *Meloidogyne arenaria*, *Meloidogyne exigua* et *Meloidogyne hapla*, comparativement aux autres espèces testées. Les analyses phylogénétiques ont montré que *M. hapla* et *M. exigua* sont plus proches entre elles qu'elles ne le sont des autres espèces, et ont illustré la divergence précoce de ces espèces à reproduction méiotique des espèces mitotiques. Afin de développer un essai pour identifier les espèces associées au café, trois marqueurs RAPD spécifiques de *M. exigua*, *Meloidogyne incognita* et *Meloidogyne paranaensis* ont été transformés en marqueurs SCAR. Après une réaction PCR avec les amorces SCAR, le polymorphisme initial s'est traduit par la présence ou l'absence d'amplification. De plus, l'utilisation simultanée des trois couples d'amorces SCAR dans une seule réaction de PCR a permis l'identification de chaque espèce, même en mélange. Par conséquent, il est conclu que la méthode développée dans cette étude est potentiellement utilisable dans des procédures de diagnostic de routine.

Mots clés : diagnostic, nématodes à galles, PCR multiplex, phylogénie, RAPD.

Introduction

Root-knot nematodes (RKN) of the genus *Meloidogyne* are major agricultural pests of a wide range of crops worldwide. So far, more than 80 species have been described (Karssen and Van Hoenselaar 1998), among which some are a serious constraint to agricultural production in tropical, subtropical, and temperate regions. The most widespread species, *Meloidogyne incognita*, is possibly the single most damaging crop pathogen in the world (Trudgill and Blok 2001). RKN are known to be highly variable from a genetic point of view. They exhibit extreme cytogenetic diversity (with frequent and variable levels of aneuploidy and polyploidy), and their mode of reproduction ranges from obligatory amphimixis to obligatory (mitotic) parthenogenesis (Triantaphyllou 1985). Taken as a whole, this genus also shows an outstanding diversity in the relationship it has evolved with its host plants: some species are extremely polyphagous, with up to 3000 host plant species (e.g., *Meloidogyne arenaria*, *Meloidogyne incognita* and *Meloidogyne javanica*; Trudgill and Blok 2001), whereas others have a host range restricted to a very few plant species like *Meloidogyne pini*, which is associated with *Pinus* spp. only (Jepson 1987). Plant resistance is currently the most efficient and environmentally safe method to control

Received 27 March 2002. Accepted 24 June 2002. Published on the NRC Research Press Web site at http://genome.nrc.ca on 21 August 2002.

Corresponding Editor: B. Golding.

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DOI: 10.1139/G02-054

these pests. However, because of both the variability in the nematode host range and the specificity of action of the resistance genes (Blok et al. 1997*a*), this strategy requires accurate and reliable preliminary detection and identification of the nematodes to optimize the use of selected resistant cultivars in adapted crop rotation systems.

In Brazil, RKN are among the most destructive plant-parasitic nematodes. They are found associated with many crops, including vegetables, soybean, rice, maize, rubber tree, ornamentals, and coffee. Indeed, these parasites are distributed widely in coffee plantations, where they cause great losses to farmers. *Meloidogyne incognita*, *Meloidogyne exigua*, and *Meloidogyne coffeicola* have been reported as the predominant species in all major coffee-producing states in the country (Campos et al. 1990). Recent surveys have shown a substantial increase in the distribution of *M. incognita* and a decrease in *M. coffeicola*, and it is speculated that *M. coffeicola* was eradicated in many places owing to the renewal of damaged coffee after the great frost of 1975 (Campos et al. 1990). More recently, an RKN parasitizing coffee in Paraná State was described as *Meloidogyne paranaenesis*, and this species was found to account for more than half of all RKN infestations in Paraná (Carneiro et al. 1996*a*). Recent surveys on coffee plantations have found *M. exigua* in 100% of infected samples from Minas Gerais State (V.P. Campos, personal communication), and a substantial increase of *M. paranaensis* and *M. incognita* in Paraná (Krzyzanowski et al. 2001) and São Paulo States (Lordello et al. 2001).

