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Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences

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Summary – The 18S rDNA of 19 populations of *Meloidogyne* spp. was amplified and directly sequenced. The region of mitochondrial DNA, located in the 3′ portion of the gene that codes for cytochrome oxidase subunit II (*COII*) through a portion of the 16S rRNA (*lRNA*) gene, from 16 of these populations was cloned and sequenced. Heteroplasmic sequences were identified from both rDNA and mtDNA regions for several taxa. Several sequences sampled from nominal taxa differed from previously published accounts. Phylogenetic trees based on alignments of these sequences were constructed using distance, parsimony and likelihood optimality criteria. For 18S rDNA data, three main clades were identified. One well supported clade (86-91% bootstrap) included the most common and widely disseminated species, *e.g*., *M. arenaria*, *M. javanica* and *M. incognita*, some recently described or redescribed species (*M. floridensis*, *M. paranaensis*, and *M. ethiopica*) plus numerous unidentified isolates. All mitotic parthenogenetic species, except for *M. oryzae*, were included in this clade. Other, less well supported clades included the amphimictic and facultative meiotic species *M. hapla*, *M. microtyla*, *M. maritima* and *M. duytsi*. One such clade comprised three meiotic parthenogens (*M. exigua*, *M. graminicola* and *M. chitwoodi*) and *M. oryzae*. This clade was moderately supported (77% bootstrap) but the relationships within this clade were poor. For mitochondrial DNA data, only the species in clade I from rDNA analysis, and *M. hapla* were analysed. These species formed a well supported clade (100% bootstrap) to the exclusion of *M. mayaguensis* and *M. hapla*. The addition of taxa and mtDNA data to publicly available records improved the discrimination sensitivity of species and atypical, non-identified, isolates.

Keywords – molecular biology, molecular diagnosis, root-knot nematodes.

Root-knot nematodes (RKN) of the genus *Meloidogyne* Goeldi, 1892 comprise more than 80 species. Many of them are important pathogens of numerous agricultural crops grown in tropical, subtropical and moderate temperate regions. The most common species, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *M. incognita* (Kofoid & White, 1919) Chitwood, 1949 and *M. javanica* (Treub, 1885) Chitwood, 1949 are extremely polyphagous, with up to 3000 plant species listed as hosts (Trudgill & Blok, 2001). There is great diversity present among these species, not only regarding their host range, but also relative to their cytogenetics (varying levels of aneuploidy and polyploidy), and their mode of reproduction (varying from obligatory amphimixis to mitotic parthenogenesis) (Triantaphyllou, 1985). Identification of species is based primarily on morphological features of females, males and second-stage juveniles (Eisenback & Triantaphyllou, 1991), as esterase and malate dehydrogenase isozyme profiles derived from single females by PAGE (Esbenshade & Triantaphyllou, 1985; Carneiro *et al*., 2000). However, precise and reliable identification based on morphological characters is a difficult task even for qualified taxonomists with expertise in the genus. Enzymatic profiles have been designated for only about 26 species, and since these can only be determined for females, the most common developmental stage, second-stage juveniles, cannot be identified by this method (Carneiro *et al.*, 2000).

The Polymerase Chain Reaction (PCR) amplification and digestion of amplified product with restriction endonucleases of the region located in the 3′ portion of the mitochondrial gene that codes for cytochrome oxi-

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dase subunit II (*COII*) through a portion of the 16S rRNA (*lRNA*) gene allowed discrimination of five major *Meloidogyne* spp. (Powers & Harris, 1993). Subsequently, the sequence of this region was used to confirm the identification of *M. mayaguensis* Rammah & Hirschmann, 1988 populations (Blok *et al.*, 2002; Brito *et al.*, 2004).

Much previous phylogenetic inference in *Meloidogyne* has been carried out. Based on cytogenetic information, Triantaphyllou (1985) made the assumption that mitotic parthenogens evolved from meiotic parthenogenetic ancestors, following suppression of meiotic process and establishing various levels of ploidy. Other phylogenetic studies, which were based on protein and DNA analysis, showed a strong relationship among ameiotic species. (Dickson *et al.*, 1971; Dalmasso & Bergé, 1978; Esbenshade & Triantaphyllou, 1987; Castagnone-Sereno *et al.*, 1993; Baum *et al*., 1994; Van der Beek *et al.*, 1998). Recently, a phylogeny was predicated on small subunit 18S rDNA sequences of 12 species of *Meloidogyne* (De Ley *et al*., 2002) and for five species using over 47 genes (Scholl *et al*., 2005). The study of De Ley *et al.* (2002) supported close relationships between the three major ameiotic species, *M. arenaria*, *M. javanica* and *M. incognita*. Again, these mitotic parthenogens were clearly different from either the meiotic or obligatory amphimictic species. The major contribution of the Scholl and Bird (2005) analysis was the elucidation of relationships among three mitotic parthenogens that had heretofore been poorly documented.

In the present study, 19 populations of *Meloidogyne* were analysed based on 18S rDNA sequences, and 16 of these were also analysed based on mitochondrial DNA sequences. Within these populations, five were unidentified and some were from different geographical regions than previously reported. For example, *M. mayaguensis* was detected for the first time in Brazil and the USA (Carneiro *et al*., 2001; Brito *et al*., 2004), and *M. ethiopica* Whitehead, 1968 was found for the first time in Brazil and Chile (Carneiro *et al*., 2003, 2004a). *Meloidogyne floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar & Higgins, 2004 is a recently described species from Florida, USA. The unidentified population *Meloidogyne* sp. (isolate 38) was detected in Brazil from yakon plants (*Polymnia sonchifolia*) and presented an atypical esterase phenotype Y3 (Carneiro *et al.*, 2000). The unidentified populations designated MS3 and MS1 are both from the same geographical region of El Salvador and are important pathogens of coffee in that country (Carneiro *et al.*, 2004b).

