IDENTIFICATION OF COFFEE ROOT-KNOT NEMATODES BASED ON PERINEAL PATTERN, SCAR MARKERS AND NUCLEAR RIBOSOMAL DNA SEQUENCES

I. Herrera*[1,2], T. Bryngelsson[1], A. Monzón[2] and M. Geleta[1]

[1] *Swedish University of Agricultural Sciences, Department of Plant Breeding and Biotechnology, P.O. Box 101, SE-23053 Alnarp, Sweden*
[2] *National Agrarian University, Department of Plant Protection, P.O. Box. 453, Managua, Nicaragua*

Summary. The present study aimed at identifying *Meloidogyne* species attacking coffee in Nicaragua. Eighteen isolates collected from coffee plantations across Nicaragua were identified. The study was based on perineal patterns of egg-laying females, SCAR markers and partial sequences of 18S and 28S nuclear rDNA. Two *Meloidogyne* species: *M. exigua* and *M. incognita* were identified and characterized. The combined sequences of 18S and 28S nuclear rDNA showed that both species were comprised of single haplotypes. *Meloidogyne exigua* was widespread and was isolated from all eighteen coffee plantations whereas *M. incognita* was identified in only one. *Meloidogyne exigua* has unique variable sites within the 18S and 28S rDNA regions when compared to several *Meloidogyne* species, including those sharing similar reproductive mechanisms. These variable sites can be used to develop new species specific SCAR markers for identification of *M. exigua*.

Key words: *Coffea arabica*, *Meloidogyne exigua*, *M. incognita*, 18S rDNA, 28S rDNA, SCAR markers.

Root-knot nematodes (RKN) of the genus *Meloidogyne* cause major damage to a wide range of crops, including coffee (*Coffea arabica* L.). Seventeen species of *Meloidogyne* have been reported to attack coffee and four of these, *M. exigua* Göldi, *M. incognita* (Kofoid *et* White) Chitw., *M. coffeicola* Lordello *et* Zamith and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos *et* Almeida, are regarded as the most common and damaging species (Carneiro and Cofcewicz, 2008). These nematodes can cause retarded plant growth and production losses of this crop (Carneiro *et al*., 1996b; Campos and Villain, 2005). Six *Meloidogyne* species have been identified in coffee in Central America of which *M. exigua* is the most widely spread species (Garcia *et al*., 2009; Muniz *et al*., 2009; Barbosa *et al*., 2010). In this region, *M. exigua* has been reported from Costa Rica, Nicaragua and Honduras, whereas *M. arenaria* (Neal) Chitw., *M. arabicida* Lopez *et* Salazar and *M. hapla* Chitw. have been reported from El Salvador, Costa Rica and Guatemala, respectively (Hernández *et al*., 2004). A study based on esterase isozymes has shown the presence of *M. incognita* in Costa Rica, Guatemala and El Salvador (Villain *et al*., 2007). *Meloidogyne enterelobii* Yang *et* Eisenback (= *M. mayaguensis* Rammah *et* Hirschmann) has been reported from coffee plantations in Cuba (Rodríguez *et al*., 1995; Hernández *et al*., 2001).

Meloidogyne exigua and *M. incognita* are probably the most damaging species on coffee in Latin America. Yield losses caused by these RKN have been estimated at up to 10% in Costa Rica (Bertrand *et al*., 1997) and 45% in Brazil (Barbosa *et al*., 2004). The wide range of RKN species reported from coffee is related to their large diversity in pathogenicity (Hernández, 1997; Villain *et al*., 1999) and research is going on to identify and characterize the species in order to allow reliable pest management strategies and coffee breeding programmes to be designed.

Traditional diagnosis of RKN is primarily based on morphological features, such as perineal patterns and morphometric data of females, males and second-stage juveniles (Jepson, 1987; Carneiro and Cofcewicz, 2008). However, morphological and morphometric data require a great deal of work and are often inconclusive as they often vary considerably within a population and may not be sufficient to distinguish closely related *Meloidogyne* species (Hirschmann, 1986; Zijlstra *et al*., 2000; Carneiro and Cofcewicz, 2008). DNA-based root-knot nematode diagnosis is an attractive solution, as it does not rely on expressed gene products, is independent of environmental influence and the stage of the life cycle and has a high discriminating power (Ziljstra *et al*., 2000). Molecular approaches used to distinguish *Meloidogyne* species include RLFP (Hugall *et al*., 1994), RAPD (Blok *et al*., 1997) and rDNA and mtDNA sequences (Blok *et al*., 2002).

