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Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) on coffee from Brazil, Central America and Hawaii

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Summary – The present study was based on 18 populations of *Meloidogyne* spp. originating from different coffee fields in Brazil, Central America and the USA (Hawaii). The identification of the main species and an outline of the diversity of root-knot nematodes parasitising coffee in these countries with respect to esterase phenotypes, morphology and molecular polymorphism, are provided. With the present electrophoretic procedure, esterase phenotypes were demonstrated to be species-specific and constitute a good tool for identifying root-knot species from coffee, *viz.*, *M. incognita* (Est I1, I2), *M. paranaensis* (Est P1, P2), *M. arenaria* (Est A2), *M. arabicida* (Est AR2), *M. exigua* (Est E1), *M. mayaguensis* (Est M2) and two unknown populations that probably represent new species (Est SA2, SA4). The perineal pattern is often an unreliable character when used alone for making diagnostic conclusions but, when used as a complementary tool together with enzyme characterisation, is essential for checking the morphological consistency of the identification. Male characters are important for confirming the diagnosis of some species, such as *M. paranaensis*, *M. konaensis* and *M. incognita*. The results showed that the RAPD markers produced are consistent with other approaches (esterase phenotypes and morphological features) for confirming species identification and for estimating genetic relationships among species and isolates. Phylogenetic analyses showed that *M. mayaguensis* and *M. exigua* are more closely related to one another than they are to the other species. This was also true for *M. javanica*, *M. arenaria* and *Meloidogyne* spp. Low levels of intraspecific polymorphism were detected in *M. exigua* (8.6%), *M. incognita* (11.2%) and *M. paranaensis* (20.3%). Conversely, *M. arenaria* and the two unknown *Meloidogyne* spp. exhibited higher levels of intra- or interspecific variability (34.9 and 29.9%, respectively).

Keywords – esterase, morphology, RAPD, root-knot nematodes.

Root-knot nematodes (*Meloidogyne* spp.) are one of the main threats to coffee plantations in almost all producing regions. During the last decade, extensive surveys and studies have shown that a wide range of species are able to parasitise coffee roots and that the situation may differ greatly from one country to another (Campos *et al.*, 1990).

Root-knot nematodes are distributed widely in coffee plantations in Brazil, where they cause great losses both to the coffee farmers and to the economy of the country. Recent surveys in coffee plantations have found *M. exigua* Goeldi, 1892 to be predominant in Minas Gerais State (V.P. Campos, pers. comm.) and an increased incidence of *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996 and *M. incognita* (Kofoid & White, 1919) Chitwood, 1949 in Paraná (Krzyzanowski *et al.*, 2001) and São Paulo States (Lordello *et al.*, 2001). *Meloidogyne coffeicola* Lordello & Zamith, 1960 has been reported in coffee plantations in Paraná and São Paulo States for many years. It is believed that *M. coffeicola* was eradicated from many plantations during the replanting of damaged coffee plantations after the great frost of 1975 (Campos *et al.*, 1990).

In the Kona area on the island of Hawaii (USA), *M. konaensis* Eisenback, Bernard & Schmitt, 1994 causes the death of coffee trees in some commercial plantations. This species has not been found either in any other crop or in the forested area of the island (Eisenback *et al.*, 1994).

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Recent surveys in Central America have clearly diagnosed five species of *Meloidogyne* and six new multienzyme phenotypes were also revealed. *Meloidogyne exigua* was found in Costa Rica, Nicaragua and Honduras and *M. arenaria* (Neal, 1889) Chitwood, 1949 in El Salvador. In Costa Rica, *M. arabicida* López & Salazar, 1989 was found and displayed a new esterase phenotype, M1F1b. In Guatemala, nematodes with the 'F1' esterase phenotype have been found, although the species was not identified. Two isolates from El Salvador presented unknown esterase phenotypes (M1F1a and Sa4). One isolate from northern Guatemala was clearly identified as *Meloidogyne hapla* Chitwood, 1949 and another from the same area was related to the *M. enterolobii* Yang & Eisenback, 1983 or *M. mayaguensis* Rammah & Hirschmann, 1988 phenotypes. Neither of these isolates was able to develop in coffee roots under growing conditions (Hernandez, 1997; Hernandez *et al.*, 2004).

Recently, *M. mayaguensis* was detected in coffee plantations in Cuba where it caused great damage in producing areas (Rodriguez *et al.*, 1995; Hernandez *et al.*, 2001).

Santos and Triantaphyllou (1992) suggested that *Meloidogyne* spp. populations on coffee from Brazil and other countries were frequently misidentified. In Central America, root-knot nematodes are often reported in coffee plantations without any attempt at species identification (Hernandez *et al.*, 2004). The wide range of root-knot species reported from coffee is linked to a large diversity in pathogenicity (Hernandez, 1997; Villain *et al.*, 1999) and therefore accurate characterisation of nematode species and types is essential for developing efficient and sustainable IPM programmes, especially those based on breeding for resistance. To this end, an assessment of the diversity of root-knot nematodes on coffee plants involving biochemical, morphological and molecular tools was undertaken in Brazil, Central America and Hawaii.

The aim of this paper was to identify the *Meloidogyne* species on coffee in Hawaii, Central America and Brazil, and to study the diversity of the major species and populations by using esterase phenotypes, morphological features and DNA analysis (PCR-RAPD).

Materials and methods

Nematode populations

Eighteen *Meloidogyne* isolates were studied (Table 1). Fifteen of these were collected from coffee roots and three from other crops in order to be used as reference species. The populations from Guatemala (isolates 5, 6 and 7) and El Salvador (isolates 16, 17 and 18) were first collected and studied by Hernandez *et al.* (2004) and were sent to us to compare with other coffee populations from Brazil and Hawaii in order to clarify their identity (Table 1). For each population, one isolate was obtained from the progeny of one female. The isolates were maintained on tomato (*Lycopersicon esculentum* var. Santa Cruz) and coffee (*Coffea arabica* var. Mundo Novo) under glasshouse conditions. Males were extracted by placing roots containing egg-masses in water, aerating them with an aquarium pump and periodically collecting the nematodes from the water. Females were hand-picked from infected tomato roots. For egg extraction to be used in PCR-RAPD, the roots were carefully washed, chopped into pieces 2-4 cm long and placed in a blender for 30 s in a 2.0% NaOCl solution. The roots were then rinsed quickly with tap water on a sieve set (32-100-500 mesh), the remaining roots on the 32-mesh sieve being processed again. The root debris and nematode eggs from the 500-mesh sieve were centrifuged at 360 *g* for 5 min in a 15 ml tube. The water supernatant was discarded and a saccharose solution (30%) was added and mixed. The solution was then centrifuged at 360 *g* for 2 min and the supernatant was rinsed for several minutes in a 500-mesh sieve with tap water to remove residual saccharose. The concentrate of nematode eggs from the 500-mesh sieve was centrifuged at 360 *g* for 3-4 min. The water was removed with a micropipette while the nematode eggs formed a pellet at the bottom of the tube. Another centrifugation in 2 ml Eppendorf tubes at 9300 *g* for 3-4 min was needed to eliminate all the water before storage of the nematode eggs at −80°C.