Most previous studies on the diversity of Brazilian *Meloidogyne* spp. have focused on morphology (e.g., the microscopic examination of female perineal patterns), and response of the populations to differential host tests. More recently, Carneiro et al. (1996*b*, 2000) have characterized RKN populations from different regions of Brazil using isoenzyme electrophoresis. However, enzymatic phenotypes provide insufficient information at the infraspecific level. Random amplified polymorphic DNA (RAPD), a single polymerase chain reaction (PCR) amplification of genomic DNA using a single oligonucleotide primer, can reveal considerable polymorphism, even between closely related organisms (Welsh and McClelland 1990; Williams et al. 1990). Because of that, it has been extensively used as a genetic marker for estimating genetic, taxonomic, and phylogenetic relationships of RKN (Cenis 1993; Baum et al. 1994; Castagnone-Sereno et al. 1994; Blok et al. 1997*b*). In this report, RAPD–PCR was applied to examine the genetic variation and relationships between RKN isolates belonging to the main species encountered in different regions of Brazil. Some of the amplified DNA fragments that were found to be specific for the major species associated with coffee (i.e., *M. exigua*, *M. incognita*, and *M. paranaensis*) were further cloned and transformed into SCAR (sequence-characterized amplified region) markers to provide reliable tools for the identification of these economically important pests.

Materials and methods

Nematodes

Twenty-five populations of *Meloidogyne* spp. originating from Brazil were sampled from the root systems of a variety of heavily infested cultivated host plants (Table 1). From each population, an isolate was raised starting from the progeny of a single female to eliminate the potential within-population heterogeneity. Owing to the parthenogenetic mode of reproduction of these nematodes, each isolate was considered to be a clonal line. Before multiplication, each isolate was specifically identified according to its isoesterase electrophoretic pattern (Dalmasso and Bergé 1978; Carneiro et al. 1996*b*).

DNA preparation

Total genomic DNA was extracted from 200 to 300 µL of nematode eggs of each available isolate that had been stored at –80°C before use. Eggs were frozen in liquid nitrogen and ground with a mortar and pestle. The DNA was purified from the resulting powder by a phenol–chloroform extraction (Sambrook et al. 1989). After ethanol precipitation, DNA was resuspended in TE buffer (10 M Tris (pH 8.0) – 1 mM EDTA (pH 8.0)). In some experiments (see below), genomic DNA was also prepared from individual RKN females as previously described (Randig et al. 2001).

RAPD analysis

Twenty-seven random 10-mer primers purchased from Eurogentec (Herstal, Belgium) were used in RAPD experiments (Table 2). RAPD–PCR was performed in a final volume of 25 µL containing 5 ng of total genomic DNA, 80 pM of primer, dATP, dCTP, dGTP, and dTTP each at 200 µM final concentration, 1× *Taq* incubation buffer, and 1.25 U *Taq* polymerase (Appligene, Illkirch, France). Each reaction mixture was overlaid with mineral oil. Amplification was performed on a PTC-100 MJ Research thermal cycler (MJ Research Inc., Waltham, Mass.). The cycling program was 1 min at 94°C; 40 cycles of 20 s at 94°C, 30 s at 36°C, and 2 min at 70°C; and a final incubation of 10 min at 70°C. Amplification products were separated by electrophoresis in 1.4% w/v agarose gels in TBE (89 mM Tris-HCl, 89 mM Borate, 2 mM EDTA) buffer at a constant current of 150 mA for approximately 3 h, and visualized with ethidium bromide (0.5 µg/mL) under UV light.

Phylogenetic analysis

Bands were scored as present or absent directly from the gels. Experiments were repeated at least once, and only DNA fragments consistently present or absent between repeats were recorded and considered as binary characters. DNA fingerprints from each isolate were converted to a 0–1 matrix, and two phylogenetic analyses were conducted using the computer program PAUP* 4.0 (Swofford 1998). First, a parsimony analysis was performed, according to the following options. Characters were run unordered with no weighting, and the heuristic search algorithm was used to find the most parsimonious tree. Characters that were phylogenetically uninformative were deleted, i.e., bands invariant between all isolates or either present or absent in one single isolate only (Li and Graur 1991). Second, the distance-based neighbor-joining algorithm (Saitou and Nei 1987) was used on the same data set, using the mean-character difference option of PAUP* to compute distances. For both analyses, 1000 bootstrap replicates were performed to test the support

Table 1. List of the *Meloidogyne* spp. isolates from Brazil used in this study.

