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Diversity of *Meloidogyne exigua* (Tylenchida: Meloidogynidae) populations from coffee and rubber tree

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Summary – Isozymes (esterase and malate dehydrogenase), SCAR and RAPD-PCR were studied in 15 populations of three races of *Meloidogyne exigua* collected in coffee-producing areas in Brazil, Bolivia and Costa Rica and one population from rubber tree plantations in Brazil. This study revealed four esterase phenotypes (E1, E2, E2a, E3) and three malate dehydrogenase phenotypes (N1, N1a, N2) for *M. exigua* populations. The most common multi-enzyme phenotype was E2N1. The enzymatic phenotypes do not separate *M. exigua* races. Sixteen populations of *M. exigua* were tested in Multiplex PCR using SCAR primers ex-D15F/R that allowed the identification of all *M. exigua* populations. Phylogenetic analyses showed high intraspecific polymorphism (25.9-59.6%) for all *M. exigua* studied. However, all populations clustered together with 100% bootstrap support, thereby demonstrating the consistency of species identification. In general, no correlation was found between enzymatic profile, race and genetic polymorphism of the studied populations.

Keywords – *Coffea arabica*, electrophoresis, *Hevea brasiliensis*, isozymes, molecular markers, RAPD, root-knot nematode, SCAR.

Root-knot nematodes (*Meloidogyne* spp.) are major agricultural pests of a wide range of crops. However, some less common species, such as *Meloidogyne exigua* Göldi, 1887, are more restricted, parasitising fewer plants within different taxonomical groups as compared to major *Meloidogyne* species (Jepson, 1987; Campos & Villain, 2005). The genus comprises more than 90 described species and is distributed worldwide. Seventeen species have been detected on coffee (*Coffea arabica* L.). In Brazil, *M. exigua*, *M. incognita* (Kofoid & White, 1919) Chitwood, 1949 and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996 are considered the main species of root-knot in coffee plantations (Campos & Villain, 2005).

Amongst the most damaging species, *M. exigua* constitutes a serious agronomic constraint because of its wide distribution in Latin America. It is the dominant species in Brazil (Campos & Villain, 2005) and Costa Rica (Flores & López, 1989) where it causes general weakening of coffee trees associated with a yield loss estimated at 10-15% in Costa Rica (Bertrand *et al*., 1997) and 45% in Brazil (Barbosa *et al*., 2004). According to Campos and Villain (2005), *M. exigua* causes typical rounded galls, mostly on newly formed roots. The galls are initially white to yellowish brown and turn dark brown as the root becomes older. Egg masses are produced either in the cortex beneath the root epidermis or protruding outside the cortex. In addition, a distinct population of *M. exigua* that parasitises only the rubber tree (*Hevea brasiliensis* Muell. Arg.) is a very important pathogen in Rondonópolis and São José do Rio Claro, Mato Grosso State, Brazil (Santos *et al*., 1992; Bernardo *et al*., 2003).

Eight populations of *M. exigua* from coffee in Brazil appeared to be very similar to each other morphologically (Lima & Ferraz, 1985) and only a few variants within this species have been reported (Eisenback & Triantaphyllou, 1991). Nevertheless, physiological variability exists and three races of *M. exigua* have been described, *viz*., race

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1 (pepper and coffee), race 2 (tomato, pepper and coffee) and race 3 (rubber tree) (Carneiro & Almeida, 2000).

Comparison of esterase patterns (Est) shows great consistency in separation of the major *Meloidogyne* species (Dalmasso & Bergé, 1978; Esbenshade & Triantaphyllou, 1985a; Kunieda de Alonso *et al*., 1995; Carneiro *et al*., 1996, 2000). The isozyme malate dehydrogenase is helpful in identification when esterases show similar patterns, *e.g*., as in *M. naasi* Franklin, 1965 and *M. exigua* (see Esbenshade & Triantaphyllou, 1985a). Three different esterase phenotypes of *M. exigua* have been reported: E1a, E1b and E2 (Carneiro *et al*., 2000, 2005; Oliveira *et al.*, 2005). Using the methodology of Carneiro and Almeida (2001), *M. exigua* can be identified by isoenzyme electrophoresis using a larger number (ten) of macerated females – the females are smaller and esterase activity is lower when compared to other *Meloidogyne* spp. (Carneiro *et al*., 1996, 2000).

Considering the difficulty in characterising *M. exigua* using esterase phenotypes, molecular markers (Sequence Characterised Amplified Region – SCAR), in multiplex PCR and satellite DNA, were developed to identify *M. exigua* populations (Randig *et al*., 2002a, b). These two techniques are the most interesting for use in routine analyses. Recently, a study of 54 populations of *Meloidogyne* spp. in coffee fields was done in São Paulo and Minas Gerais States using a combination of six SCAR primers in a single reaction. The multiplex PCR allowed the unambiguous differentiation of the three main *Meloidogyne* species from coffee (including *M. exigua*), alone or in a mixture, and its potential for application in routine diagnostic procedure has been confirmed (Carneiro *et al*., 2005).

Polymerase chain reaction (PCR) based methods are relatively fast and very reliable, and are also independent of the nematode life cycle stage (Zijlstra, 2000). The random amplified polymorphic DNA (RAPD) technique can reveal considerable polymorphism, even between closely related species (Williams *et al*., 1990) and it is mostly used for intra- or interspecific variability studies (Randig *et al*., 2002a; Carneiro *et al*., 2004). These papers also showed that two races of *M. exigua* (race 1 and race 3, from coffee and rubber trees, respectively) exhibited higher levels (67.5%) of genetic variability (Randig *et al*., 2002a), whereas races 1 and 2 from coffee showed only 8.6% polymorphic fragments (Carneiro *et al*., 2004).

The objectives of the present study were: *i*) to examine the genetic variability and relationships among the *M. exigua* populations encountered in different regions from Brazil; *ii*) to look for any possible association between enzymatic/molecular markers and host races in *M. exigua*; and *iii*) to validate the specificity of the previously described SCAR markers on a wide variety of *M. exigua* populations.