Electrophoresis

Electrophoresis was performed in 7% polyacrylamide gel slabs (11 × 18 cm, 1 mm thick) in a CL18 Permatron apparatus using the technique proposed by Carneiro and Almeida (2001). *Meloidogyne javanica* was used as a reference for esterase phenotypes. Enzyme phenotypes were designated by a letter suggestive of the species it specified and a numeral indicating the number of bands (Esbenshade & Triantaphyllou, 1985, 1990). For the unknown phenotypes, the name of the country together with the number of bands was used.

Table 1. *Identification of different* Meloidogyne *spp. isolates from coffee based on esterase phenotypes, perineal patterns and morphological features of males.*

Isolate/Species (population or race)	Geographical origin	Host plant	Esterase phenotypes	Male features	Perineal pattern	Diagnosis of *Meloidogyne* species
1. *M. paranaensis* (MP5)	Pompéia, SP, Brazil	coffee, tomato	P1	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
2. *M. paranaensis* (MP71)	Apucarana, PR, Brazil	coffee, tomato	P1	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
3. *M. paranaensis* (MP73)	Londrina, PR, Brazil	coffee, tomato	P1	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
4. *M. konaensis*	Hawaii, USA	coffee, tomato	P1	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
5. *Meloidogyne* sp. (MG03)	La Providência, Palin, Guatemala	coffee, tomato	P1	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
6. *Meloidogyne* sp. (MG06)	Panorama, San Marcos, Guatemala	coffee, tomato	P2	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
7. *Meloidogyne* sp. (MG07)	Los Manaques Quezaltenango, Guatemala	coffee, tomato	P2	*M. paranaensis*	*M. paranaensis* *M. konaensis*	*M. paranaensis*
8. *M. incognita* (race 1)	Londrina – PR, Brazil	coffee, tomato	I2	*M. incognita*	*M. incognita*	*M. incognita*
9. *M. incognita* (race 3)	Londrina – PR, Brazil	coffee, tomato	I1	*M. incognita*	*M. incognita*	*M. incognita*
10. *M. arabicida*	Juan Vinas, Cartago, Costa Rica	coffee	AR2	–	*M. arabicida*	*M. arabicida*
11. *M. exigua*	Lavras – MG, Brazil	coffee, tomato	E1	–	*M. exigua*	*M. exigua*
12. *M. exigua*	Quatimi – MG, Brazil	coffee	E1	–	*M. exigua*	*M. exigua*
13. *M. javanica*	Ponta, Porã – MS, Brazil	Soybean, tomato	J3	–	*M. javanica*	*M. javanica*
14. *M. arenaria* (race 1)	Gainesville, Florida, USA	Peanut, tomato	A2	*M. arenaria*	*M. arenaria*	*M. arenaria*
15. *M. mayaguensis*	Petrolina – PE, Brazil	Guava, tomato, coffee	M2	–	*M. mayaguensis*	*M. mayaguensis*
16. *Meloidogyne* sp. (MS01)	Cruz Grande, Izalco, El Salvador	Coffee, tomato	SA2	*Meloidogyne* sp.	*M. incognita.*	*Meloidogyne* sp.
17. *Meloidogyne* sp. (MS02)	Monte Belo, Santiago Maria, El Salvador	Coffee, tomato	A2	*M. arenaria*	*M. arenaria*	*M. arenaria*
18. *Meloidogyne* sp. (MS03)	El Rosario, Izalco El Salvador	Coffee, tomato	SA4	*Meloidogyne* sp.	*M. incognita*	*Meloidogyne* sp.

MORPHOLOGICAL STUDIES

Males were killed in cold 2% formalin and measured immediately under light microscopy (LM). Perineal patterns were cut from live young females in 45% lactic acid and mounted in glycerin (Taylor & Netscher, 1974). At least 30 specimens of each sex were examined.

Table 2. *Measurements (range in μm) of 30 males of* Meloidogyne *spp. isolates from Brazil, Hawaii, Guatemala and El Salvador.*

Characters	*M. paranaensis* Isolate 3* (Est P1)	*M. konaensis* Isolate 4 (Est P1)	*Meloidogyne* sp. Isolate 7 (Est P2)	*Meloidogyne* sp. Isolate 7 (Est P2)	*M. incognita* Isolate 9 (Est I1)	*M. incognita* Isolate 8 (Est I2)	*M. arenaria* Isolate 14 (Est A2)
Stylet length	20.0-27.0	23.0-27.0	23.0-25.0	22.5-25.0	22.0-24.5	22.5-24.5	20.0-28.0
Stylet knob width	4.5-7.0	5.0-6.0	4.5-6.0	4.5-6.5	5.5-7.0	4.5-5.7	4.0-6.0
Stylet knob height	2.0-6.0	2.5-4.0	2.5-5.0	3.0-4.5	3.0-3.5	3.0-3.5	3.0-5.0
DGO	3.5-5.5	5.0-5.5	3.5-4.0	3.5-4.0	1.7-2.5	2.5-4.0	4.0-8.0
Head end to excretory pore	130.0-205.0	141.0-200.0	142.0-185.0	146.0-206.0	124.0-182.0	104.0-182.0	119.0-213.0
Spicule length	22.0-38.0	31.0-36.0	29.0-35.0	29.0-38.0	30.0-35.0	29.5-40.0	27.0-39.0
Body diam. at stylet knob	17.0-22.5	17.5-18.0	17.0-20.5	17.5-20.0	20.0-24.0	19.5-22.5	15.0-22.0

* Population codes are given in Table 1.

RAPD ANALYSES

The total genomic DNA was extracted from 200-300 μl of eggs, using the adapted methodology proposed by Randig *et al.* (2002). The RAPD-PCR reactions were performed in 30 μl volume, containing 6 ng of total genomic DNA, 2.5 U of Taq polymerase (Phoneutria Biotecnologia & Serviços Ltda.), 1× Taq polymerase reaction buffer, 200 μM of each deoxynucleotides triphosphate (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and 80 pM of primer (Operon Technologies, Inc., Alameda, CA, USA). Forty-nine 10-mer oligonucleotide primers were used in the analysis (Table 3). The amplifications were made using the PTC-100 programmable thermal controller (MJ Research, Waltham, MA, USA), and the temperature profile described by Randig *et al.* (2002).