Species	Isolate	Geographical origin[a]	Host plant
M. arenaria	are1	Pato Branco, PR	*Cucurbita* sp.
	are2	Gramado, RS	*Cucumis melo*
	are3	Pelotas, RS	*Lycopersicon esculentum*
	are4	Nova Soure, BA	*Malpighia punicifolia*
M. exigua	ex1	Lavras, MG	*Coffea arabica*
	ex2	Rondonopolis, MS	*Hevea brasiliensis*
	ex3	Lavras, MG	*Coffea arabica*
	ex4	unknown, SP	*Coffea arabica*
M. hapla	ha1	Caxias, RS	*Actinidia deliciosa*
	ha2	Botucatu, SP	*Fragaria* sp.
M. incognita	inc1	Londrina, PR	*Phaseolus vulgaris*
	inc2	Londrina, PR	*Coffea arabica*
	inc3	Londrina, PR	*Coffea arabica*
	inc4	Londrina, PR	*Citrullus vulgaris*
M. javanica	jav1	Pelotas, RS	*Solanum tuberosum*
	jav2	Ponta Porã, MS	*Glycine max*
M. paranaensis	par1	Londrina, PR	*Coffea arabica*
	par2	Pompeia, SP	*Coffea arabica*
	par3	Nova Esperança, PR	*Coffea arabica*
	par4	Ourizona, PR	*Coffea arabica*
	par5	Apucarana, PR	*Coffea arabica*
	par6	Londrina, PR	*Coffea arabica*
Meloidogyne spp.	sp1	Vacaria, RS	*Actinidia deliciosa*
	sp2	Caxias, RS	*Lavandula spica*
	sp3	Capão Bonito, SP	*Polymnia sonchifolia*

[a]State codes from Brazil as follows: BA, Bahia; MG, Minas Gerais; MS, Mato Grosso do Sul; PR, Parána; RS, Rio Grande do Sul; SP, São Paulo.

of nodes for the most parsimonious tree (Felsenstein 1985) and a consensus dendrogram was computed.

Cloning, sequencing, design of SCAR primers, and SCAR analysis

To design primers that could be used to amplify DNA fragments specific for each of the three species present on coffee roots (i.e., *M. exigua*, *M. incognita*, and *M. paranaensis*), RAPD bands of interest were selected, excised from the gel, and cloned into the pGEM-T vector (Promega, Charbonnières, France) according to the manufacturer's instructions. In each case, sequencing of the inserts was done on two independant clones by Genome Express (Grenoble, France). From each sequence obtained, a pair of putative specific primers was designed longer than the original RAPD primer and with a G+C content greater than 50 percent (Table 3).

PCRs using the SCAR primers and either nematode genomic DNA or single female DNA as template were performed as described for the RAPD analysis according to the following amplification conditions: 5 min at 94°C; 30 cycles of 30 s at 94°C, 45 s at 64°C, and 1 min at 70°C; and a final incubation of 8 min at 70°C.

Results

RAPD profiles and isolate differentiation

Owing to limitations in the biological material available, RAPD fingerprinting was performed on 18 of the 25 *Meloidogyne* spp. isolates (*M. exigua* isolates exi3 and exi4 and *M. paranaensis* isolates par2–par6 could not be used). On these isolates, the RAPD profiles analyzed showed abundant polymorphisms (Fig. 1). With the 27 random primers used, the number of reproducible amplified fragments varied from 2 to 21 per isolate, and their size ranged from ~200 to ~3000 bp. The global results of the RAPD analysis are provided in Table 2. Over the whole experiment, each primer produced from 25 to 59 polymorphic bands, among which 18–48 appeared phylogenetically informative. Globally, 1125 fragments were amplified and scored as RAPD markers. No single fragment was found to be amplified in the 18 isolates tested (i.e., monomorphic), and 829 were informative. Using RAPD patterns alone or in combination, all the root-knot nematode isolates studied could be unambiguously identified.

For the five species for which more than one isolate was available (i.e., *M. arenaria*, *M. exigua*, *M. hapla*, *M. incognita*, and *M. javanica*), 261–460 reproducible fragments were amplified (Table 4). For *M. arenaria*, *M. exigua*, and *M. hapla*, the rate of amplified bands that were polymorphic was very similar and ranged from 67.5 to 69.8%. Conversely, in the case of either *M. incognita* or *M. javanica*, fewer RAPD bands were scored as polymorphic (30.1 or 19.5%, respectively).

All the scorable amplified bands were recorded to build a 0–1 matrix, on which two phylogenetic analyses were performed. Parsimony and neighbor-joining algorithms were run on the 0–1 matrix, and a bootstrap analysis with 1 000 replicates was conducted in both cases, which resulted in two comparative majority-rule consensus dendrograms (Fig. 2).

Table 2. RAPD primers used and number of amplified fragments scored.