Materials and methods

Nematode populations

Twenty-one populations of *Meloidogyne* spp. were examined (Table 1), 13 being collected in coffee fields from the Brazilian States of Minas Gerais, São Paulo and Rio de Janeiro, and another from rubber tree (Rondonópolis, MT, Brazil). Two populations from other countries (*i.e*., populations 11 and 12 from Costa Rica and Bolivia, respectively) were included from the nematode collection of Embrapa Recursos Genéticos e Biotecnologia. Five populations from coffee and tomato (Brazil and Costa Rica), which had already been identified through esterase and malate dehydrogenase phenotypes as *M. incognita*, *M. paranaensis*, *M. javanica* (Treub, 1885) Chitwood, 1949 and *M. mayaguensis* (Rammah & Hirschmann, 1988) (Table 1), were used as a reference for DNA analysis. All populations studied were maintained on tomato (*Lycopersicon esculentum* Santa Cruz cv. Kada) or coffee cv. Catuaí plants in the glasshouse. The population from rubber tree was propagated on its respective host.

Isozyme analyses: esterase and malate dehydrogenase

Isozyme characterisations were done for esterase, Est (EC 3.1.1.1) and malate dehydrogenase, Mdh (EC 1.1.1.37), in 7% polyacrylamide gel slabs (11 × 18 cm, 1 mm thick) in a Cl-18 Permatron apparatus, using a modification of the technique proposed by Smithies (1955) according to the technique described by Carneiro and Almeida (2001). *Meloidogyne exigua* females were dissected from coffee, tomato or rubber tree roots under a stereomicroscope and macerated (30 females for esterase studies and 15 for Mdh) in 3 μl of extraction buffer containing sucrose-Triton (Esbenshade & Triantaphyllou, 1985b) or Tris/HCl (Trudgill & Carpenter, 1971) for Est or Mdh, respectively. Several females were used to ensure good resolution of secondary bands. The nematode suspension was absorbed by Whatman 3 MM filter paper (1.5 × 4.0 mm) and each piece of paper was loaded, using fine forceps, into wells in the separating gel.

Table 1. *Geographical origin, host preference, enzyme phenotypes and identification of* Meloidogyne *spp.*

Geographical origin	Population code	Reproduction on		Enzyme phenotypes		Species/race
		Coffee	Tomato	Est	Mdh	
Varginha, MG, Brazil	7	+	+	E1	N1	*M. exigua* Race 2
São Sebastião do Paraíso, MG, Brazil	4	+	+	E3	N1	*M. exigua*
	16	+	+	E3	N1	Race 2
	17	+	+	E2	N2	
Lavras, MG, Brazil	13	+	−	E1	N1	*M. exigua*
	14	+	+	E2	N1	Race 1, 2
São Francisco do Glória, MG, Brazil	10	+	+	E1	N1a	*M. exigua* Race 2
São Sebastião da Vargem Alegre, MG, Brazil (9)	9	+	+	E3	N1	*M. exigua* Race 2
Canaã, MG, Brazil (6)	6	+	−	E2	N2	*M. exigua* Race 1
Itirapuã, SP, Brazil (15)	15	+	+	E2	N1	*M. exigua* Race 2
Campinas, SP, Brazil	23	+	−	E2	N1	*M. exigua* Race 1
Varre-Sai, RJ, Brazil	18[1]	+	+	E2	N1a	*M. exigua*
	19	+	−	E2	N1	Race 1, 2
Rondonópolis, MT, Brazil	24[2]	−	−	E2a	N1	*M. exigua* Race 3
Bolivia	12	+	+	E1	N1	*M. exigua* Race 2
Costa Rica	11	+	+	E2	N1	*M. exigua* Race 2
Avilândia, SP, Brazil	25	+	+	I1	N1	*M. incognita*
Londrina, PR, Brazil	26	+	+	I2	N1	*M. incognita*
Londrina, PR, Brazil	27	+	+	P1	N1	*M. paranaensis*
Petrolina, PE, Brazil	30[3]	−	+	J3	N1	*M. javanica*
Costa Rica	32	+	+	M2	N1	*M. mayaguensis*

[1] Population 18 is virulent to the Mex-1 coffee resistant gene (Barbosa *et al*., 2007).
[2] Population collected from rubber tree.
[3] Population collected from tomato. The remaining populations were collected from coffee plantations. Est = esterase, Mdh = malate dehydrogenase.

Protein extracts of five females of pure populations of *M. hapla* Chitwood, 1949 and *M. javanica* were used as reference phenotypes in each gel. When all the samples had been loaded, a drop of Bromophenol Blue solution was added to the samples. Electrophoresis was run for approximately 2 h until the Bromophenol Blue dye had migrated 5.5 cm. Gels were stained for 30 min for esterase activity (Scandalios, 1969) and 45 min for malate dehydrogenase (Harris & Hopkinson, 1976). Gels were washed, stored and placed in 10% acetic acid and 40% methyl alcohol solution for 30 min. Gels were pressed between two cellophane sheets to dry. The values of the rate of migration (Rm) of the various bands in the gels were measured. Enzyme phenotypes were designated by a suggestive letter for the species and an indicative number for the bands according to Esbenshade and Triantaphyllou (1985a, 1990).

Differential host test

Considering previous work (Carneiro & Almeida, 2000; Oliveira *et al*., 2005), a differential host test was done with

the following host plants: coffee Catuaí Vermelho IAC 144, tomato Santa Cruz cv. Kada and rubber tree clone GD1, following the methodology described by Hartman and Sasser (1985).

Egg extraction and DNA preparation

The extraction of eggs for PCR-SCAR-RAPD was done according to the protocol described by Carneiro *et al*. (2004). Total genomic DNA was extracted from 200-300 μl of nematode eggs of each population that had been stored at −80°C before use, as described by Randig *et al*. (2002a). Eggs were crushed in liquid nitrogen with a mortar and pestle. DNA was extracted from the resulting fine powder and purified by phenol-chloroform extraction. Samples of DNA were precipitated by mixing with absolute ethanol. The pellet was collected, rinsed with 70% (v/v) ethanol, dried at room temperature and then stored at −20°C in sterile Milli-Q water. The quantity of DNA was estimated in a 1% agarose gel compared to the total DNA on five concentrations of lambda DNA. DNA samples were then diluted in sterile Milli-Q water to obtain a DNA concentration of 6 ng/μl. The aliquots were stored at −20°C for further analyses.