Bands were scored directly from the gels as present or absent. For each isolate, two independent PCR reactions were electrophoresed in the same gel and only DNA fragments consistently present or absent between repeats were recorded and considered as binary characters. Moreover, experiments were repeated at least once. 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). In parsimony analysis, characters were run unordered with no weighting and the heuristic search algorithm was used to find the most parsimonius 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 & Graur, 1991). Distance-based neighbour-joining algorithm (Saitou & Nei, 1987) analysis was used on the same data set, using the mean-character difference option of PAUP* to compute distance. For both analyses, 1000 bootstrap replicates were performed to test the node support of the tree (Felsenstein, 1985) and a consensus dendrogram was computed.

Results

ESTERASE PHENOTYPES

Eighteen bands with an esterase activity and 12 phenotypes were detected among the 18 populations of *Meloidogyne* spp. from coffee and other crops (Table 1; Fig. 1). A distinct phenotype was associated with every population of the major and minor species. Phenotype J3 (Rm: 1.0, 1.24, 1.36) was characteristic of *M. javanica* from soybean (reference). Phenotype I1 (Rm: 1.0) and I2 (Rm: 1.05, 1.1) were observed in two isolates of *M. incognita* from coffee, Brazil. The P1 phenotype (Rm: 1.32) was displayed by four isolates that were sampled in Brazil, Hawaii (USA) and in Guatemala. This phenotype is linked to *M. paranaensis*. A new phenotype, P2 (Rm: 0.9, 1.32), was detected in two populations from Guatemala (Fig. 2). The identification of these isolates will be clarified using morphological and DNA approaches. The A2 phenotype (Rm: 1.20, 1.30), corresponding to *M. arenaria*, was observed in two isolates: one sampled on peanut (reference) from Florida, USA, and the other from the southern region of El Salvador. The unknown phenotypes SA2 (Rm: 1.24, 1.30) and SA4 (Rm: 0.86, 0.96, 1.24, 1.30) were observed in two isolates MS01 and MS03, respectively, from El Salvador, sampled on different farms in the same area. Another phenotype AR2 (Rm: 1.20, 1.40) was associated with *M. arabicida* extracted from coffee plants

Table 2. *(Continued).*

M. arenaria Isolate 17 (Est A2)	*Meloidogyne* sp. Isolate 16 (Est SA2)	*Meloidogyne* sp. Isolate 18 (Est SA4)
20.0-22.0	23.5-25.0	25.0-26.0
4.0-5.0	5.0-5.5	5.0-6.0
3.0-3.5	3.5-4.0	3.5-4.0
5.0-7.0	5.0-6.0	4.5-5.0
141.0-192.0	172.0-230.0	145.0-193.0
27.0-31.0	30.0-36.0	28.0-35.0
16.0-20.0	22.0-23.5	19.0-21.0

showing 'corchosis' die-back symptoms from a farm in Costa Rica. The phenotype E1 (Rm: 1.5) is related to two populations of *M. exigua* from coffee from Brazil. The phenotype M2 with two major bands (Rm: 0.68; 0.96) and two minor bands (Rm: 0.79, 1.08) was isolated from one population of *M. mayaguensis* from guava from Petrolina, Brazil. This isolate was used as reference of this species, which also occurs in coffee in Cuba and parasitises coffee cv. Mundo Novo in glasshouse conditions in Brazil (data not shown).

MORPHOLOGY AND MORPHOMETRICS

Perineal patterns

Although the perineal patterns were quite variable, detailed observation of 30 females from each isolate allowed us to compare them with the descriptions of the species identified by esterase phenotypes. The *M. incognita* type was observed in four isolates from coffee: *M. incognita* (Est I1, I2) and in two unknown esterase populations (SA2, SA4). The *M. arenaria* type was observed in two isolates, one from peanut (reference) and the other from coffee from El Salvador (Est A2). The *M. paranaensis* type, which was similar to *M. incognita* and generally had smooth striae, was present in the populations on coffee from Brazil, Guatemala and Hawaii (Est P1 and P2). The populations from Guatemala were studied first by Hernandez *et al.* (2004). The perineal pattern of *M. konaensis*, which is similar to the *M. incognita* and *M. arenaria* types (Eisenback *et al.*, 1994), was observed in a few specimens in isolates from Brazil, Hawaii and Guatemala. *Meloidogyne mayaguensis* types (Est M2) frequently showed atypical features, varying from *M. incognita* to *M. arenaria* and *M. javanica*, as reported by Fargette and Braaksma (1990) and by Carneiro *et al.* (2001). The *M. arabicida* type (Est AR2) was associated with an isolate sent from Costa Rica. The patterns were quite variable between females and these differences reflected those reported by Lopez and Salazar (1989) in their original description of *M. arabicida*. The *M. exigua* type was observed in two isolates from Brazil which presented the same Est phenotype E1 first characterised by Carneiro *et al.* (2000).

Males

The males were studied only for those species which were difficult to identify using esterase phenotypes and perineal patterns. Only useful, stable, morphometric data with a CV below 10% (Rammah & Hirschmann, 1988) were used to evaluate our isolates. The measurements of males were in the range established for *M. paranaensis* (Table 2), including the population sent to us as *M. konaensis*. The males of these populations differ from males of *M. konaensis* especially in stylet length (20-27 *vs* 20-24 μm), stylet knob width (4.5-7.0 *vs* 3.4-5.0 μm) and DGO (3.5-5.5 *vs* 5.9-8.4 μm). The male stylets of the isolates with esterase phenotypes P1 or P2 were typical of *M. paranaensis* (Carneiro *et al.*, 1996), the knobs being transversely elongated, broad and set off from the shaft, with sometimes one or two projections surrounding the shaft, whereas *M. konaensis* (Eisenback *et al.*, 1994) has knobs that are not set off, are backward sloping and have six to 12 large projections surrounding the shaft. This character represents the most useful criterion for differentiating *M. konaensis* from *M. paranaensis* (Carneiro *et al.*, 1996).

The head shape of *M. incognita* (Est I1 and I2) males is very characteristic and not easily confused with any other species. The labial disc is large and rounded, centrally concave and raised above the medial lips. The medial lips are as wide as the head region which is generally marked by two or three incomplete annulations. The stylets have the same characteristic morphology described by Eisenback and Triantaphyllou (1991). The measurements were in the range established by Chitwood (1949) for *M. incognita* (*incognita* and *acrita*).