Primer	Sequence (5′→3′)	No. amplified fragments			
		Min./isolate	Max./isolate	Polymorphic	Informative
2	ATGGATCCGC	2	11	38	22
A04	AATCGGGCTG	3	11	27	23
C02	GTGAGGCGTC	3	12	29	25
C09	CTCACCGTCC	3	11	33	21
C16	CACACTCCAG	2	16	44	27
D05	TGAGCGGACA	6	17	38	33
D13	GGGGTGACGA	5	16	39	33
D15	CATCCGTGCT	5	17	41	29
E07	AGATGCAGCC	7	13	31	21
F06	GGGAATTCGG	3	15	42	33
J20	AAGCGGCCTC	7	16	54	46
K01	CATTCGAGCC	3	14	45	28
K04	CCGCCCAAAC	5	15	52	38
K06	CACCTTTCCC	5	11	25	22
K07	AGCGAGCAAG	8	17	56	40
K09	CCCTACCGAC	5	14	33	23
K14	CCCGCTACAC	8	17	40	32
K19	CACAGGCGGA	8	14	46	32
K20	GTGTCGCGAG	5	18	56	37
L08	AGCAGGTGGA	5	14	46	36
M10	TCTGGCGCAC	3	12	44	33
M20	AGGTCTTGGG	10	19	50	42
N07	CAGCCCAGAG	4	12	34	24
N10	ACAACTGGGG	2	12	41	27
P01	GTAGCACTCC	3	13	31	18
P02	TCGGCACGCA	6	16	51	36
P05	CCCCGGTAAC	3	21	59	48
Total		—	—	**1125**	**829**

Table 3. Characteristics of the SCAR markers developed for *Meloidogyne exigua*, *M. incognita*, and *M. paranaensis*.

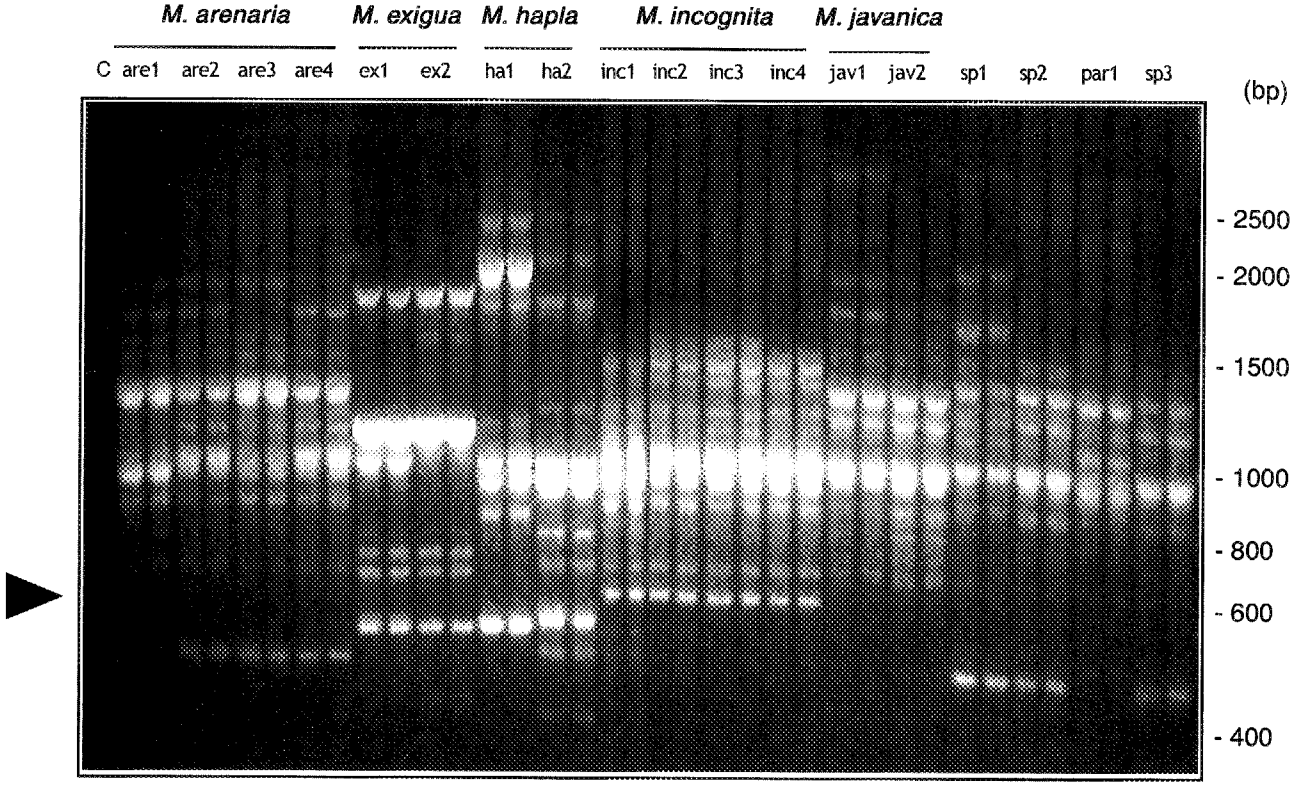
Species	SCAR primers	SCAR primer sequence (5′→3′)	Size of the SCAR (bp)
M. exigua	ex-D15-F	CATCCGTGCTGTAGCTGCGAG	562
	ex-D15-R	CTCCGTGGGAAGAAAGACTG	
M. incognita	inc-K14-F	GGGATGTGTAAATGCTCCTG	399
	inc-K14-R	CCCGCTACACCCTCAACTTC	
M. paranaensis	par-C09-F	GCCCGA CTCCATTTGA CGGA	208
	par-C09-R	CCGTCCAGATCCATCGAAGTC	