Molecular markers specific to a single species can be identified from band profiles generated by PCR techniques, such as RAPD and AFLP (Geleta *et al*., 2007). These markers can be isolated and sequenced to design species-specific primers (Ou *et al*., 2008), which can then be used to generate Sequence Characterized Amplified Regions (SCAR) markers (Paran and Michelmore, 1993). For example, the development of SCAR markers has been achieved for rapid identification of the soybean cyst nematode *Heterodera glycines* Ichinoe (Subbotin *et al*., 2001) and potato cyst nematodes, *Globodera pallida* Stone and *G. rostochiensis* (Woll.) Skar-

* Corresponding author: Isabel.Herrera@slu.se

bilovich (Mulholland *et al*., 1996). SCAR markers have also been developed for the identification of the three most common nematodes on coffee, *M. incognita, M. exigua* and *M. paranaensis* (Randig *et al*., 2002).

Several regions of the nuclear genome, such as 18S and 28S ribosomal DNA (rDNA), have been used for phylogenetic analyses and characterization of *Meloidogyne* species. Ribosomal DNA has highly informative regions for diagnostic and phylogenetic studies of plant-parasitic nematodes (Blok, 2005). The 18S rDNA region was used for phylogenetic analysis of 12 *Meloidogyne* species, which revealed three well supported clades (De Ley *et al*., 2002). Landa *et al*. (2008) used DNA sequences from the ITS, 18S and 28S rDNA regions to characterize *M. hispanica* isolates of different geographic origin, and could differentiate *M. hispanica* Hirschmann from other *Meloidogyne* species. The 18S rDNA region was also used in combination with a mtDNA region for phylogenetic analysis of these species, which revealed three major clades (Tigano *et al*., 2005).

In Nicaragua, the presence of RKN in coffee plantations has been reported previously (Sequeira, 1977; Rosales-Mercado and Reyes-García, 1991), although the species were not identified. The first species report on RKN from Nicaraguan coffee plantations was by Hernández *et al*. (2004), who reported the presence of *M. exigua* in the Carazo province in the South Pacific region. The identification and characterization of coffee RKN at the species level is a significant step towards developing efficient approaches for crop protection. It also helps to assess the species and population diversity of RKN and their impact on coffee plantations. Hence, the objective of this study was to identify and characterize RKN in Nicaraguan coffee plantations using morphological and molecular markers and DNA sequence data.

MATERIALS AND METHODS

Isolates of Meloidogyne species. Eighteen *Meloidogyne* isolates were collected from two coffee growing regions in Nicaragua (Table I). Fifteen isolates were from the South Pacific region and three isolates were from the North Central region. All isolates were collected from coffee roots and maintained on coffee and tomato plants (*Solanum lycopersicum* L.) in a greenhouse with a mean temperature of 27 ºC.

Perineal pattern. The perineal patterns of ten egg-laying females from each isolate were prepared according to Taylor and Netscher (1974) and Hartman and Sasser (1985). This was followed by analysis of the perineal pattern of the ten specimens. When high levels of variation were observed, up to twenty perineal patterns were analyzed.

DNA extraction. DNA was extracted both from single females and bulk samples of female. Single female DNA

Table I. Isolates of *Meloidogyne* species from eighteen coffee plantations in Nicaragua used in this study and their geographic description.

Isolate code	Source of coffee variety	Farm	Province	Position	masl*	*Meloidogyne* species identified based on SCAR markers
M-1	Caturra	Aguacatal	Masatepe	N11º 52´ W 86º 08´	479	*M. exigua*
M-2	Catrenic	Sn. Dionisio	San Marcos	N11º 52´ W 86º 10´	606	*M. exigua*
M-3	Caturra	La Escoba	Granada	N11º 52´ W 86º 08´	486	*M. exigua*
M-4	Pacas	J. Botánico	Masatepe	N11º 53´ W 86º 08´	454	*M. exigua*
M-5	Caturra roja	St. Elisa	Jinotepe	N11º 09´ W 86º 10´	470	*M. exigua*
M-6	Caturra	P. Guerrero	Masatepe	N11º 51´ W 86º 07´	470	*M. exigua*
M-7	Caturra	Sta. Emilia	Matagalpa	N12º 58´ W 85º 49´	817	*M. exigua*
M-8	Catimor	La Canavalia	Matagalpa	N12º 59´ W 85º 48´	796	*M. exigua*
M-9	Catuaí roja	St. Francisca	Masatepe	N11º52´ W 86º 08´	565	*M. exigua*
M-10	Caturra	Concepción	Masatepe	N11º 52´ W 86º 08´	476	*M. exigua*
M-11	Catuaí roja	Sn. Filemón	Dipilto	N13º 45´ W 86º 30´	1100	*M. exigua*
M-12	Pacas	Sn. Miguel	Carazo	N11º 52´ W 86º 09´	511	*M. exigua*
M-13	Caturra	Sn. Pedro	Masatepe	N11º 54´ W 86º 07´	479	*M. exigua*
M-14	Pacas	El Rosal	Carazo	N11º 45´ W 86º 31´	303	*M. exigua*
M-15	Pacas	El Danubio	Masatepe	N11º 52´ W 86º 12´	470	*M. exigua* and *M. incognita*
M-16	Bourbon	St. Francisca	Masatepe	N11º 52´ W 86º 08´	498	*M. exigua*
M-17	Caturra	Sn. Rafael	Carazo	N11º 38´ W 86º 11´	460	*M. exigua*
M-18	Pacas	Sn. Enrique	Masaya	N11º 51´ W 86º 08´	477	*M. exigua*