The males of the other atypical isolates (Est SA2, SA4) had features similar to *M. javanica* and *M. arenaria* and measurements within the range of *M. arenaria*.

DNA ANALYSIS

The genetic variability of the 18 *Meloidogyne* spp. populations were analysed using the RAPD technique. Fifteen populations were analysed from coffee crops, including two unidentified isolates, and 13 which had al-

Table 3. *RAPD primers used and number of amplified fragments scored.*

Primer	Sequence	Number of amplified fragments			
		Min./isolate	Max./isolate	Polymorphic	Informative
A01	CAG GCC CTT C	5	11	23	18
A04	AAT CGG GCT G	3	9	18	15
A09	GGG TAA CGC C	2	9	27	22
A12	TCG GCG ATA G	1	5	14	5
B01	GTT TCG CTC C	1	4	13	9
B06	TGC TCT GCC C	2	5	20	15
B07	GTT GAC GCA G	1	7	22	18
B11	GTA TAC CCG T	2	5	19	10
B12	CCT TGA CGC A	1	4	12	8
B15	GGA GGG TGT T	3	9	28	22
B17	AGG GAA CGA G	1	5	11	8
C02	GTG AGG CGT C	2	6	17	12
C09	CTC ACC GTC C	4	6	15	12
C16	CAC ACT CCA G	1	5	13	9
D05	TGA GCG GAC A	2	6	15	12
D13	GGG GTG ACG A	3	10	22	18
D15	CAT CCG TGC T	3	7	24	18
E07	AGA TGC AGC C	2	6	13	11
F06	GGG AAT TCG G	2	6	11	10
G02	GGC ACT GAG G	2	7	18	15
G03	GAG CCC TCC A	4	10	20	17
G04	AGC GTG TCT G	1	4	13	9
J10	AAG CCC GAG G	2	9	24	20
J20	AAG CGG CCT C	2	4	10	8
K01	CAT TCG AGC C	3	6	17	13
K04	CCG CCC AAA C	4	7	16	13
K06	CAC CTT TCC C	3	6	13	10
K07	AGC GAG CAA G	1	7	13	11
K09	CCC TAC CGA C	2	4	11	9
K10	CAC CTT TCC C	1	6	13	11
K14	CCC GCT ACA C	3	5	16	13
K16	GAG CGT CGA A	2	4	7	4
K19	CAC AGG CGG A	1	4	11	7
K20	GTG TCG CGA G	1	6	12	10
M10	TCT GGC GCA C	3	9	27	18
M20	AGG TCT TGG G	3	7	21	14
N07	CAG CCC AGA G	1	5	11	10
P01	GTA GCA CTC C	2	8	21	16
P02	TCG GCA CGC A	1	5	10	9
P05	CCC CGG TAA C	2	9	20	17
R03	ACA CAG AGG G	1	7	24	18
R04	CCC GTA GCA C	2	6	16	13
R05	GAC CTA GTG G	2	9	23	16
R07	ACT GGC CTG A	1	6	16	11
R08	CCC GTT GCC T	1	6	17	15
AB02	GGA AAC CCC T	1	7	21	19
AB04	GGC ACG GGT T	1	5	14	11
AB05	CCC GAA GCG A	1	4	16	11
AB06	GTG GCT TGG A	3	9	23	19
Total				**831**	**639**

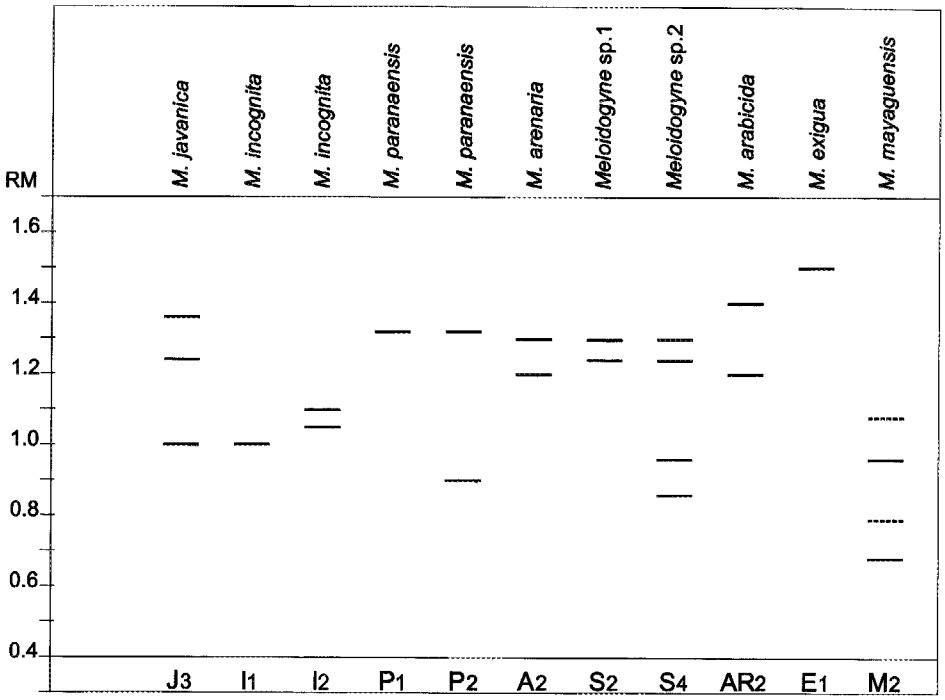

Fig. 1. *Esterase phenotypes observed in different species of* Meloidogyne *from coffee in Brazil, Central America and Hawaii.*

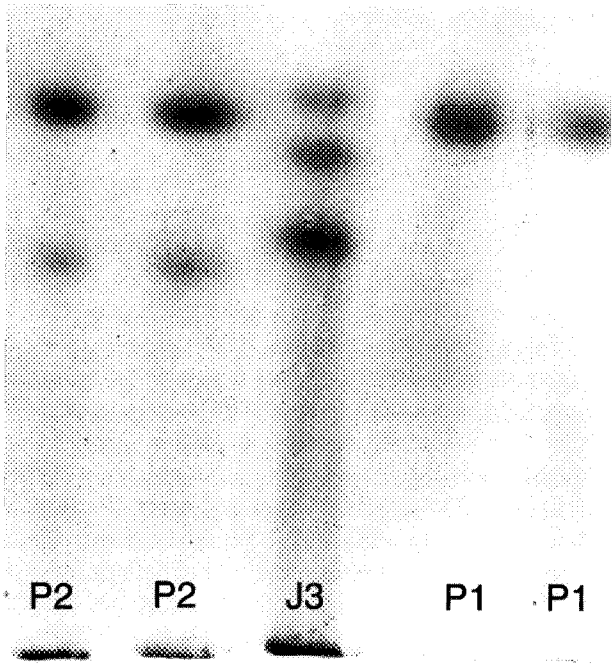

Fig. 2. *Gel slab showing relative positions of new esterase phenotype of* Meloidogyne paranaensis *from Brazil (P1) and Guatemala (P2), compared to reference phenotype J3 of* M. javanica.