Materials and methods

Populations and DNA extraction

Nineteen populations (13 collected in Brazil) were sampled for 18S DNA sequencing (Table 1). Sixteen of these populations were also used in the analysis of the mitochondrial DNA sequence. The latter analysis did not include *M. exigua* Goeldi, 1887, *M. oryzae* Maas, Sanders & Dede, 1978 and the semaphoronts of *Meloidogyne* sp. (isolates 35, 47) were reduced to isolate 35.

From each population, an isolate was cultured starting from the progeny of a single female. Identification of most taxa was based on isozyme phenotypes, resolved by PAGE from young, egg-laying females (Carneiro *et al.*, 2000, 2004a). Genomic DNA was extracted from 200-300 μl of eggs (Randig *et al.*, 2002).

Amplification of 18S RNA gene by PCR

The 18S rDNA was partially amplified using two sets of primers: 1A (forward) and 3B (reverse; Baldwin *et al.*, 1997), and two primers specially designed for this study: MelF (forward) and MelR (reverse) (Table 2). A 50 μl reaction was used containing 5.0 μl of 10$\times$ reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 pM of forward and reverse primers of each set, 1.25 U of *Taq* polymerase (CLP, San Diego, CA, USA) and 2 μl of genomic DNA. DNA was amplified using the PTC-100 Thermocycler (MJ Research, Inc., Waltham, MA, USA), under the following cycling parameters: 1 cycle at 94°C for 7 min followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The last step was 72°C for 10 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc., Santa Clara, CA, USA).

Mitochondrial DNA amplification by PCR

Primers C2F3 5′-GGTCAATGTTCAGAAATTTGT-GG-3′ and 1108 5′-TACCTTTGACCAATCACGCT-3″ (Powers & Harris, 1993) were used to amplify the mitochondrial region located between the 3′ portion of cytochrome oxidase subunit II (*COII*) gene and the 5′ portion of 16S rRNA (*lRNA*) gene. Long PCR or 'high fidelity' PCR (Barnes, 1994; Jeyaprakash & Hoy, 2000) was performed in a 50 μl volume containing 50 mM Tris (pH 9.2), 16 mM ammonium sulphate, 1.75 mM $MgCl_2$, 350 μM dNTPs, 400 pM of primers, 3 μl of DNA, 0.2 U

Table 1. Meloidogyne *species/isolates, esterase phenotypes, origins, original host plants, source of species or isolate and the mtDNA region size.*

Species (isolate)	Esterase phenotype	Origin	Host plant	Source of material	MtDNA region size (bp)
M. arabicida	AR2 (Carneiro *et al*., 2004)	Costa Rica	*Coffea arabica* L.	P. Topard, Cirad, Costa Rica	1683-1684
M. arenaria (36)	A3 (Carneiro, pers. obs.)	Petrolina/PE, Brazil	*Lycopersicon esculentum* Mill. cv. Moneymaker	W. Moreira, Embrapa, Petrolina, PE, Brazil	1112
M. ethiopica	E3 (Carneiro *et al*., 2004)	Encruzilhada/RS, Brazil	*Actinidia deliciosa* (A. Chev)	R.M.V. Sanhueza, Embrapa, Bento Gonçalves, RS, Brazil	1639
M. exigua (17)	E1 (Carneiro *et al*., 2000)	Lavras/MG, Brazil	*Coffea arabica* L.	V.P. Campos, UFLA, Lavras, MG, Brazil	Not analysed
M. floridensis	Fl3 (Carneiro *et al*., 2000)	Florida, USA	*Prunus persica* (L.) Batsch	A.P. Nyczepir, USDA-ARS, SE Fruit & Tree Nut Research Lab, Byron, GA, USA	1111
M. hapla (19)	H1 (Carneiro *et al*., 2000)	Farropilha/RS, Brazil	*Actinidia deliciosa*	C.B. Gomes, Embrapa, Pelotas, RS., Brazil	528
M. incognita (10)	I1 (Carneiro *et al*., 2000)	Londrina/PR, Brazil	*Coffea arabica* L.	R.G. Carneiro, IAPAR, Londrina, PR, Brazil	1502
M. javanica (13)	J3 (Carneiro *et al*., 2000)	Londrina/PR, Brazil	*Glycine max* Merr.	J.F. Veloso, Embrapa, Londrina, PR, Brazil	1640
M. mayaguensis	M2 (Carneiro *et al*., 2003)	Petrolina/PE, Brazil	*Psidium guajava* L.	W. Moreira, Embrapa, Petrolina, PE, Brazil	705
M. morocciensis	A3 (Rammah & Hirschmann, 2003)	Morocco	*Prunus persica* (L.) Batsch	G. Karssen, Plant Protection Service, Wageningen, The Netherlands	1112
M. oryzae	O1 (Carneiro *et al*., 2000)	Surinam	*Oryza sativa* L.	P. Quénéhervé, IRD, Fort de France, Martinique	Not analysed
M. paranaensis	P1 (Carneiro *et al*., 2000)	Pompeia/SP, Brazil	*Coffea arabica*	W. Gonçalves, IAC, Campinas, SP, Brazil	1255
Meloidogyne sp. (35)	S1 (Castro *et al*., 2003)	Diorama/GO, Brazil	*Glycine max*	J.M.C. Castro, UFV, Viçosa, MG, Brazil	1514
Meloidogyne sp. (47)	S1 (Cofcewciz *et al*., 2004)	Cajati/SP, Brazil	*Musa* spp.	E. Cofcewciz, UFEPEL, Pelotas, RS, Brazil	Not analysed
Meloidogyne sp. (MS1)	SA2 (Carneiro *et al*., 2004)	El Salvador	*Coffea arabica*	J.-L. Sarah, Cirad-Amis, Montpellier, France	1656
Meloidogyne sp. (MS3)	SA4 (Carneiro *et al*., 2004)	El Salvador	*Coffea arabica*	J.-L. Sarah, Cirad-Amis, Montpellier, France	1656
Meloidogyne sp. (38)	Y3 (Carneiro *et al*., 2000)	Capão Bonito/SP, Brazil	*Polymnia sonchifolia* Poepp.	M. Mendes, UNESP-Botucatu, SP, Brazil	1639
Meloidogyne sp. (40)	G3 (this study)	Brasilia/DF, Brazil	*Pfaffia glomerata* (Spreng.)	V. Gonzaga, Embrapa, Brasília, DF, Brazil	1626-1627
Meloidogyne sp. (50)	L3 (Carneiro *et al*., 2000)	Caxias do Sul/RS, Brazil	*Lavandula spica* Loisel.	C.B. Gomes, Embrapa, Pelotas, RS, Brazil	1641