Multiplex SCAR-PCR analysis

The analysis was done using four sets of SCAR primers (produced by Invitrogen Life Technologies, São Paulo, SP, Brazil; Table 2) in a single PCR reaction, according to Randig *et al*. (2002a) and Zijlstra *et al*. (2000). The multiplex PCR was done in a final volume of 25 μl containing 6 ng of the total genomic DNA, in addition to 4 μM of primer, dATP, dCTP, dGTP and dTTP (Fermentas Life Sciences, Hanover, MD, USA) each at 200 μM final concentration, 1× *Taq* incubation buffer and 1 unit of *Taq* polymerase (Phoneutria Biotecnologia & Serviços, Belo Horizonte, MG, Brazil). Control reaction with all reagents except template DNA was included in the test. Each reaction mixture was overlaid with mineral oil to prevent evaporation. Amplification was performed on a PTC-100 MJ Research thermal cycler (MJ Research, Watertown, MA, USA), according to the following amplification conditions: 10 min at 94°C, 30 cycles of 30 s at 94°C, 45 s at 62°C and 1 min at 72°C plus a final incubation of 8 min at 72°C. Amplification products were separated by electrophoresis in 1.3% w/v agarose gels in TBE buffer (90 mM Tris base, 89 mM boric acid and 2 mM EDTA, pH 8.3). A 1 kb Plus DNA Ladder was used as the molecular weight standard. Gels were stained with ethidium bromide (0.2 μg/ml) and DNA fragments were photographed under ultraviolet light.

PCR-RAPD analysis

Thirty-three random 10-mer primers, obtained from Invitrogen Life Technologies or Operon Technologies (Almeida, CA, USA), were used in RAPD trials (Table 3). Two replicates of the reactions were done. RAPD-PCR was performed in a final volume of 25 μl containing 6 ng of total genomic DNA, in addition to 8 μM of primer, dATP, dCTP, dGTP and dTTP each at 200 μM final concentration, 1× *Taq* incubation buffer and 1 unit *Taq* polymerase (Phoneutria Biotecnologia & Serviços). To confirm that the observed bands were amplified from the genomic DNA and were not primer artefacts, the genomic DNA was then omitted from control reactions for each primer. The cycling program was 5 min at 94°C; 40 cycles of 30 s at 94°C, 45 s at 36°C and 2 min at 70°C plus a final incubation of 10 min at 70°C. Amplification

Table 2. *Characteristics of the SCAR markers developed for* Meloidogyne exigua*,* M. incognita*,* M. paranaensis *and* M. javanica *according to Randig* et al. *(2002a) and Zijlstra* et al. *(2000).*

Species	SCAR primer	SCAR primer sequence (5′ → 3′)	Size of the SCAR (bp)
M. exigua	EX-D15-F	CAT CCG TGC TGT AGC TGC GAG	562
	EX-D15-R	CTC CGT GGG AAG AAA GAC TG	
M. incognita	INC-K14-F	GGG ATG TGT AAA TGC TCC TG	399
	INC-K14-R	CCC GCT ACA CCC TCA ACT TC	
M. paranaensis	PAR-C09-F	GCC CGA CTC CAT TTG ACG GA	208
	PAR-C09-R	CCG TCC AGA TCC ATC GAA GTC	
M. javanica	JAV A01-F	CAG GCC CTT CAG TGG AAC TAT AC	670
	JAV A01-R	GCC CGA CTC CAT TTG ACG GA	

bp = base pairs.

Table 3. *Oligonucleotide primers used for RAPD analysis and number of amplified fragments scored for* Meloidogyne *spp.*

RAPD primer	RAPD primer sequence ($5' \rightarrow 3'$)	GC (%)	Minimum/ population	Maximum/ population	Polymorphic	Total
A04	AAT CGG GCT G	60	1	7	22	22
AB04	GGA ACG GGT T	60	3	11	31	31
OPAB-06	GTG GCT TGG A	60	3	8	23	23
OPB-11	GTA GAC CCG T	60	3	9	31	31
OPB-12	CCT TGACGC A	60	1	5	12	12
OPB-13	TTC CCC CGC T	70	1	10	19	19
C07	GTC CCG ACG A	70	2	11	27	27
C09	CTC ACC GTC C	70	4	13	31	31
C16	CAC ACT CCA G	60	2	11	25	25
OPC-18	TGA GTG GGT G	60	1	6	17	17
OPF-06	GGG AAT TCG G	60	1	8	22	22
G03	GAG CCC TCC A	70	3	9	21	21
G05	CTG AGA CGG A	60	2	11	29	29
G06	GTG CCT AAC C	60	3	12	37	37
G13	CTC TCC GCC A	70	2	6	15	15
OPJ-19	GGA CAC CAC T	60	4	17	27	27
K01	CAT TCG AGC C	60	1	5	26	26
K04	CCG CCC AAA C	70	4	9	30	30
K06	CAC CTT TCC C	60	3	10	19	19
K07	AGC GAG CAA G	60	1	10	23	23
OPK-13	GGT TGT ACC C	60	1	6	19	19
K16	GAG CGT CGA A	60	1	7	23	23
OPK-17	CCC AGC TGT G	70	3	12	25	25
K19	CAC AGG CGG A	70	1	9	29	29
K20	GTG TCG CGA G	70	3	6	31	31
L08	AGC AGG TGG A	60	2	8	17	17
M20	AGG TCT TGG G	60	4	12	26	26
OPN-07	CAG CCC AGA G	70	1	9	27	27
P01	GTA GCA CTC C	60	1	9	22	22
P02	TCG GCA CGC A	70	1	10	28	28
P05	CCC CGG TAA C	70	1	11	29	29
R07	ACT GGC CTG A	60	1	7	27	27
R08	CCC GTT GCC T	70	2	10	24	25
Total			–	–	814	815

products were separated by electrophoresis as previously described.

PHYLOGENETIC ANALYSES

DNA bands were scored as 1 (present) or 0 (absent) directly from the gels. For each population, two independent PCR reactions were electrophoresed in the same gel. Only DNA fragments consistently present or absent in these replicated samples were recorded and considered as binary characters. DNA fingerprints from each population were converted to a 0-1 matrix, and two phylogenetic analyses were performed using PAUP* 4.0 (Swofford, 1998). For the parsimony analysis, 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.*, invariant bands among all populations, and bands either present or absent in a single population only (Li & Graur, 1991). The distance-based neighbour-joining algorithm (Saitou & Nei, 1987) was used on the same dataset using the mean-character difference option of PAUP* to compute distances. For both analyses, 1000 bootstrap replicates were performed to test the support of

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

Results

Isozyme characterisation

Four phenotypes for esterase (Est) activity were recognised among 16 *Meloidogyne* populations: 15 being from coffee fields and one from rubber tree (Table 1). We detected the phenotype E1 with a single band (Rm 1.5), the phenotype E2 with two bands (Rm 1.5, 1.9) and a new phenotype designated E3 showing one strong band (Rm 1.5) and two weak bands (Rm 1.7, 1.8) (Fig. 1). In addition, the *M. exigua* population from rubber tree gave a different enzymatic profile with two bands phenotype (E2a – Rm 1.5, 1.6). All phenotypes confirmed the diagnosis of *M. exigua* because of the presence of the main band (Rm 1.5) (Fig. 1).