Basically, they appeared largely similar in their topology, with isolates belonging to a given species always clustered together. First, *M. hapla* and *M. exigua* were clustered together, and well separated from all the other species and (or) isolates, with high bootstrap support in both analyses. Second, although the support of their clustering was lower than for the two previous species (77 and 74% in the parsimony and the neighbor-joining analyses, respectively), *M. arenaria* and *M. javanica* appeared closer to one another than to *M. incognita*. Third, the non-identified isolates sp1 and sp2 clustered together, with variable confidence levels (89% in the parsimony tree and 52% in the neighbor-joining tree).

SCAR primers for the specific identification of *Meloidogyne* spp.

Some of the random primers used in this study allowed the amplification of RAPD fragments that were specific for one of the species tested only. As exemplified in Fig. 1, a ~600-bp band was specifically generated with primer K14 in the RAPD patterns from the *M. incognita* isolates inc1, inc2, inc3, and inc4. No band of the same size was observed for all the other isolates and (or) species tested. The band was further cloned and sequenced. A computer-assisted sequence similarity search revealed no significant homology with any known sequence in databases. A pair of putative specific SCAR primers, inc-K14-F and inc-K14-R, was designed that should allow the amplification of a fragment of 399 bp (Table 3). Using this primer set, a band of the expected size was amplified with DNA from the four *M. incognita* isolates available (Fig. 3*a*). By contrast, no amplification could be observed when DNA from any of the other isolates was used as template (Fig. 3*a*). The same overall procedure allowed the cloning and sequencing of two other RAPD fragments specific for *M. exigua* and *M. paranaenesis*, respectively

Fig. 1. RAPD patterns for 18 *Meloidogyne* spp. isolates generated with primer D15. For each isolate, two duplicate amplifications were loaded side by side on the gel. The arrowhead indicates the ~600-bp band specific for *M. incognita* isolates. C, control reaction without template DNA. Populations codes are given in Table 1.



(data not shown). As previously observed for *M. incognita*, the two sequences did not show any significant homology in databases. Two new pairs of SCAR primers were designed, (*i*) ex-D15-R and ex-D15-F and (*ii*) par-C09-F and par-C09-R, which should amplify specific bands of 562 and 208 bp for isolates of *M. exigua* and *M. paranaenesis*, respectively (Table 3). Similarly, use of each of the two primer sets led to the amplification of a specific band of the expected size for either *M. exigua* (Fig. 3*b*) or *M. paranaenesis* (Fig. 3*c*) isolates. Apart from the species to be detected, no amplification was observed with any of the other species tested. With the three primer sets defined above, the same specific amplified fragments were obtained with DNA from single individuals of *M. exigua*, *M. incognita*, and *M. paranaensis*, respectively, thus demonstrating the sensitivity of the detection (data not shown).

Multiplex PCR amplifications were performed using all six SCAR primers (inc-K14-F, inc-K14-R, ex-D15-R, ex-D15-F, par-C09-F, and par-C09-R) together in a single PCR according to the conditions previously used for each individual pair of SCAR primers. The following templates were used: *M. exigua*, *M. incognita*, and *M. paranaensis* genomic DNA; 1:1 mixtures of *M. exigua* and *M. incognita*, *M. exigua* and *M. paranaensis*, and *M. incognita* and *M. paranaensis* genomic DNA; and a 1:1:1 mixture of *M. exigua*, *M. incognita*, and *M. paranaensis* genomic DNA. As shown in Fig. 4, the multiplex PCR allowed the unambiguous differentiation of each species from the two others, taken alone or in mixtures. The same result was obtained using DNA from single females as template (data not shown).

Table 4. Rate of RAPD polymorphisms observed at species level.