Table 2. *List of amplification and sequencing primers used in the 18S rDNA analyses.*

Primer	Sequence	Location based on *Meloidogyne arenaria* U42342
1A	5′-GGCGATCGAAAAGATTAAGCC-3′	779-790
3B	5′-GGCGATCGATTGGCAAATGCTTTCGC-3′	1668-1684 (Reverse)
MelF	5′-TACGGACTGAGATAATGGT-3′	1574-1592
MelR	5′-GGTTCAAGCCACTGCGA-3′	2459-2475 (Reverse)

of *Tgo* DNA polymerase (Roche Applied Science, Indianapolis, IN, USA) and 5 U of *Taq* DNA polymerase (Roche Applied Science).

Amplification was carried out using a linked profile consisting of three different cycles: *i*) one cycle consisting of denaturation at 94°C for 2 min; *ii*) ten cycles consisting of denaturation at 94°C for 10 s, annealing at 48°C for 30 s and extension at 68°C for 2 min; and *iii*) 25 cycles consisting of denaturation at 94°C for 10 s, annealing at 48°C for 30 s and extension at 68°C for 2 min plus 20 s added for every consecutive cycle. Electrophoresis was performed on a 1% TAE agarose gel to detect and inspect the amplified DNA product.

DNA SEQUENCING

The mitochondrial DNA PCR products were cloned into plasmid pCR2.1-TOPO using a procedure and reagents provided by the manufacturer (Invitrogen, Carlsbad, CA, USA). Purified PCR products of 18S rDNA and clones of mitochondrial DNA were sequenced, in both strands, using an ABI PRISM™ Dye Terminator Cycling Sequencing Ready Reaction Kit (Perkin-Elmer Corp., Foster City, CA, USA). The primers used for the direct sequencing of 18S rDNA were the same used for amplification. Sequences were assembled using Sequencher 4.1 (Genes Codes Corp., Ann Arbor, MI, USA). At least two clones of each isolate were sequenced.

SEQUENCE ALIGNMENTS

All sequences obtained in this study were aligned using the default parameters of Clustal X v. 1.83 (Thompson *et al*., 1997) and adjusted in MacClade 4.02 (Sinauer Associates, Inc., Sunderland, MA, USA). The sequences of 18S RNA gene were aligned against the alignment produced by De Ley *et al.* (2002) and available in GenBank (NCBI REF 1107784) using the profile alignment mode in Clustal X. In the final dataset, sequences were reduced by 31 bp characters downstream of 5′ of 1 A primer, and 100 bp upstream of 3′ MelR primer, in order to adjust our sequences to the GenBank-downloaded sequences that were shorter.

PHYLOGENETIC ANALYSES

Models of sequence evolution were evaluated using ModelTest 3.06 (Posada & Crandall, 1998). For the rDNA sequences dataset, ModelTest favoured the TrNef+I+G model, by Hierarchical Likelihood Ratio Tests (hLRTs) and the GTR+I+G model, by Akaike Information Criterion (AIC), with a 0.6633 and 0.6488 gamma distribution shaped parameter. For the mtDNA sequences dataset, both hLRTs and AIC tests, favoured the Hasegawa-Kishino-Yano (HKY+G) model (Hasegawa *et al*., 1985), with a 0.1674 gamma distribution shaped parameter.

Phylogenetic relationships were reconstructed using distance-based (neighbour-joining, NJ; Saitou & Nei, 1987), and discrete character-based (maximum parsimony and maximum likelihood, respectively MP and ML) algorithms using PAUP* m4.0v10 (Swofford, 2002). Maximum parsimony heuristic searches were performed twice, once with 3000 bootstrap replicates, using the fast default settings, and once without bootstrapping, using more exhaustive settings (100 replicates of random branch addition). A neighbour-joining tree employing the BioNJ option was obtained using the Log Determinant distance measure to account for possible positional rate heterogeneity artefacts (Steel *et al.*, 2000), and subjected to 3000 bootstrap replications. Optimal models of sequence substitution as determined by Akaike and likelihood ratio tests (Posada & Buckley, 2004) were used for maximum likelihood searches, conducted heuristically with 100 replicates of random branch addition. The trees were compared statistically using the Kishino-Hasegawa and Shimodaira-Hasegawa pairwise tests. Gaps were treated as missing data for most of the analyses, except for MP with the mtDNA dataset where gaps were also treated as 5th state, considering that in this dataset gaps occurred in conserved blocks and could be inferred positionally with

greater confidence. In the case of the rDNA dataset, *Subanguina radicicola* (Greef, 1872) Paramonov, 1967 was used as the outgroup taxon, as suggested by De Ley *et al.* (2002). For the mtDNA dataset, phylogenetic trees were rooted with *M. hapla* Chitwood, 1949.