ready identified through esterase phenotypes and morphological characters. Populations of *M. mayaguensis*, *M. arenaria* and *M. javanica* obtained from other plants were used as a reference in this analysis. An example of the RAPD profiles obtained for the 18 *Meloidogyne* spp. populations analysed is shown in Figure 3. With the 49 random primers used the number of reproducible amplified fragments varied from seven to 28 per isolate, their size ranging from *ca* 200 to 3000 bp. The global results of the RAPD analysis are provided in Table 3. Over the whole experiment, each primer produced from one to 11 polymorphic bands, among which four to 22 appeared phylogenetically informative. Globally, 831 fragments were amplified and scored as RAPD markers. Three single fragment was found to be amplified in the 18 isolates tested (*i.e.*, monomorphic), and 639 were informative. Using RAPD patterns alone or in combination, all the root-knot nematode isolates studied could be unambiguously identified (Fig. 4).

For the five species for which more than one isolate was available (*i.e.*, *M. arenaria*, *M. exigua*, *M. incognita*, *M. paranaensis* and *Meloidogyne* sp.), 151-278 reproducible fragments were amplified (Table 4). For *M. arenaria* and *Meloidogyne* sp. the proportion of amplified bands that were polymorphic was 34.9% and 29.9%, respectively.

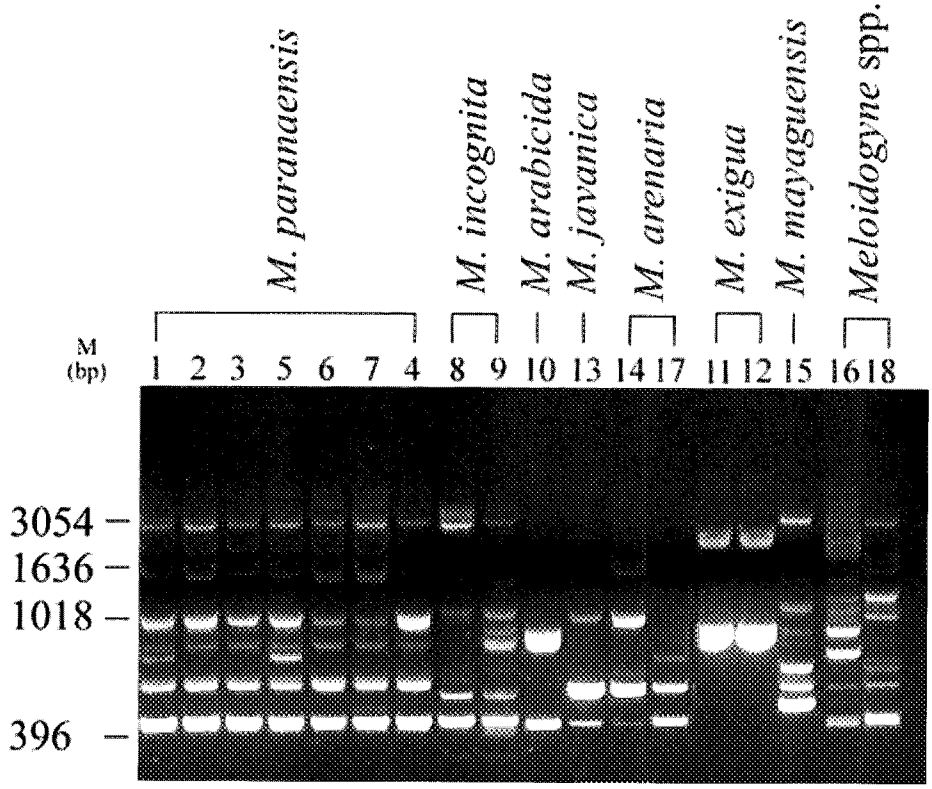

Fig. 3. *RAPD patterns for* Meloidogyne *spp. isolates generated with primer OPA-4.*

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

Species	RAPD fragments		
	Amplified	Polymorphic (%)	Informative
M. arenaria	278	97 (34.9)	97
M. exigua	151	13 (8.6)	13
M. incognita	224	25 (11.2)	25
M. paranaensis (Est P1, P2)	271	55 (20.3)	45
Isolates 1, 2, 3, 4, 5 (Est P1)	254	25 (9.8)	11
Isolates 6 and 7 (Est P2)	248	12 (4.8)	12
Meloidogyne spp.	261	78 (29.9)	78

Conversely, for *M. exigua*, *M. incognita* and *M. paranaensis*, fewer RAPD bands scored as polymorphic (8.6, 11.3 and 20.3%, respectively).

All the scorable amplified bands were recorded to build a 0-1 matrix, on which phylogenetic analyses were performed. Parsimony and neighbour-joining algorithms were run on the 0-1 matrix, and bootstrap analysis with 1000 replicates was conducted in both cases, resulting in two comparative majority-rule consensus dendrograms (Fig. 4).

The dendrograms were basically similar in their topology, isolates belonging to a given species always clustering together with very high bootstrap support in the analyses (Fig. 4). Firstly, all isolates of *M. paranaensis* clustered together with 100% bootstrap support in the analyses and were separated from all the other species with 20.3% of polymorphism (Table 4). A low intraspecific variability was observed between the isolates with Est P2 (6 and 7), 4.8% polymorphic fragments. The isolate sent as *M. konaensis* (Est P1) from Hawaii, USA, which did not show the typical esterase profile K3 (Carneiro *et al.*, 2000) and morphological characters of this species (Table 2), showed high homogeneity (9.8% of polymorphic fragments) and clustered together with the Brazilian populations of *M. paranaensis* (Fig. 4). Secondly, *M. mayaguensis* and *M. exigua* clustered together and were well separated from the other species with 100% bootstrap support in both analyses. Thirdly, *M. arenaria* and *M. ja-*