Three distinct malate dehydrogenase phenotypes were observed. The N1 phenotype (Rm 1.0) was detected in almost all populations of *M. exigua* from coffee and that from the rubber tree. Two undescribed phenotypes (N1a, Rm 1.3; N2 Rm 1.0, 1.3) were found in four *M. exigua* populations obtained from coffee fields (Table 1; Fig. 2).

Differential host test

Under glasshouse conditions four *M. exigua* populations reproduced on coffee plants but not on tomato and were characterised as race 1 (Table 1). By contrast, 11 *M. exigua* populations reproduced on coffee and tomato (race 2) and one population reproduced only on rubber tree (race 3).

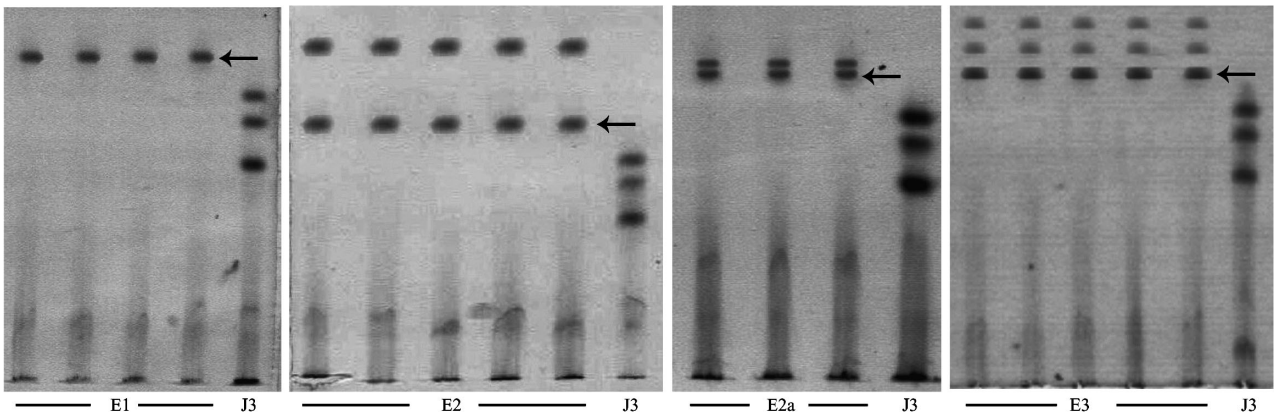

Fig. 1. *Esterase phenotypes of* Meloidogyne exigua*: E1, E2, E3 (from coffee); E2a (from rubber tree) compared to reference phenotype J3 for* M. javanica*. The main band (Rm 1.5) is marked by an arrow.*

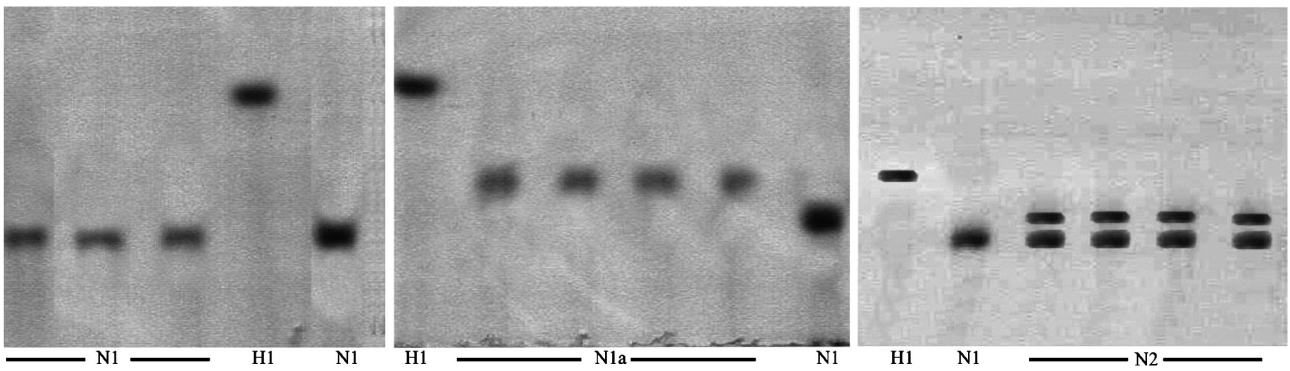

Fig. 2. *Malate dehydrogenase phenotypes of* Meloidogyne exigua*: N1, N1a, N2 compared to phenotypes N1 and H1 for* M. javanica *and* M. hapla *used as standard, respectively.*