A partition-homogeneity test was carried out to explore the combinability of the rDNA and mtDNA datasets, as among the different gene regions of the mtDNA dataset.

Results

rDNA sequences

The nearly complete 18S rDNA sequences obtained from the 19 taxa varied from 1111 (*M. floridensis*) to 1714 (*M. incognita*) bp. Sequences were deposited in GenBank (Table 3) and the aligned data matrix is available from the corresponding author.

Identical rDNA sequences were obtained for *M. floridensis* and the two unidentified populations, *Meloidogyne* sp. (isolates 35, 47); thus only the sequence of *M. floridensis* was used for further analyses. The same occurred with the two unidentified populations from El Salvador, *Meloidogyne* sp. (isolates MS1, MS3). In this case, only the sequence of MS3 was used. A total of 16 sequences were aligned to the previously published alignment (De Ley *et al.*, 2002). The final alignment contained 1623 positions with 369 variable characters, 227 of which were phylogenetically informative characters under parsimony analysis. A+T content ranged from 49 to 53% in the sequences analysed.

Table 3. *List of GenBank accession numbers for the material studied.*

Species (isolate)	18S rDNA[1]	mtDNA region[2]
M. arabicida	AY942625	AY942852
M. arenaria (36)	AY942623	AY635610
M. ethiopica	AY942630	AY942848
M. exigua (17)	AY942627	–
M. floridensis	AY942621	AY635609
M. hapla (19)	AY942628	AY942850
M. incognita (10)	AY942624	AY635611
M. javanica (13)	AY942626	AY635612
M. mayaguensis	AY942629	AY635613
M. morocciensis	AY942632	AY942849
M. oryzae	AY942631	–
M. paranaensis	AY942622	AY942851
Meloidogyne sp. (35)	–	AY942853
Meloidogyne sp. (47)	–	–
Meloidogyne sp. (MS1)	–	AY942854
Meloidogyne sp. (MS3)	AY942636	AY942855
Meloidogyne sp. (38)	AY942633	AY942856
Meloidogyne sp. (40)	AY942634	AY942857
Meloidogyne sp. (50)	AY942635	AY942858

[1] Identical sequences were obtained for *Meloidogyne* sp. isolates 35 and 47 and *M. floridensis*; also for *Meloidogyne* sp. isolates MS1 and MS3.
[2] Sequences not analysed for *M. exigua*, *M. oryzae* and *Meloidogyne* sp. isolate 47.

Our populations of *M. arenaria, M. incognita, M. javanica*, *M. exigua* and *M. hapla* did not give identical 18S rDNA sequences that corresponded with the published sequences for the same species (De Ley *et al.*, 2002). Between the two sequences from *M. arenaria* (present study *vs* accession number AF535867), the differences were related to two substitutions, being one with ambiguity, and four insertions, of which two were an unknown nucleotide (N). The two *M. incognita* isolates (present study *vs* accession number AF535868) presented one insertion and ten substitution differences between them. The sequence for *M. javanica* previously reported (accession number AF442193), and the one used in the present study differed by 24 nucleotide substitutions, 18 ambiguities, and four insertions. The two *M. exigua* isolates (present study *vs* accession number AF442200) differed by 16 nucleotide substitutions and four insertions. Finally, the two sequences obtained for *M. hapla* differed by two insertions involving unidentified nucleotides (N) in the published sequence (accession number AF442194).

The pairwise distances among the *Meloidogyne* spp. 18S rDNA sequences is shown in Table 4, differences between species varying from 0 to 151 bp. The absence of difference among taxa is due to the fact that some sequences presented character ambiguity, which is not counted by the distance analysis.

The topology of the ML trees obtained with the two models favoured by ModelTest, TrNef+I+G and GTR+I+G, was consistent, except for the position of *M. mayaguensis*. This species appears as a sister taxon to *M. ethiopica* and *Meloidogyne* sp. (isolate 38) in the model TrNef+I+G, whereas in the model GTR+I+G it stood separately from the other species. The pairwise tests showed no statistically difference between the topologies of the two ML trees. The topology of ML tree using the model GTR+I+G and NJ and MP trees were congruent, represented by the ML tree, with bootstrap values obtained via MP and NJ in Figure 1. The tree is divided artificially into three clades for ease of discussion.