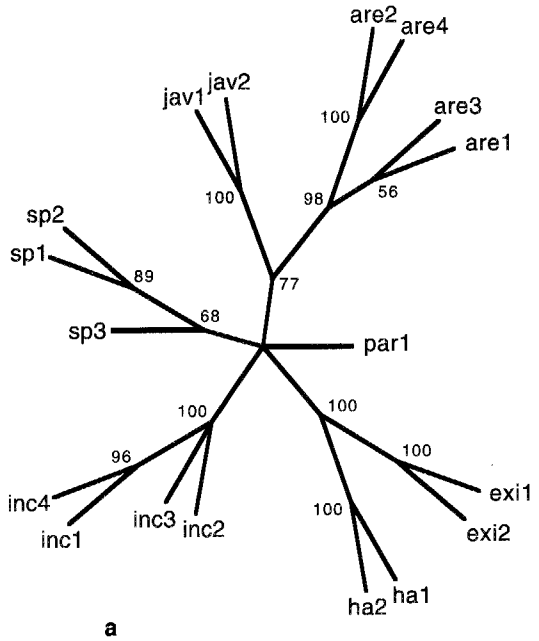
	RAPD fragments		
Species	Amplified	Polymorphic (%)	Informative
M. arenaria	460	321 (69.8)	97
M. exigua	302	204 (67.5)	204*
M. hapla	388	262 (67.5)	262*
M. incognita	286	86 (30.1)	25
M. javanica	261	51 (19.5)	51

*Because only two isolates were analyzed from this species, all the polymorphic RAPD fragments were considered to be informative.

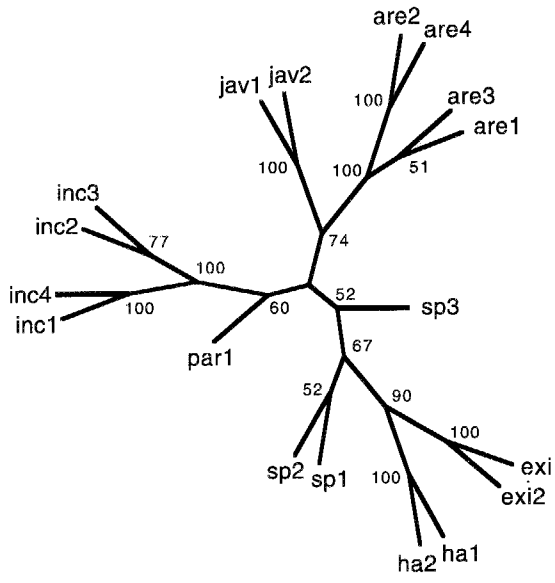
Discussion

The first objective of this study was to estimate the genetic diversity and relationships among *Meloidogyne* spp. isolates collected from the Southern part of Brazil using RAPD markers. A total of 27 random oligonucleotide primers were used that allowed us to generate 1125 polymorphic markers. To our knowledge, this is the most detailed and extensive study of genetic variability among Brazilian isolates of RKN and the first to use molecular analysis for such a purpose. Using RAPD amplification patterns alone or in combination, each nematode genotype (i.e., isolate) tested could be differentiated from all the others, which showed that the RAPD technique can be successfully applied to the genus *Meloidogyne* for DNA fingerprinting.

Fig. 2. Majority-rule consensus dendrograms of relationships of *Meloidogyne* spp. isolates. (*a*) Parsimony-based tree. (*b*) Neighbor-joining tree. Bootstrap percentages based on 1000 replicates are given on each node. Populations codes are given in Table 1.