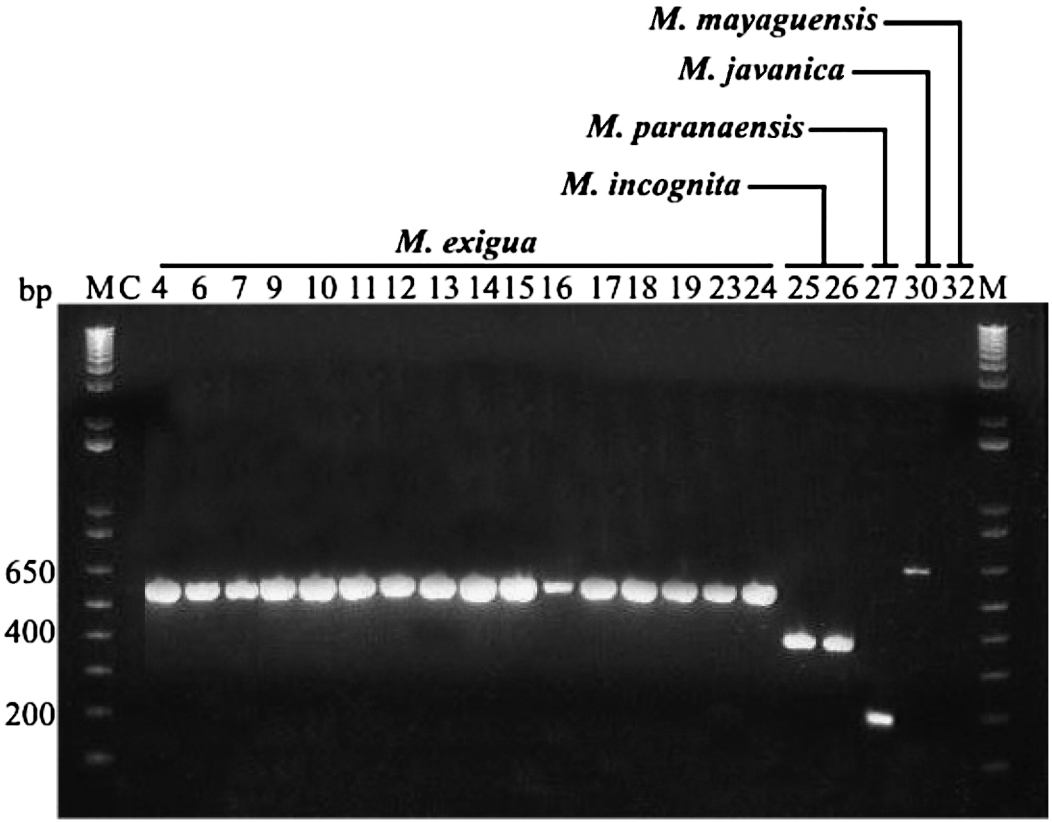

Fig. 3. *Typical amplification products of* Meloidogyne *spp. using SCAR primers in multiplex-PCR. M = 1 kb size marker DNA in base pairs (bp); C: Negative control without DNA. Population codes are given in Table 1.*

SCAR ANALYSIS

The populations of *Meloidogyne* spp. were investigated using SCAR primers (Table 2) in Multiplex-PCR. These reactions always resulted in species-specific fragments of 562 bp for 16 *M. exigua* populations, 399 bp for *M. incognita*, 208 bp for *M. paranaensis* and 670 bp for *M. javanica*. No band could be observed when DNA from one population of *M. mayaguensis* was used as a template (Fig. 3). The specificity of the SCAR primers was confirmed for different populations (enzymatic phenotypes and races) of *M. exigua* from coffee and that from rubber tree.

RAPD ANALYSES

All primers produced good amplification products. An example is shown in Figure 4. As can be seen, PCR with primer B13 produced identical patterns for all populations of *M. exigua* and could be considered diagnostic. On the other hand, the primer G6 contained species-specific bands but showed a lot of intraspecific variation. The majority of the random primers tested in this study produced common bands that discriminated *M. exigua* populations from each other.

With the 33 random primers used, the number of amplified fragments ranged from 1-17/population and varied in size from *ca* 150 to 4000 bp. The global results of the RAPD analysis are given in Table 3. In the overall trial, each primer produced from 12-37 polymorphic bands. Overall, 815 fragments were amplified and considered as RAPD markers, 814 of them being polymorphic and one monomorphic. In general, the *M. exigua* populations were not grouped according to their geographical distribution, enzymatic profile or race. For *M. exigua* populations from coffee, the effects of the geographical origin, isozyme esterase and race, ranged from 25.9-46.4%, 28.9-59.6% and 28.3-56.1% of rate of RAPD polymorphisms, respectively. Considering virulence, population 18 (virulent, according to Barbosa *et al*., 2007) was compared with populations 11, 13, 14 and 23 (not virulent)