Table 4. *Adjusted character distance matrix based on 18S rDNA sequence comparison between* Meloidogyne *species. Isolate numbers are given in parentheses.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1 *M. ichinohei*	–																											
2 *M. artiellia*	136	–																										
3 *M. hapla*	139	110	–																									
4 *M. hapla* (19)	141	110	3	–																								
5 *M. microtyla*	143	107	26	24	–																							
6 *M. duytsi*	140	103	42	40	29	–																						
7 *M. chitwoodi*	144	100	56	54	46	39	–																					
8 *M. maritima*	148	97	47	45	34	27	45	–																				
9 *M. graminicola*	144	101	55	53	45	39	12	45	–																			
10 *M. oryzae*	145	101	54	52	44	37	12	43	4	–																		
11 *M. exigua*	146	102	56	52	45	38	18	44	17	14	–																	
12 *M. exigua* (17)	144	100	49	47	37	27	24	40	23	20	16	–																
13 *M. arenaria*	150	109	51	49	42	33	46	44	48	46	46	30	–															
14 *M. arenaria* (36)	147	108	50	48	41	31	44	42	46	44	44	28	3	–														
15 *M. javanica*	151	109	49	47	40	29	45	42	47	45	45	29	8	8	–													
16 *M. javanica* (13)	148	109	50	48	41	31	44	42	46	44	44	28	4	0	8	–												
17 *M. incognita*	144	110	48	46	39	30	44	40	44	42	40	32	12	11	16	12	–											
18 *M. incognita* (10)	147	107	49	47	40	30	43	4	45	43	43	27	4	1	10	2	12	–										
19 *M. morocciensis*	143	105	50	48	39	29	42	39	44	42	42	26	3	0	9	1	11	1	–									
20 *M. floridensis*	147	108	50	48	41	31	44	42	46	44	44	28	3	0	9	1	11	1	0	–								
21 *Meloidogyne* sp. (40)	147	108	48	46	40	30	43	41	45	43	43	27	4	0	8	0	12	1	1	1	–							
22 *Meloidogyne* sp. (50)	145	107	48	46	42	30	43	43	45	43	43	27	6	3	12	4	14	2	3	3	2	–						
23 *M. mayaguensis*	144	106	50	48	38	23	37	38	41	39	39	23	23	21	21	21	27	20	19	21	20	20	–					
24 *M. ethiopica* (37)	143	102	45	43	35	20	36	38	38	36	36	20	18	16	18	16	26	15	14	16	14	13	9	–				
25 *Meloidogyne* sp. (38)	144	102	46	44	35	21	37	38	39	37	37	21	18	16	18	16	26	15	14	16	14	14	10	0	–			
26 *M. paranaensis*	146	107	47	45	39	27	40	40	42	40	40	24	14	12	16	11	20	13	12	12	13	13	15	12	13	–		
27 *Meloidogyne* sp. (MS3)	144	105	45	43	37	25	38	38	40	38	38	22	12	10	14	10	18	11	10	10	11	11	13	10	11	2	–	
28 *M. arabicida*	143	104	43	41	34	23	36	35	38	36	36	20	8	6	10	6	14	7	6	6	7	8	13	9	10	2	0	–

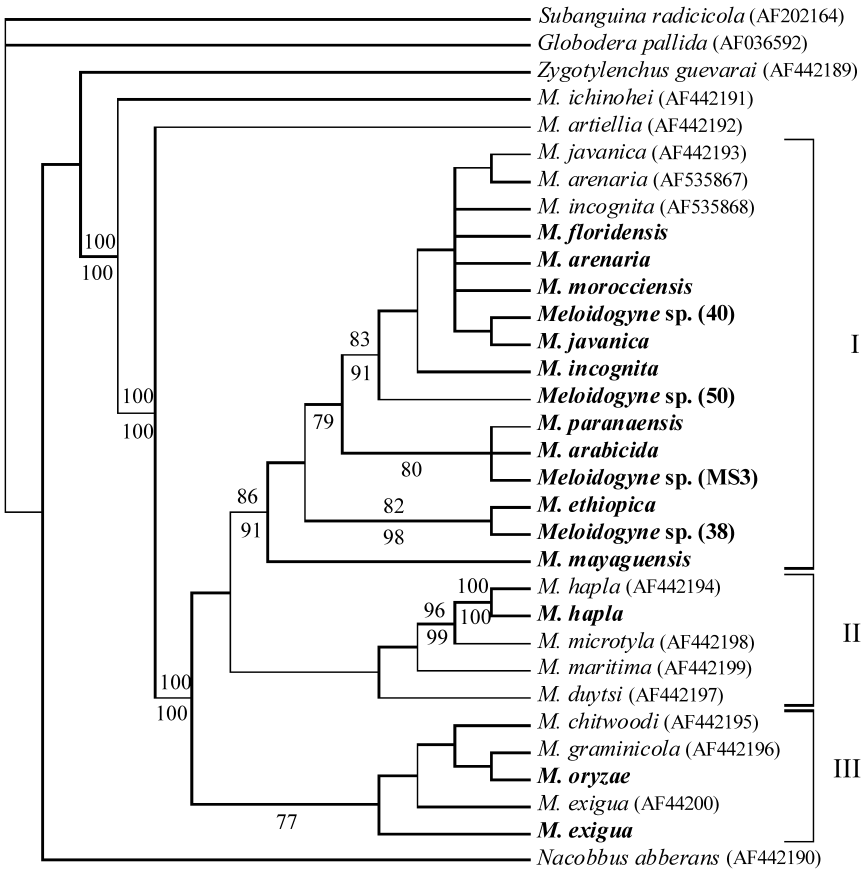

Fig. 1. *Maximum likelihood tree based on 18S rRNA gene sequences. Identical rDNA sequences were obtained for* Meloidogyne floridensis *and the two* Meloidogyne *sp. (isolates 35 and 37), so only the sequence of* M. floridensis *was used in the analyses. The same occurred with the two unidentified populations from El Salvador,* Meloidogyne *sp. (MS1 and MS3). In this case, only the sequence of MS3 was used. The accession numbers of the GenBank-downloaded sequences are show in parentheses. Numbers next to branches are bootstrap values (>70%) from MP analysis (above) and NJ analysis (below). Isolates unique to this study are shown in bold. Monophyletic groups (I, II, III) are arbitrarily delimited and presented for ease of discussion only.*