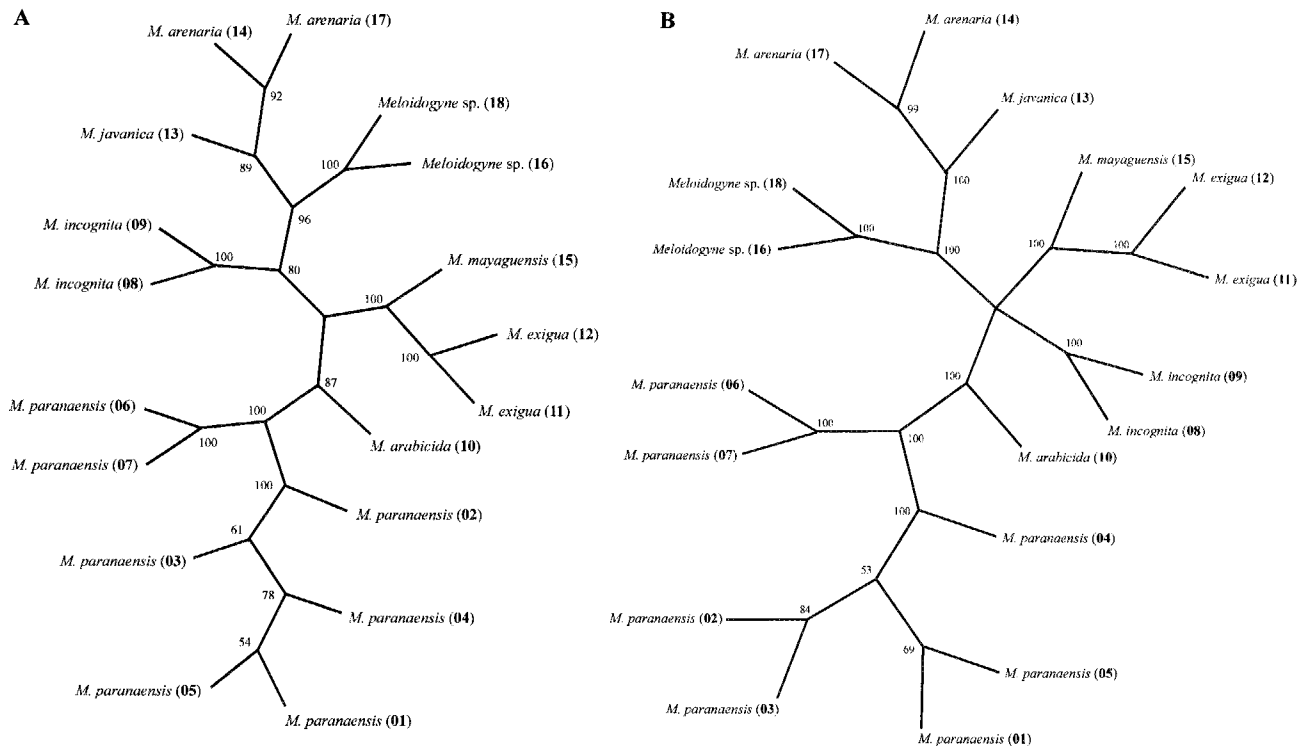

Fig. 4. *Majority-rule consensus dendrograms of relationships of* Meloidogyne *spp. isolates. A: Parsimony-based tree; B: Neighbour-joining tree. Bootstrap percentages based on 1000 replicates are given on each node. Coffee populations codes are given in Table 1.*

vanica appeared closer to one another than to the unidentified species (isolates 16 and 18) which also clustered together with 100% bootstrap support in the two analyses.

Discussion

The present study included populations of *Meloidogyne* spp. originating from coffee fields in Brazil, Central America and Hawaii and, as such, provides species identification and an outline of the diversity of root-knot nematode isolates parasitising coffee with respect to enzyme phenotypes, morphology and molecular polymorphism.

With the electrophoretic procedure used herein, esterase phenotypes are species-specific and are a good tool for identifying root-knot nematodes species from coffee, *i.e.*, *M. incognita* (Est I1, I2), *M. paranaensis* (Est P1, P2), *M. arenaria* (Est A2), *M. arabicida* (Est AR2), *M. exigua* (Est E1) and *M. mayaguensis* (Est M2). These phenotypes were previously reported by Carneiro *et al.* (2000) and Hernandez *et al.* (2004). A new Est-phenotype, P2 (isolates 6, 7), was detected in two populations of *M. paranaensis* from Guatemala which presented the same Est-band F1 observed by Hernandez *et al.* (2004) and a supplementary band with Rm: 0.9. The intraspecific variability among the isolates of *M. paranaensis* (isolate MGUO7) was first detected using other isozymes (MDH, SOD and GOT) by Hernandez *et al.* (2004). The two isolates from El Salvador (16, 18), collected in the same geographical area, showed unknown esterase phenotypes (SA2, SA4), which were first detected by Hernandez *et al.* (2004). As the perineal patterns of these populations are typical of *M. incognita* and the males showed features of *M. javanica* and *M. arenaria* with measurements in the range of *M. arenaria*, detailed morphological and morphometrical study using SEM and other techniques will be necessary to clarify the identity of these isolates. They probably represent new species.

Although some intraspecific variability was observed in the different populations of *M. incognita* and *M. paranaensis* using esterase phenotype analysis, it was observed that intraspecific variation in the enzymatic level was usually low. This observation is supported by the fact that enzymes are produced *via* the expression of genes which are often highly conserved between closely related

taxa and which represent only a minor fraction of the total genome, whereas non-encoding regions are more abundant and subjected to extensive evolutionary changes due to the absence of, or low, selection pressure (McLain *et al.*, 1987).

The perineal pattern character represents a difficult criterion when used alone for making diagnostic conclusions on populations from coffee since it involves subjective observation of morphological patterns and their comparison to figures included in species descriptions. Frequently, different species such as *M. paranaensis* and *M. konaensis* have perineal patterns similar to *M. incognita* or *M. arenaria*. *Meloidogyne mayaguensis*-type patterns have been frequently characterised as varying from *M. incognita* to *M. arenaria* and *M. javanica* (Fargette & Braaksma, 1990; Carneiro *et al.*, 2001). Populations of other species, such as *M. exigua*, appear to be very similar and present typical perineal patterns (Lima & Ferraz, 1985). Using only this approach it is impossible to accurately identify *Meloidogyne* spp. in coffee plantations. For example, *M. paranaensis* was misidentified as *M. incognita* for 22 years in Brazil (Carneiro *et al.*, 1996), and also for many years in Guatemala (Anzueto *et al.*, 1991; Hernandez, 1997). Conversely, the perineal pattern can be used as a complementary tool to enzyme characterisation and is important for checking the morphological consistency of the identification. Comparing the perineal patterns of the two isolates of *M. incognita* (Est I1 and I2) it is possible to see clearly the differences observed by Chitwood (1949) for *M. incognita* var. *acrita* and *M. incognita*, respectively.