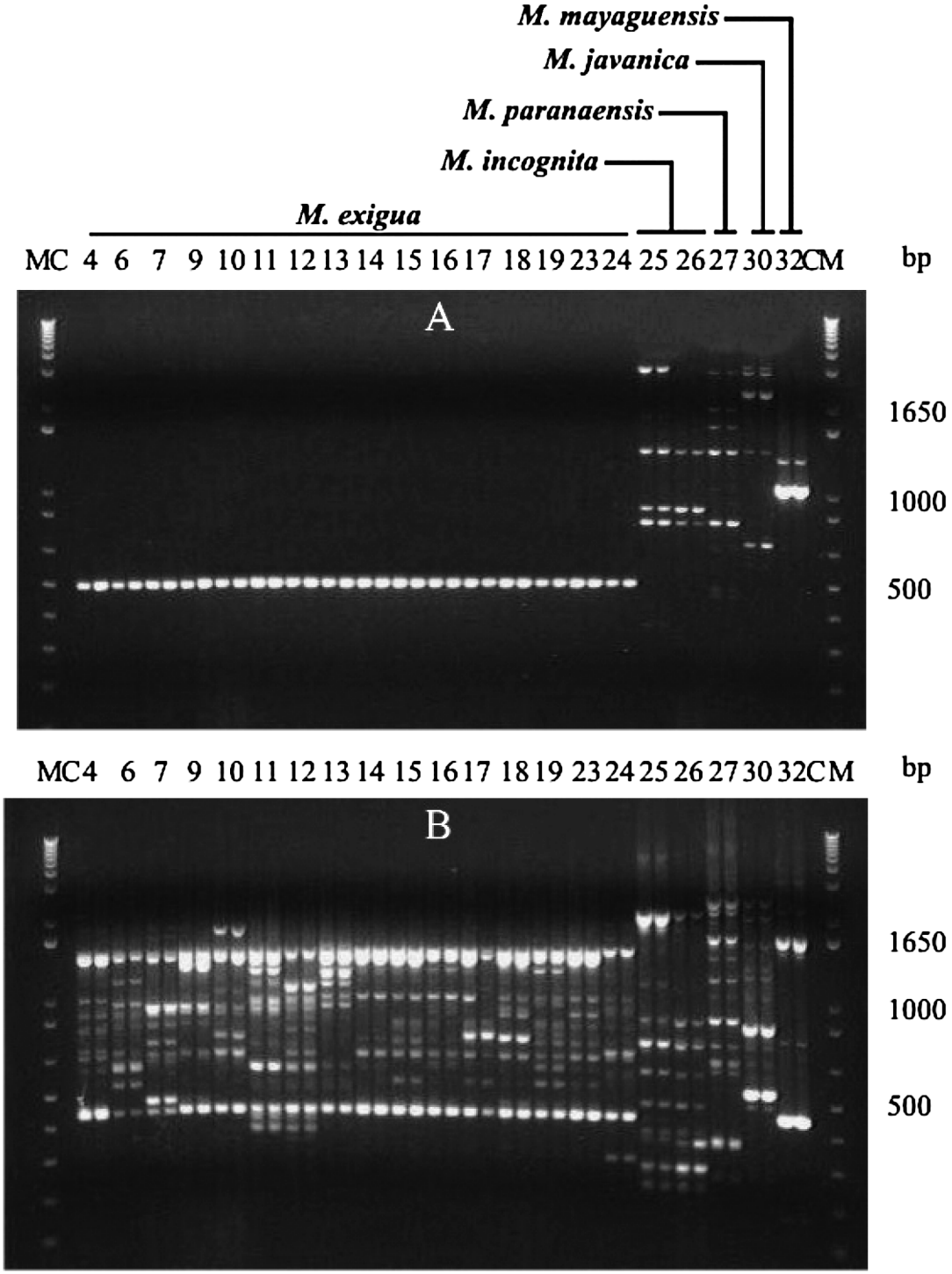

Fig. 4. *RAPD patterns for 21* Meloidogyne *spp. populations generated with primers B13 (A) and G6 (B). For each population, two duplicate amplifications were loaded side by side on the gel. M = 1 kb size marker DNA in bp; C: Negative control without DNA. Population codes are given in Table 1.*

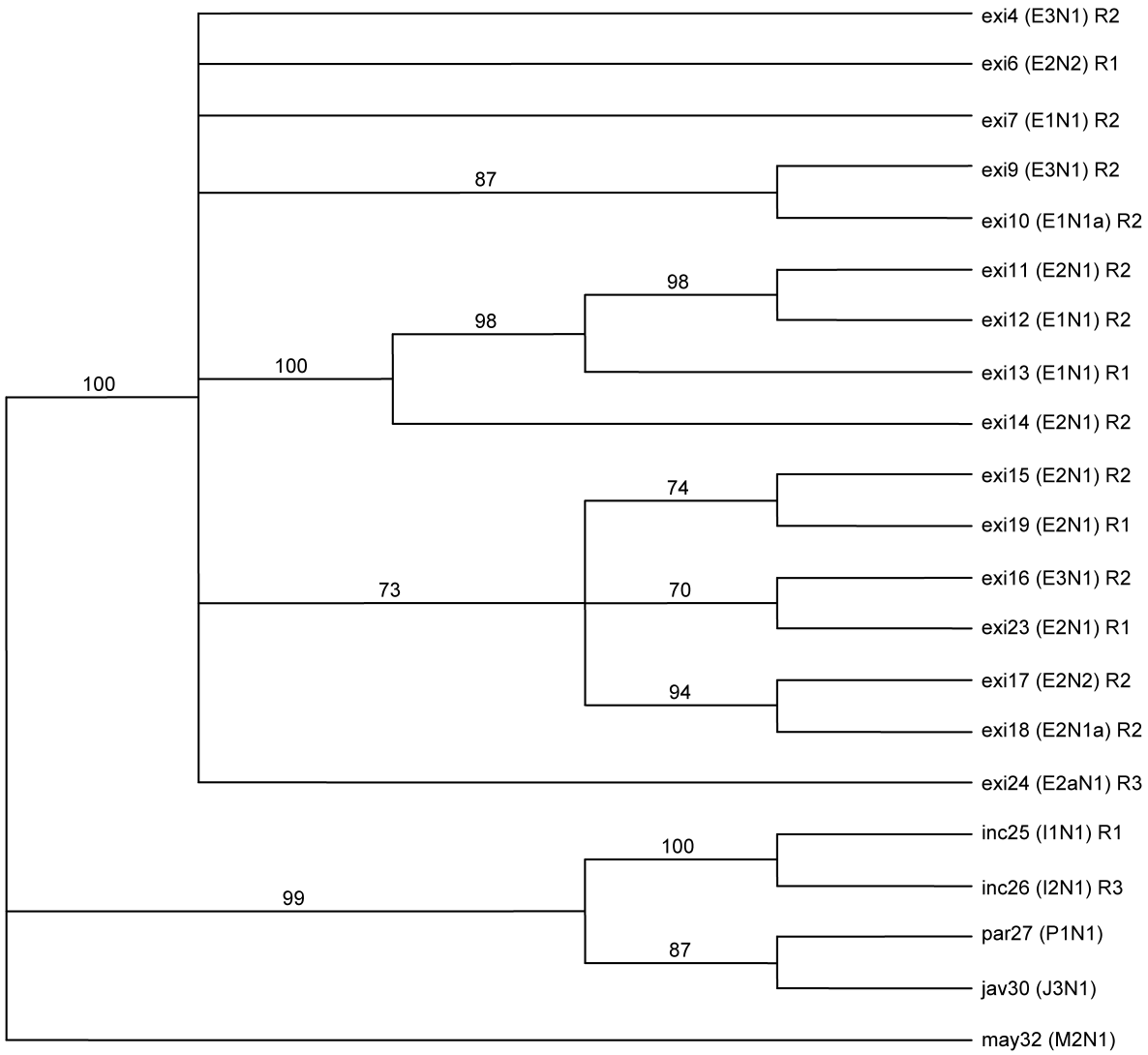

Fig. 5. *Consensus dendrograms of relationship of* Meloidogyne *spp. populations. Parsimony-based tree. Bootstrap percentages based on 1000 replicates are given on each node. Populations 25 to 32 were considered as the outgroup. Population codes are given in Table 1. Abbreviations: exi* = M. exigua*; inc* = M. incognita*; par* = M. paranaensis*; jav* = M. javanica*; may* = M. mayaguensis*; R1 = race 1; R2 = race 2; R3 = race 3.*

(Noir *et al*., 2003; Muniz *et al*., 2007). In this case, 33.6-54.8% of RAPD bands were polymorphic. Moreover, when comparing the population from rubber tree with those from coffee, 43.1-57.8% of RAPD bands were polymorphic.