a



b

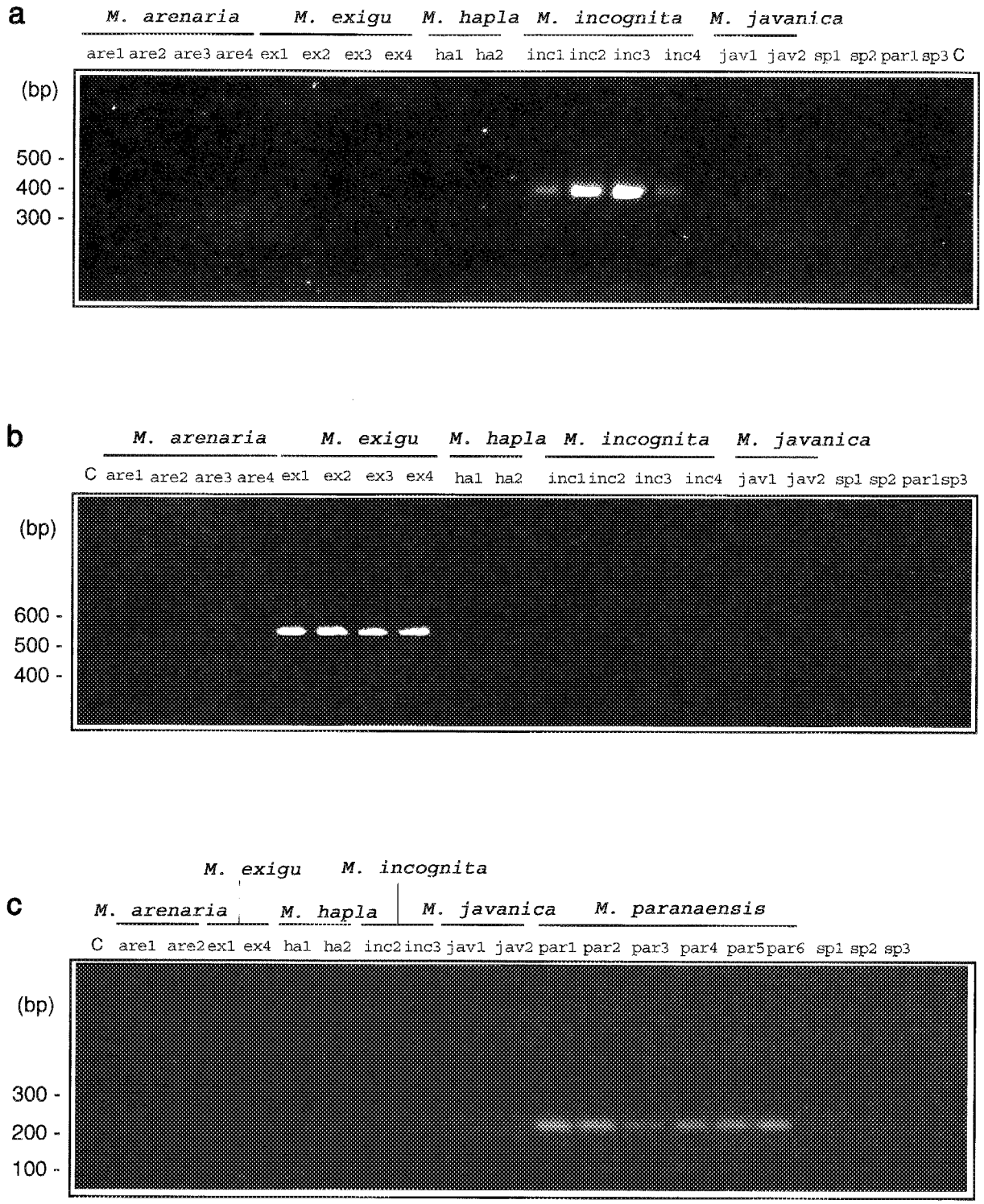
Variable degrees of polymorphism were detected at the intraspecific level. In *M. incognita* and *M. javanica*, a low proportion of the RAPD fragments appeared to be polymorphic (30.1 and 19.5%, respectively). This rather low variability among isolates could be related to the mitotic parthenogenetic mode of reproduction of these two species that should theoretically lead to clonal progenies (Triantaphyllou 1985). Moreover, such a relative lack of genetic diversity in *M. incognita* and *M. javanica* is in good agreement with other RAPD studies (Castagnone-Sereno et al. 1994 ; Blok et al. 1997*b*), and suggests that these species may have been spread recently from a unique source rather than representing independent lineages. Alternatively, *M. exigua*, *M. hapla*, and *M. arenaria* exhibited higher levels of infraspecific variability, with 67.5, 67.5, and 69.8% of polymorphic amplified fragments, respectively. In the case of *M. exigua* and *M. hapla*, it seems reasonable to correlate again such a high level of infraspecific polymorphism with the mode of reproduction of the two species, i.e., amphimixis and facultative meiotic parthenogenesis, respectively (Triantaphyllou 1985). Although reproducing by mitotic parthenogenesis, *M. arenaria* showed the highest level of genetic variability, which was not unexpected because this result is in good agreement with previous molecular analyses performed on the mitochondrial (Hugall et al. 1994) or nuclear (Castagnone-Sereno et al. 1994; Semblat et al. 1998) genome of this taxon.

The relationships among and between Brazilian species of *Meloidogyne* were inferred from either a parsimony or a distance-based analysis, and the two resulting dendrograms proved to be largely similar at the species level. Overall, the fact that *M. hapla* and *M. exigua* were more closely related to one another than they were to the three obligatory mitotic parthenogenetic species is consistent with RAPD studies conducted on RKN from other origins (Baum et al. 1994; Blok et al. 1997*b*; Castagnone-Sereno et al. 1994). This result illustrates the early separation of meiotic and mitotic species and supports the hypothesis that amphimixis is the ancestral reproductive state of the genus (Triantaphyllou 1985). Within *M. incognita*, isolates inc1 and inc4 and isolates inc2 and inc3 were clustered together. The same distribution had previously been obtained on the same isolates based on their esterase phenotypes (Carneiro et al. 2000), which indicated some infraspecific variability. The fact that isolates inc2 and inc3 originated from coffee, and the two others did not (Table 1), suggests that host-specific specialization might explain this observation. However, more detailed studies, including a large number of *M. incognita* isolates sampled from coffee and from other plants, should be conducted to strengthen this hypothesis. The clustering of the three undescribed isolates, sp1, sp2, and sp3, with the mitotic or meiotic species was not resolved, which makes it impossible to hypothesize their mode of reproduction. Further cytogenetic investigations are needed to determine this point.