Our populations of *M. arenaria, M. incognita, M. javanica*, *M. exigua* and *M. hapla* did not give identical sequences to the published sequences for the same species (De Ley *et al.*, 2002). The recently described *M. floridensis* and *M. morocciensis* Rammah & Hirschmann, 1990 were sister taxa to *M. arenaria*, *M. incognita* and *M. javanica*, and also to the unidentified populations of *Meloidogyne* sp. (isolates 35, 37, 40, 50). Relationships among these species cannot be resolved unambiguously as they share few synapomorphies among their subtle differences (Table 4), but together they comprise a robust clade (83-91% bootstrap support; Fig. 1). The unidentified populations, *Meloidogyne* sp. (isolates MS1, MS3), which have identical sequences, form a fairly robust clade with *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996 and *M. arabicida* López & Salazar, 1989 (80% bootstrap support; 0-2 bp differences). *Meloidogyne ethiopica* and *Meloidogyne* sp. (isolate 38) were sister taxa in another subgroup (82-98% bootstrap support) of clade I. *Meloidogyne mayaguensis* is depicted as a sister taxon to the remaining taxa in clade I, maintaining the greatest number of changes (19-27 bp) relative to the other isolates in clade I (Table 4). Clade II was not supported, except for the two isolates of *M. hapla* and their sister taxon relationship to *M. microtyla* (96-99% bootstrap support), *M. duytsi* Karssen, Aelst & Van der Putten, 1998 and *M. maritima* Jepson, 1987, all of which are also depicted as members of clade II. Support for this relationship is poor. *Meloidogyne oryzae*, *M. graminicola* Golden & Birchfield, 1965, *M. chitwoodi* Golden,

O'Bannon, Santo & Finley, 1980 and *M. exigua* comprise a clade (clade III; 77% bootstrap support), but relationships within this clade are not supported by bootstrap analyses. The *M. exigua* isolates were paraphyletic.

Mitochondrial DNA sequences

The size of the amplified PCR products for the mitochondrial region spanning portions of *COII* through *lRNA* genes, determined by the sequence without the sequence of the primers, varied from 528 to 1684 bp for *M. hapla* and *M. arabicida*, respectively (Table 1). Sequences were deposited in GenBank (Table 3). This region included partial *COII* and 16S rRNA sequences, the complete tRNA-His sequence and AT-rich region sequences, located between the *COII* and tRNA-His genes, and varied in length. AT-rich regions were completely absent in *M. hapla* sequences, which has been shown previously (Hugall *et al.*, 1997). The Brazilian *M. hapla* isolate produced the smallest product, as previously reported for other isolates of this species (Powers & Harris, 1993; Blok *et al.*, 2002). Also, the Brazilian population of *M. mayaguensis* produced a 705 bp product, which was in agreement with the size previously reported for this species (Blok *et al.*, 2002). Isolates of *M. arenaria* and *M. morocciensis* produced fragments of the same size (1112 bp). The same occurred with *M. ethiopica* and the unidentified isolate, *Meloidogyne* sp. (isolate 38), which produced a PCR product of 1639 bp, and the unidentified populations from El Salvador, MS1 and MS3, each with 1656 bp products. The *M. floridensis* and *M. paranaensis* isolates produced products of 1110 and 1255 bp, respectively, which is similar in size to *M. arenaria* and *M. morocciensis*. *Meloidogyne* sp. (isolate 35) and *M. incognita* isolates produced products of 1514 and 1502 bp, respectively. *Meloidogyne incognita* has been reported as having populations that produce two sizes of PCR product of *ca* 1500 and 1700 bp (Powers & Harris, 1993; Blok *et al.*, 2002). The *M. javanica* isolate produced a product with a smaller size of 1640 bp than obtained from another isolate of the same species with 1700 bp (Powers & Harris, 1993; Blok *et al.*, 2002).

We detected clone sequence polymorphisms for most of the species, except *M. paranaensis*, *M. javanica*, *M. arenaria*, *M. hapla* and *Meloidogyne* sp. (isolate 38). The maximum sequence divergence among clones of the same isolate was only 0.3% and, upon phylogenetic analyses, all intraspecific polymorphic clones were more closely related to each other than to a clone from any other taxon in the analysis. Thus, only one sequence (clone) from each isolate was used for subsequent analyses. The final alignment contained 1890 positions with 53 phylogenetically informative characters for parsimony analyses. A+T content ranged from 84 to 88% in the sequences analysed. Distances among the *Meloidogyne* spp. mtDNA sequences is shown in Table 5, and the differences between species varied from 1 bp between *M. ethiopica* and *Meloidogyne* sp. (isolate 38), and the two

Table 5. *Adjusted character distance matrix based on mtDNA sequence comparison between* Meloidogyne *species. Isolate numbers are given in parentheses.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *M. morocciensis*	–															
2 *M. arenaria*	2	–														
3 *M. javanica*	4	4	–													
4 *Meloidogyne* sp. (40)	5	5	10	–												
5 *M. ethiopica*	6	6	9	9	–											
6 *Meloidogyne* sp. (38)	7	7	10	10	1	–										
7 *Meloidogyne* sp. (50)	7	7	13	13	3	4	–									
8 *M. floridensis*	7	7	7	7	5	6	6	–								
9 *Meloidogyne* sp. (35)	16	15	22	22	18	18	22	12	–							
10 *M. incognita*	10	10	16	16	12	13	16	7	10	–						
11 *Meloidogyne* sp. (MS3)	7	7	15	13	12	13	16	5	23	17	–					
12 *Meloidogyne* sp. (MS1)	7	7	16	14	13	14	17	6	24	18	1	–				
13 *M. paranaensis*	9	9	13	14	11	11	12	9	22	13	13	14	–			
14 *M. arabicida*	12	12	29	29	26	27	30	10	34	29	28	29	13	–		
15 *M. mayaguensis*	60	59	60	60	57	57	59	60	59	59	59	59	58	58	–	
16 *M. hapla*	104	104	105	103	102	102	103	105	104	104	102	102	103	102	98	–

unidentified isolates MS1 and MS3, to 105 bp between *M. hapla* and *M. javanica*, and between *M. hapla* and *M. floridensis* (Table 5).