All *M. exigua* populations from coffee and rubber tree clustered together with a high confidence level (bootstrap value of 100%) in the two resulting dendrograms (Figs 5, 6) but the population from rubber tree was distinctly separated from the others. In the dendrograms, *M. incognita*, *M. paranaensis*, *M. javanica* and *M. mayaguensis* were analysed as outgroups. The two *M. incognita* populations clustered together with 100% bootstrap support. *Meloidogyne paranaensis* and *M. javanica* clustered together with 87 and 90% bootstrap support in the parsimony and neighbour-joining methods, respectively. By contrast, *M. mayaguensis* was the most genetically distinct from the other species.

The dendrograms generated through RAPD data showed one homogeneous cluster within the *M. exigua*

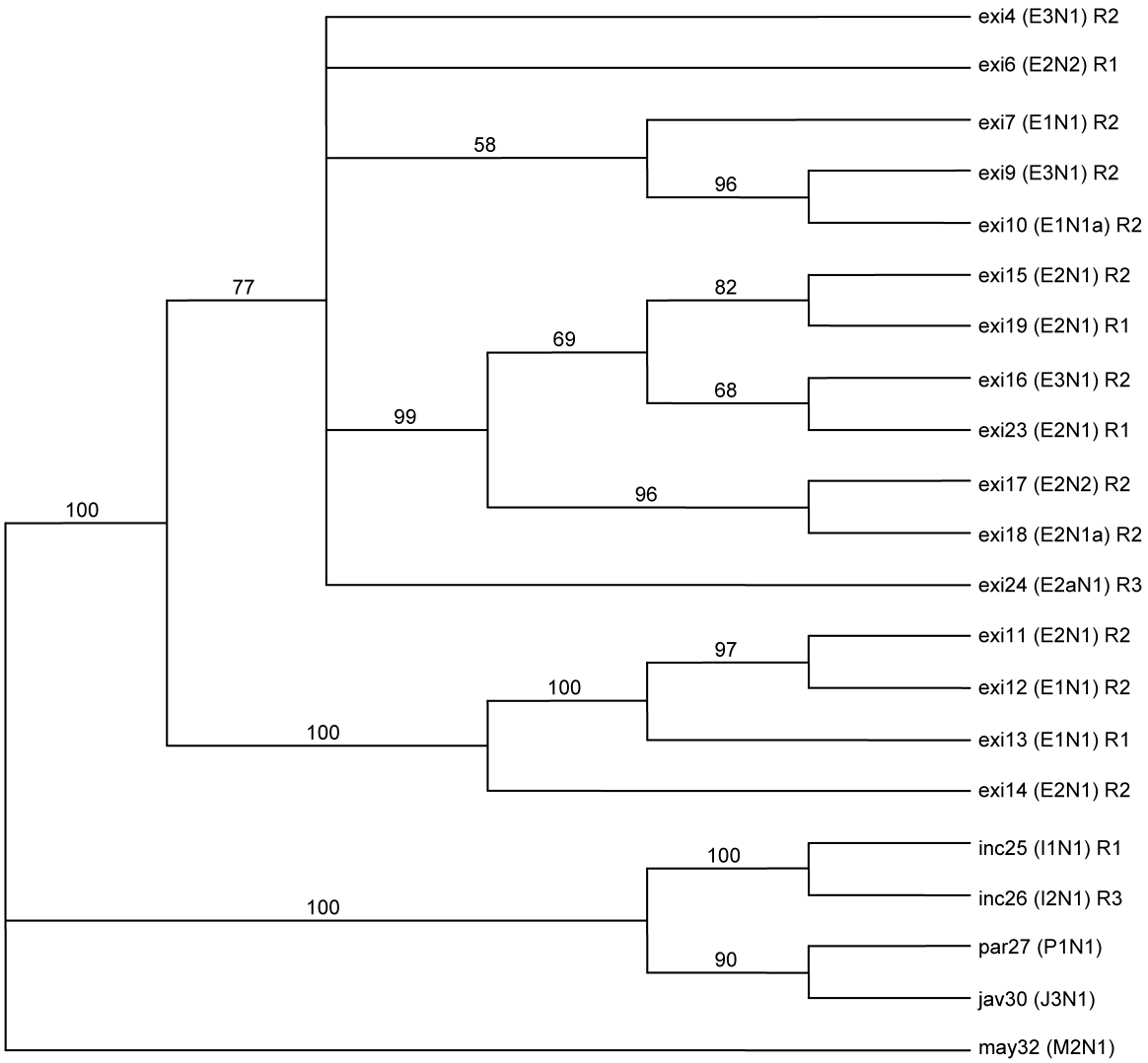

Fig. 6. *Consensus dendrograms of relationship of* Meloidogyne *spp. populations. Neighbour-joining tree. Bootstrap percentages based on 1000 replicates are given on each node. Populations 25 to 32 were considered as the outgroup. Population codes are given in Table 1. Abbreviations: exi* = M. exigua*; inc* = M. incognita*; par* = M. paranaensis*; jav* = M. javanica*; may* = M. mayaguensis*; R1 = race 1; R2 = race 2; R3 = race 3.*

populations (11, 12, 13, 14) with 100% bootstrap support in the analyses (Figs 5, 6). The *M. exigua* populations from Costa Rica (sample 11, E2N1 race 2) and Bolivia (sample 12, E1N1 race 2) were clustered together (97 and 98% bootstrap support) in the neighbour-joining and the parsimony methods, respectively. This cluster was close to the populations from Lavras, Minas Gerais State, Brazil (samples 13 and 14; E1N1 race 1 and E2N1 race 2, respectively).

In addition, the populations from São Sebastião da Vargem Alegre (sample 9, E3N1 race 2) and São Francisco do Glória (sample 10, E1N1a race 2), both from Minas Gerais State, clustered together with 87 and 96% bootstrap support. Populations from Itirapuã, São Paulo State (E2N1 race 2) and Varre-Sai, Rio de Janeiro State (E2N1 race 1) (samples 15 and 19, respectively) were clustered together with 74 and 82% bootstrap support, and this cluster was close to the populations from São Sebastião do Paraíso, Minas Gerais State (sample 16, E3N1 race 2) and Campinas, São Paulo State (sample 23, E2N1 race 1) and also to the populations from São Sebastião do Paraíso, Minas Gerais State (sample 17, E2N2 race 2) and

Varre-Sai, Rio de Janeiro State, Brazil (sample 18, E2N1a race 2). Conversely, the populations from São Sebastião do Paraíso (sample 4, E3N1 race 2), Canaã (sample 6, E2N2 race 1) and Varginha (sample 7, E1N1 race 2), all obtained from coffee, and from rubber tree (sample 24, E2aN1 race 3) appear in different branches.