Currently, routine identification of the most agronomically important RKN species in Brazil, including those associated with coffee, is made by microscopic examination of perineal patterns of females and (or) by differential host tests. Owing to the inherent limitations of such methods *Meloidogyne* spp. populations have frequently been misidentified. For example, based on perineal patterns, a new pathotype of *M. incognita*, biotype IAPAR, was reported on coffee and later described as *M. paranaensis* (Carneiro et al. 1996*a*). On the basis of our results, it is clear that RAPD patterns allow reliable identification of RKN at the species level. However, it has been shown in a number of studies that reproducibility of RAPD patterns may be dependent upon the experimental conditions and (or) that non-reproducible fragments may be amplified, which precludes the use of such a method for routine diagnostic purposes (Perez et al. 1998; Rieseberg 1996).

To develop a PCR-based assay that could be used on a routine basis to specifically identify RKN associated with coffee, RAPD markers of interest were further transformed

Fig. 3. Amplification patterns for 20 *Meloidogyne* spp. isolates generated with the specific SCAR primers. (*a*) Primers inc-K14-R and inc-K14-F. (*b*) Primers ex-D15-R and ex-D15-R. (*c*) Primers par-C09-F and par-C09-R. C, control reaction without template DNA. Populations codes are given in Table 1.



into SCAR markers. Such an approach had previously been tested for the three main tropical RKN species (Zijlstra et al. 2000). The RAPD analysis resulted in the identification of three DNA fragments that were specifically amplified in *M. exigua*, *M. incognita* and *M. paranaensis*, respectively. After cloning and sequencing these fragments, three pairs of SCAR primers were designed. After PCR amplification using these SCAR primers, the initial polymorphism was retained as the presence or absence of the amplification, thus providing a simple yes or no answer. Because the SCAR primers were longer than the RAPD primers, the annealing temperature in the amplification procedure was significantly

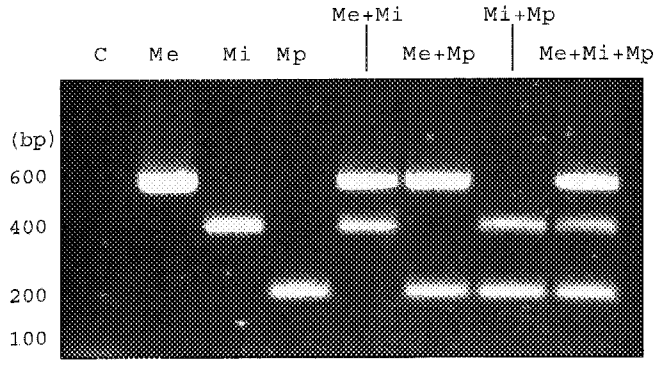


Fig. 4. Multiplex PCR amplification patterns for *Meloidogyne exigua* (Me), *M. incognita* (Mi) and *M. paranaenesis* (Mp) alone or in mixtures. C, control reaction without template DNA.

higher (64 instead of 36°C), resulting in more specific amplification conditions that eliminated the problems of sensitivity to conditions and reproducibility mentioned above (Perez et al. 1998; Rieseberg 1996). Moreover, the sensitivity of the detection was demonstrated with positive identification of single nematodes. In coffee plantations, *M. exigua*, *M. incognita*, and *M. paranaensis* are often found associated in separate or mixed populations with fluctuations in the predominance of each species over the others (Campos et al. 1990; Carneiro et al. 1996*a*). To identify nematode species in such mixtures, we used the SCAR primers in multiplex assays. Unambiguous characterization of the three species was possible either on genomic DNA or on single nematodes by direct visualization of the ethidium bromide stained electrophoresis gel. Using single nematodes, this multiplex procedure is very simple compared with similar assays previously developed for RKN species that required either digestion of the amplified products with restriction enzymes (Zijlstra 1997) or a subsequent multiplex nested PCR (Zijlstra 2000).

In conclusion, the SCAR primers and their use in multiplex PCR as developed in the present study have potential for application in routine diagnostic procedures. Moreover, because specific RAPD fragments are easy to identify in any RKN species without prior sequence knowledge, the approach described here could easily be generalized, either for known species or for phylogenetic lineages of particular interest.

Acknowledgements

We wish to thank all the Brazilian colleagues who provided us with *Meloidogyne* spp. isolates. O.R. is the recipient of a Ph.D. scholarship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil, which is gratefully acknowledged.

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