The partition-homogeneity among the genes and intergenic regions on mitochondrial DNA sequences showed that the genes and intergenic region were probably all inherited together, and all share a common evolutionary history, so in the further analyses all sequence will be used.

The MP (gaps treated as missing data), NJ and ML trees were congruent, and are represented here by the parsimony tree. Bootstrap values obtained with MP and NJ are shown in Figure 2. All the species grouped in clade I from the rDNA analysis (Fig. 1) formed a well-supported clade (100% bootstrap) to the exclusion of *M. mayaguensis* and *M. hapla*. Both species were the most divergent from other species with 57-105 bp differences (Table 5). In this clade, some sister taxa, identified in the 18S analysis, were observed. The unidentified isolates *Meloidogyne* sp. (MS1 and MS3), *M. incognita* and the unidentified isolate *Meloidogyne* sp. (isolate 35), and *M. ethiopica* and the unidentified isolates *Meloidogyne* sp. (isolates 38, 50), each appeared well-supported as sister taxa.

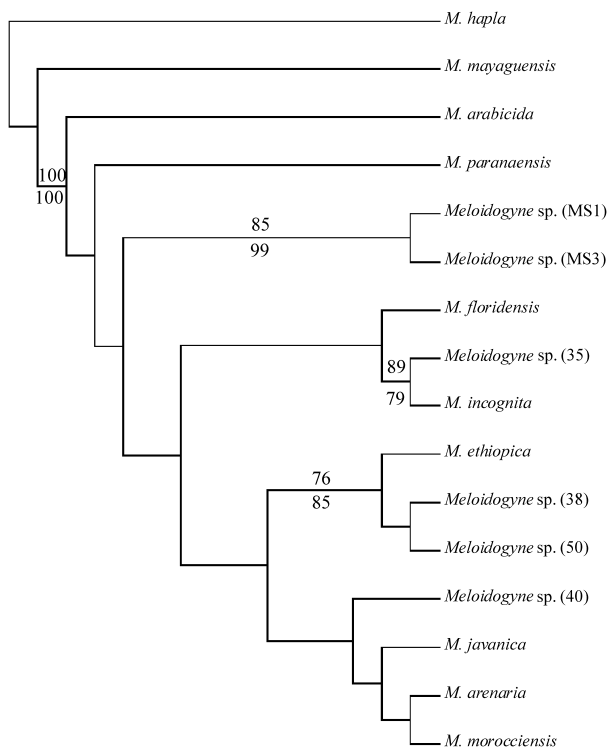

Fig. 2. *Maximum parsimony tree based on mtDNA sequences. Numbers next to branches are bootstrap values from MP analysis (above) and NJ analysis (below).*

For the isolates sampled for both 18S rDNA and mtDNA, the dataset failed a homogeneity partition test, which suggest that these two regions have different evolutionary history ($P \leqslant 0.01$) and should not be combined for phylogenetic analysis.

Discussion

Our results using 18S rDNA sequences for phylogenetic analyses were consistent with the results obtained by De Ley *et al.* (2002). Interestingly, several of the sequences from the same species (as deposited in public databases) were not identical to those we isolated, identified and sequenced. This implies that these species are more molecularly heterogenous than previously thought. Clearly, greater effort needs to be directed to more extensive and intensive sampling of populations in order to improve the likelihood that intraspecific and phylogenetic variation is accurately partitioned.

We report the same three main clades as observed by De Ley *et al.* (2002). In our study, several species and unidentified isolates were included in clade I. With the introduction of new species or isolates, three subgroups were identified in this clade. In the previous study (De Ley *et al.*, 2002), clade I included the mitotic parthenogens, namely, *M. arenaria*, *M. incognita* and *M. javanica*. In this study we were able to include other mitotic parthenogenetic species: *M. mayaguensis*, *M. morocciensis*, *M. paranaensis* and *M. ethiopica.* These taxa are shown to be members of clade I, which includes the supposedly mitotic parthenogen *M. arabicida*. The mode of reproduction for *M. arabicida* was not given in species description, but was later reported to have *ca* 30 chromosomes and to reproduce by mitotic parthenogenesis (Hernandez, 1997). Clade I also included all unidentified species that we subjected to analyses. Their mode of reproduction has not yet been studied. In the case of *Meloidogyne* sp. (isolates 35, 37), their esterase profiles are the same as other unidentified populations detected in USA that have 35 chromosomes (S1 M1 = Br2) (Carneiro *et al.*, 2004b), and reproduce by mitotic parthenogenesis (Esbenshade & Triantaphyllou, 1985). The mode of reproduction of the species in this clade is probably by obligatory mitotic parthenogenesis, except for *M. floridensis* which was described as showing evidence for a meiotic parthenogenetic pathway (Handoo *et al.*, 2004). Also, another probable mitotic parthenogen, *M. oryzae*, was not included in clade I.