Discussion

Four populations of *M. exigua* collected in Brazil reproduced only on coffee (race 1) and 11 on coffee and tomato (race 2). The ability of *M. exigua* from coffee to reproduce on tomato has already been noted (Carneiro & Almeida, 2000; Carneiro *et al*., 2004; Hernandez *et al*., 2004). This result confirms the physiological variability of this species and the prevalence of *M. exigua* race 2 in Brazil, a fact which agrees with the observations made by Oliveira *et al.* (2005).

The analyses of esterase enzyme showed four phenotypes (E1, E2 and E3 from coffee and E2a for rubber tree). *Meloidogyne exigua* populations with the Est phenotype E2 are the most widespread in the coffee areas of Brazil, according to Oliveira *et al*. (2005) and Carneiro *et al*. (2005). These results agree with the data obtained in the present paper. The occurrence of more than one phenotype for the same enzyme is known in other *Meloidogyne* species (Esbenshade & Triantaphyllou, 1985a; Santos & Triantaphyllou, 1992; Carneiro *et al*., 1996, 2000). In fact, the esterase band E1 (Rm 1.5) seems to be the most species-specific band present in all *M. exigua* populations. The lack of resolution of other *M. exigua* bands was apparently related to the low esterase activity of those bands (Carneiro *et al*., 1996).

The *M. exigua* population from rubber tree, studied previously by Carneiro *et al*. (2000), exhibited the E1a phenotype. In this study, band E1 is the same but a secondary band appeared in position Rm: 1.6. The N1 phenotype for Mdh was found in 57 *M. exigua* populations from Minas Gerais State, Brazil (Oliveira *et al*., 2005) and also in populations from Honduras and Costa Rica (Hernandez *et al*., 2004). In this study, three different phenotypes were observed. Consequently, the isozyme profiles of Mdh detected intraspecific variability among populations of *M. exigua*.

Analysing the two enzymatic profiles (Est and Mdh) together, seven different phenotypes were detected and showed high diversity among *M. exigua* populations. The occurrence of similar variability has been detected only for *M. arenaria*: A3N1, A2N1, A2N3, A1N1 (Cofcewicz *et al*., 2004, 2005).

Randig *et al*. (2002a, 2004) and Carneiro *et al*. (2005) tested the diagnostic potential of SCAR markers for a few *M. exigua* populations collected from Minas Gerais State, Brazil. The present study, which included 14 populations of *M. exigua* originating from different States of Brazil (different races and enzymatic phenotypes) and also two populations from Bolivia and Costa Rica, validated the SCAR-PCR markers for different *M. exigua* populations. For the SCAR markers, a simple visual evaluation of the gels is enough to give an easy and rapid assessment of a great number of samples. The SCAR-PCR technique used in this study proved to be very sensitive and was a reliable tool for routine work in laboratories interested in identification of root-knot nematode populations.

RAPD has been shown to be valuable in discriminating *Meloidogyne* species (Cenis, 1993; Castagnone-Sereno *et al.*, 1994; Handoo *et al*., 2004) and to study intraspecific variability (Blok *et al*., 1997, 2002; Randig *et al*. 2002a; Carneiro *et al*., 2004; Cofcewicz *et al*., 2004, 2005). Our results showed that *M. exigua* populations from coffee and rubber tree displayed high diversity (43.1-57.8% of polymorphic amplified fragments). These results agree with Randig *et al*. (2002a) who observed abundant polymorphism (67.5% of polymorphic amplified fragments) between one *M. exigua* population from coffee and another from rubber tree (the latter being the same one used in the present study). However, both populations clustered together with 100% bootstrap support in the parsimony and the neighbour-joining analyses. On the other hand, studies using light microscopy and scanning electron microscopy showed that the *M. exigua* population from rubber tree presented similarities with the coffee population with regard to female perineal patterns and male head region (Santos, 1997). According to Carneiro *et al*. (2000) the population from rubber tree did not affect coffee or tomato plants in experiments done under glasshouse conditions. This population also presented a new esterase phenotype not previously detected.

Carneiro *et al*. (2004) observed low levels (8.6%) of intraspecific polymorphism in race 1 and race 2 of two *M. exigua* (Est E1) populations from coffee. Conversely, for the 15 populations from this host (Est E1, E2, E3) used in this study, the number of RAPD bands scored as polymorphic ranged from 25.9 to 59.6%. More variability is expected in field populations of *M. exigua* which reproduces by facultative meiotic parthenogenesis (n =

18) rather than mitotic parthenogenesis (Triantaphyllou, 1985).

Meiotic parthenogenesis in *Meloidogyne* is facultative in that a single population can reproduce by cross-fertilisation when males are present or by meiotic parthenogenesis when males are absent (Triantaphyllou, 1985). According to Cook and Evans (1987) sexual reproduction permits recombination between homologous chromosomes and so facilitates some reassortment of genes. This reassortment may activate previously masked genes.

Two populations of *M. exigua* (population 18 included in this study) from Varre Sai and another from Bom Jesus de Itabapoana, both from Rio de Janeiro State, were of particular concern because they can reproduce on coffee cv. Iapar 59 with the Mex-1 resistance gene and on genotype H 419-5-4-5-2 (Paraíso) which probably has the same gene (Barbosa *et al*., 2007; Muniz *et al*., 2007). Enzymatic phenotypes or RAPD markers were not able to differentiate the virulent populations of *M. exigua*. In addition, RAPD markers did not show consistency in the separation of races 1 and 2. This result is in agreement with previous investigations that showed *M. arenaria* and *M. incognita* populations did not cluster according to host race designation (Cenis, 1993; Baum *et al*., 1994). This result suggests that either host races do not form monophyletic groups or that they do not originate from a common ancestor, *i.e*., the notion of races was not sustained by genetic determinism.

This study demonstrated that RAPD-PCR is a powerful methodology for the detection of genetic variability among populations of *M. exigua*. The clustering derived from RAPDs was fully consistent with that obtained from enzymatic and SCAR-PCR analyses. In general, no relationships were found between enzyme profiles, races and genetic polymorphism. The high genetic variability among *M. exigua* revealed in this study is a factor that should be systematically considered in breeding programmes for durable resistance of coffee genotypes in fields infested by root-knot nematodes.

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