Features of the male are essential for assisting in the diagnosis of some species, such as *M. paranaensis*, *M. konaensis* and *M. incognita*. Using this approach it is possible to confirm that the populations from Guatemala (MGU03, MGU06 and MGU07) are *M. paranaensis*. The population from Hawaii, which was sent as *M. konaensis*, did not have males similar to those described by Eisenback *et al.* (1994). Instead, the males presented the morphological and morphometrical characters of *M. paranaensis*. The esterase phenotype P1 is the same as that characterised in *M. paranaensis*, not the Est K3 that was first detected in *M. konaensis* (Carneiro *et al.*, 2000). The DNA analysis confirms that the population from Hawaii is very close to *M. paranaensis* isolates (polymorphism of 9.8%). Recently, a survey of coffee fields in Hawaii showed at least four esterase phenotypes, thereby indicating the presence of a mixture of species on the island (B.S. Sipes, pers. comm.).

The results obtained with RAPD markers were consistent with other approaches for confirming species identification and estimating genetic relationship among isolates. Analysing the dendrograms deduced from the RAPD data we can characterise different clusters of species with high bootstrap support in the analysis: *i*) *M. paranaensis* and *M. arabicida*; *ii*) *M. exigua* and *M. mayaguensis*; *iii*) *M. arenaria*, *M. javanica* and two *Meloidogyne* spp.; *iv*) *M. incognita*. We can see different intraspecific groups with a low degree of polymorphism, such as *M. paranaensis* (polymorphism of 20.3%) from Brazil, Central America and Hawaii which is quite separated from *M. incognita* (Est I1 and I2) (polymorphism of 11.2%). Similar results were observed by Randig *et al.* (2002) within *M. incognita* isolates Est I1 and Est I2 which clustered separately and had a relatively low degree of polymorphism. These observations confirm the existence of two types of *M. incognita* in Brazil. The same observation can be inferred for *M. paranaensis* with the presence of two groups (P1 and P2) which presented separate topological positions in the dendrogram. The phenotype P2 was never detected in Brazil, suggesting a genetic divergence of *M. paranaensis* in Guatemala. These results confirm the detection of *M. paranaensis* in Guatemala and Hawaii and the genetic proximity of these populations to Brazilian isolates, and also clarify the results obtained by Hernandez *et al.* (2004).

The two isolates of *M. exigua* sampled in different coffee farms of Minas Gerais State, Brazil, showed only 8.6% genetic diversity. These populations can be separated by their ability to parasitise coffee and tomato (isolate 11) or only coffee (isolate 12). Randig *et al.* (2002), studying two populations of *M. exigua* from coffee and the rubber tree (*Hevea brasiliensis*) detected a high polymorphism of 67.5%. The population from rubber trees did not parasitise coffee or tomato in studies made in glasshouse conditions (data not shown).

In the dendrograms, *M. javanica* and *M. arenaria* are seen to be the most closely related species and are close to *M. incognita* in a parsimony-based tree. Similar results were observed by Guirao *et al.* (1995), Blok *et al.* (1997) and Randig *et al.* (2002). At the intraspecific level, a high degree of polymorphism (34.9%) was detected within *M. arenaria* isolates compared to the other species analysed. This highest level of genetic variability in *M. arenaria* was not unexpected, since that result is in good agreement with previous molecular analysis (Castagnone-Sereno *et al.*, 1994; Semblat *et al.*, 1998; Randig *et al.*, 2002).

The two isolates from El Salvador which presented unknown esterase phenotypes (SA2 and SA4), and which were collected in the same geographical area, clustered together with 100% bootstrap support and presented about 30% of polymorphic fragments. They are related to *M. arenaria* and *M. javanica* in the dendrograms. Recently, surveys in El Salvador showed that these two populations always appeared together with the predominant phenotype Est SA4 occurring in more them 90% of the samples (A. Hernandez, pers. comm.), suggesting a recent genetic divergence of these two isolates.

The results presented in this paper demonstrate that it is possible to identify the species of *Meloidogyne* on coffee and to detect intraspecific variability using esterase phenotypes, morphological features and molecular approaches. This PCR-RAPD technique should be a useful tool both for diagnosis and for resolving questions of genetic variation, thus helping to accurately identify species and populations, an essential prerequisite to designing IPM strategies utilising nematode resistance and crop rotation and adapted to local pathogenic situations.

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References

ANZUETO, F., ESKES, A.B., SARAH, J.L. & DECAZY, B. (1991). Recherche de la résistance à *Meloidogyne* sp. dans une collection de *Coffea arabica*. *Proceedings of the 14th International Scientific Colloquium on Coffee (ASIC), 14-19 July 1991, San Francisco, USA*. pp. 534-543. [Abstr.]

BLOK, V.C., PHILLIPS, M.S., MCNICOL, J.W. & FARGETTE, M. (1997). Genetic variation in tropical *Meloidogyne* spp. as shown by RAPDs. *Fundamental and Applied Nematology* 20, 127-133.

CAMPOS, V.P., SIVAPALAN, P. & GNANAPRAGASAM, N.C. (1990). Nematode parasites of coffee, cocoa and tea. In: Luc, M., Sikora, R.A. & Bridge, J. (Eds). *Plant parasitic nematodes in subtropical and tropical agriculture*. Wallingford, UK, CAB International, pp. 387-430.

CARNEIRO, R.M.D.G. & ALMEIDA, M.R.A. (2001). Técnica de eletroforese usada no estudo de enzimas dos nematóides de galhas para identificação de espécies. *Nematologia Brasileira* 25, 35-44.

CARNEIRO, R.M.D.G., CARNEIRO, R.G., ABRANTES, I.M.O., SANTOS, M.S.N.A. & ALMEIDA, M.R.A. (1996). *Meloidogyne paranaensis* n. sp. (Nematoda: Meloidogynidae) a root-knot nematode parasitizing coffee in Brazil. *Journal of Nematology* 28, 177-189.

CARNEIRO, R.M.D.G., ALMEIDA, M.R.A. & QUÉNÉHERVÉ, P. (2000). Enzyme phenotypes of *Meloidogyne* spp. populations. *Nematology* 2, 645-654.

CARNEIRO, R.M.D.G., MOREIRA, W.A., ALMEIDA, M.R.A. & GOMES, A.C.M.M. (2001). Primeiro registro de *Meloidogyne mayaguensis* em goiabeira no Brasil. *Nematologia Brasileira* 25, 223-228.

CASTAGNONE-SERENO, P., VANLERBERGHE-MASUTTI, F. & LEROY, F. (1994). Genetic polymorphism between and within *Meloidogyne* species detected with RAPD markers. *Genome* 37, 904-909.