Meloidogyne oryzae was described as having 2n = 52-54 chromosomes (Esbenshade & Triantaphyllou, 1985), which indicates that this species probably reproduces by obligatory mitotic triploid parthenogenesis. This species is a member of clade III, which includes the three meiotic pathogens (*M. graminicola*, *M. chitwoodi* and *M. exigua*). In this clade, *M. oryzae* and *M. graminicola* are sister taxa. Both of these species are important in rice plantations, and were included in the morphological *graminis*-group because they have similar morphological characters, *e.g.*, the second-stage juvenile tail tapering to a long narrow hyaline portion and marked clavate terminus. Qualitative differences between *M. oryzae* and *M. graminicola* are small and the species are not easily separated by morphology alone (Jepson, 1987). The isolates of *M. arenaria* and *M. morocciensis* produced amplified 18S fragments of the same size (1112 bp). They are very similar in morphology, and have the same esterase phenotype (A3; Cliff & Hirschmann, 1985; Rammah & Hirschmann, 1990). *Meloidogyne incognita* and the unidentified isolates *Meloidogyne* sp. (isolates 35, 37), also produced similar PCR products and recent studies showed they clustered together in PCR-RAPD with 100% of bootstrap support (Cofcewicz *et al*., 2004). This suggests that these two species diverged recently.

Clear separation of the ameiotic species from other species has been shown in previous phylogenetic studies (Esbenshade & Triantaphyllou, 1987; Castagnone-Sereno *et al.*, 1993; Van der Beek *et al*., 1998; De Ley *et al*., 2002; Castillo *et al*., 2003). The mode of reproduction from the *M. hapla* isolate used in this study was not determined, but De Ley *et al*. (2002) have showed that both meiotic and mitotic isolates of *M. hapla* had identical 18S DNA sequences. Our results, in general, agree with the previous assumptions that the evolution in *Meloidogyne* is related to the mode of reproduction (Triantaphyllou, 1985). However, if the mode of reproduction is confirmed for the species analysed, character mapping suggests that mitotic parthenogenesis in the genus may have multiple origins.

In order to understand more fully the relationships among the species in clade I, we have also subjected them to analyses based on the mtDNA region between the *COII* and *lRNA* genes. This region has been used for diagnostics of *Meloidogyne* spp. (Powers & Harris, 1993; Blok *et al.*, 2002; Brito *et al.*, 2004). Phylogenetic analysis of the complete region, including partial *COII* and partial *lRNA* sequences, the complete sequence for tRNA-His and the AT rich region, produced one strongly supported clade. All the species that appear in clade I of the rDNA analyses were from a similar clade in the mtDNA analysis with the exception of *M. mayaguensis*. Although this species was included in clade I, it stood apart from the others. The size of the PCR product obtained with the *COII*/*lRNA* mtDNA primers (*ca* 700 bp), distinguishes *M. mayaguensis* from the other widespread species with mitotic parthenogenetic reproduction. Some sister taxa identified by mtDNA analysis are discordant from the rDNA results. The unidentified isolate, *Meloidogyne* sp. (isolate 50) was showed to be in the same subgroup of the common root-knot nematodes, *M. arenaria*, *M. javanica* and *M. incognita*, based on the rDNA phylogenetic trees (83-91 bs). However, in the mtDNA phylogenetic trees, this isolate appeared as a sister taxon to the unidentified *Meloidogyne* sp. (isolate 38) and *M. ethiopica* (76-85 bootstrap support). Also, *M. arenaria*, *M. javanica*, *M. morocciensis* and *Meloidogyne* sp. (isolate 40), were closer to *M. ethiopica* and *Meloidogyne* sp. (isolates 38, 50) than to *M. floridensis*, *M. incognita* and *Meloidogyne* sp. (isolate 35). Clade membership between *M. incognita*, *M. javanica* and *M. arenaria* were resolved, but not supported. However, the mtDNA phylogenetic trees are concordant with those obtained by PCR-RAPD analyses (Randig *et al*., 2002; Carneiro *et al.*, 2004b). *Meloidogyne arabicida*, *M. paranaensis* and the unidentified isolates, *Meloidogyne* sp. (isolates MS1 and MS3), were shown to be sister taxa in the rDNA trees (80% bs), but in the mtDNA trees they were not resolved. Although, these three species are different morphologically and have different esterase phenotypes, they are all pathogens of coffee in Central America (Carneiro *et al.*, 2004b). Regarding the unidentified isolates from El Salvador, they appear to be very closely related. They were identical for 18S rRNA sequence and presented only 1 bp difference in their mtDNA sequence. These two isolates clustered together with 100% bootstrap support in PCR-RAPD studies, and they are probably a recent genetic divergence of the same new species (Carneiro *et al.*, 2004b). The same was observed for *Meloidogyne* sp. (isolate 38) and *M. ethiopica*, but these two species are very different in PCR-RAPD studies (Randig *et al.*, 2002) and morphology (data not included). High levels of nucleotide sequence identity in the 18S rRNA gene are commonly found among species of other genera, such as *Caenorhabditis* and *Heterorhabditis*, suggesting that these species may have diverged from each other recently (Fitch *et al.*, 1995; Liu *et al.*, 1997). Considering both regions analysed, *Meloidogyne* sp. (isolate 40) appeared to be more closely related to *M. arenaria*, *M. morocciensis* and *M. javanica* than to the other

species studied. The relationship of *Meloidogyne* sp. (isolate 50) and the other species analysed was discordant between the 18S rDNA and mtDNA datasets. Detailed morphological analyses aimed at clarifying the relationship among these atypical isolates and nominal species are currently underway.

The rDNA and mtDNA tree topologies revealed phylogenetic discordance among several lineages and, as interpreted by the homogeneity partition test, likely reflect different evolutionary histories. The phylogenetic utility of both molecular markers is frustrated by taxon sampling, the information content of mtDNA and rDNA regions and cytonuclear genomic dissociation. However, the addition of taxa and mtDNA data to publicly available 18S rDNA records improved the discrimination sensitivity of species and atypical unidentified isolates, and highlighted several instances of diagnostic and taxonomic discord.

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