CHITWOOD, B.G. (1949). Root-knot nematodes – Part I. A revision of the genus *Meloidogyne* Goeldi, 1887. *Proceedings of the Helminthological Society of Washington* 16, 90-104.

EISENBACK, J.D. & TRIANTAPHYLLOU, H.H. (1991). Root-knot nematodes *Meloidogyne* sp. and races. In: Nickle, W.R. (Ed.). *Manual of agricultural nematology*. NY, USA, Marcel Dekker, pp. 191-274.

EISENBACK, J.D., BERNARD, E.C. & SCHMITT, D.P. (1994). Description of the kona coffee root-knot nematode, *Meloidogyne konaensis* n. sp. *Journal of Nematology* 26, 363-374.

ESBENSHADE, P.R. & TRIANTAPHYLLOU, A.C. (1985). Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* 17, 6-20.

ESBENSHADE, P.R. & TRIANTAPHYLLOU, A.C. (1990). Isoenzyme phenotypes for the identification of *Meloidogyne* species. *Journal of Nematology* 22, 10-15.

FARGETTE, M. & BRAAKSMA, R. (1990). Use of the esterase phenotypes in the taxonomy of the genus *Meloidogyne*. 3. A study of some 'B' race lines and their taxonomic position. *Revue de Nématologie* 13, 375-386.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791.

GOELDI, E.A. (1892). Relatório sobre a moléstia do cafeeiro na Província do Rio de Janeiro. *Archivos do Museu Nacional do Rio de Janeiro* 8, 7-123.

GUIRAO, P., MOYA, A. & CENIS, J.L. (1995). Optimal use of random amplified polymorphic DNA in estimating the genetic relationship of four major *Meloidogyne* spp. *Phytopathology* 85, 547-551.

HERNANDEZ, A. (1997). *Étude de la variabilité intra et interspécifique des nématodes du genre* Meloidogyne *parasites des caféiers en Amérique Centrale*. Thèse de Doctorat, Académie de Montpellier II Science et Techniques du Languedoc, Montpellier, France, 98 pp.

HERNÁNDEZ, M.G.R., SANCHEZ, L., AROCHA, Y., PETEIRA, B., SOLÓRZANO, E. & ROWE, J. (2001). Identification and characterization of *Meloidogyne mayaguensis* from Cuba. *33rd Annual Meeting of Organization of Nematologists*

of Tropical America, Cuba, Varadero, 11-15 June 2001, pp. 6-7. [Abstr.]

HERNANDEZ, A., FARGETTE, M. & SARAH, J.L. (2004). Characterisation of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) from coffee plantations in Central America and Brazil. *Nematology* 6, 193-204.

KRZYZANOWSKI, A.A., FIGUEIREDO, R., SANTIAGO, D.C. & FAVORETO, L. (2001). Levantamento de espécies e raças de *Meloidogyne* em cafeeiros no estado do Paraná. In: da Rocha, A.N., Rufino, J.L.S. & Giusti, W.M. (Eds), *Segundo Sinpósio de Pesquisas dos Cafés do Brasil, 24-27 September 2000, Vitória, Brazil*. Brasília DF, Brazil, Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), pp. 1175-1181.

LI, W. & GRAUR, D. (1991). *Fundamentals of molecular evolution*. Sunderland, MA, Sinauer Associates, 284 pp.

DE LIMA, R.D'A. & FERAZ, S. (1985). Análise comparativa das variações morfométricas entre diferentes populações de *Meloidogyne exigua* (Nematoda: Meloidogynidae). *Revista Ceres* 32, 362-372.

LOPEZ, R. & SALAZAR, L. (1989). *Meloidogyne arabicida* sp. n. (Nemata: Heteroderidae) nativo de Costa Rica: un nuevo y severo patógeno del cafeto. *Turrialba* 39, 313-323.

LORDELLO, L.G.E. & ZAMITH, A.P.L. (1960). *Meloidogyne coffeicola* sp. n., a pest of coffee trees in the state of Paraná, Brazil (Nematoda, Heteroderidae). *Revista Brasileira de Biologia* 20, 375-379.

LORDELLO, A.I.L., LORDELLO, R.R.A. & AZUOLLI, L.C. (2001). Levantamento de espécies de *Meloidogyne* em cafeeiros no estado de São Paulo. In: da Rocha, A.N., Rufino, J.L.S. & Giusti, W.M. (Eds). *Segundo Simpósio de Pesquisas dos Cafés do Brasil, 24-27 September 2000, Vitória, Brazil*. Brasília DF, Brazil, Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), pp. 1182-1187.

MCCLAIN, D.K., RAI, K.S. & FRASER, J.M. (1987). Intraspecific and interspecific variation in the sequence and abundance of highly repeated DNA among mosquitoes of *Aedes albopictus* subgroup. *Heredity* 58, 373-381.

RAMMAH, A. & HIRSCHMANN, H. (1988). *Meloidogyne mayaguensis* n. sp. (Meloidogynidae), a root-knot nematode from Puerto Rico. *Journal of Nematology* 20, 58-69.

RANDIG, O., BONGIOVANNI, M., CARNEIRO, R.M.D.G. & 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, 862-870.

RODRIGUEZ, M.G., RODRIGUEZ, I. & SANCHEZ, L. (1995). Especies del genero *Meloidogyne* que parasitan el cafeto en Cuba. Distribución geográfica y sintomatologia. *Revista de Protección Vegetal* 10, 123-128.

SAITOU, N. & NEI, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

SANTOS, J.M. & TRIANTAPHYLLOU, H.H. (1992). Determinação dos fenótipos isoenzimáticos e estudos comparativos da morfologia de 88 populações de *Meloidogyne* spp., parasitas do cafeeiro. *Nematologia Brasileira* 16, 88. [Abstr.]

SEMBLAT, J.P., WAJNBERG, E., DALMASSO, A., ABAD, P. & CASTAGNONE-SERENO, P. (1998). High-resolution DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis. *Molecular Ecology* 7, 119-125.

SWOFFORD, D.L. (1998). *PAUP*. Phylogenetic analyses using parsimony (* and other methods). Version 4.* Sunderland, MA, Sinauer Associates.

TAYLOR, D.P. & NETSCHER, C. (1974). An improved technique for preparing perineal patterns of *Meloidogyne* spp. *Nematologica* 20, 268-269.

VILLAIN, B., ANZUETO, F., HERNANDEZ, A. & SARAH, J.L. (1999). Los nematodos parásitos del cafeto. In: Bertrand, B. & Rapidel, B. (Eds). *Desafíos de la caficultura en Centroamérica*. San Jose, Costa Rica, ICCA-PROMECAFE, pp. 327-368.