* metres above sea level.

was extracted from each of ten females using Extract-N-Amp™ Tissue PCR Kits (SIGMA) following the instructions provided with the kit. For bulk extractions, DNA was extracted from forty bulked females using a protocol originally developed for DNA extraction from insects (Reineke *et al.*, 1998), with minor modifications. The samples were transferred to Eppendorf tubes containing 25 µl of extraction buffer [1M Tris (pH 8), 0.5 M EDTA, 10% SDS and 0.1 ml of 20 mg/ml of Proteinase K] and frozen in liquid nitrogen for 10 seconds before being manually ground using a pestle. Then, 75 µl of extraction buffer was added and the samples were completely homogenized using a pestle connected to cordless motor. Once the samples were completely homogenized, 300 µl of extraction buffer and 100 µl of 10% SDS were added and the samples were incubated at 58 ºC for 1 h. After the addition of 140 µl of 5M NaCl and 65 µl of 10% CTAB the samples were incubated at 65 ºC for 10 min. This was followed by the addition of 700 µl of chloroform-isoamyl alcohol (24:1), incubation on ice for 30 min and centrifugation at 4 ºC for 20 min. The supernatant was transferred to a new Eppendorf tube, 225 µl of 5M NH_4Ac was added and the samples were incubated on ice for 30 min before centrifugation at 4 ºC for 20 min. For precipitation, 500 µl of cold isoprapanol was added, followed by centrifugation at 4 ºC for 20 min and washing with 70% ethanol. In all cases, centrifugation was at 14000 rpm. The pellet was finally dissolved in 30 µl of TE buffer. The quality of the extracted DNA was analyzed by agarose gel electrophoresis and DNA concentration was determined using a Nanodrop® ND-1000 spectrophotometer (Saveen Werner, Sweden).

Polymerase chain reaction and DNA sequencing. The 18S nuclear rDNA gene fragment was amplified using the primer-pair *MelF* and *MelR* (Tigano *et al.*, 2005; Table II). The PCR amplification was done in a 20 µl reaction volume containing 10 µl of Extract-N-Amp PCR reaction mix (SIGMA), 0.75 µM of each primer and 3 µl of genomic DNA. The amplification temperature profile was: 7 min initial denaturing at 94 ºC and 10 min final extension at 72 ºC with the intervening 35 cycles of 1 min at 94 ºC, 1 min primer annealing at 50 ºC and 1 min primer extension at 72 ºC. The 28S nuclear rDNA gene fragment was amplified using primer-pair *D3A* and *D3B* (Chen *et al.*, 2003). PCR amplification was done in a 20 µl reaction volume containing 10 µl of Extract-N-Amp PCR reaction mix (SIGMA), 0.4 µM of each primer and 4 µl of genomic DNA. The amplification temperature profile was as follows: initial 3 min denaturing at 95 ºC and final 5 min primer extension at 72 ºC with the intervening 40 cycles of 30 sec denaturing at 95 ºC, 30 sec primer annealing at 55 ºC and 1 min primer extension at 72 °C.

Partial sequences of the 18S and 28S rDNA were amplified and sequenced using DNA extracted from single females. SCAR marker analyses were performed using bulk female DNA; when bulk DNA based SCAR markers revealed the presence of more than one *Meloidogyne* species, single female DNA was also used to confirm the results obtained using bulk DNA.

In the case of SCAR marker analysis, three primer-pairs specific to *M. exigua*, *M. incognita* and *M. paranaensis* were used (Randig *et al.*, 2002; Table II). PCR amplification was done using DNA from both single and bulked female nematodes. For single female DNA, PCR was done in a 20 µl reaction volume containing 10 µl of Extract-N-Amp PCR reaction mix (SIGMA), 0.75 µM of each primer and 3 µl of genomic DNA. For bulk samples, the PCR amplification was done in a 50 µl reaction volume containing 10× PCR buffer (Saveen Warner AB), 2 mM $MgCl_2$, 0.1 mM dNTPs, 1 U of Taq DNA polymerase, 0.5 µM of each primer and 10 ng of genomic DNA. Both groups of samples were amplified under the following temperature profile: 5 min at 94 ºC, followed by 30 cycles of 30 sec at 94 ºC, 45 sec at 64 ºC, and 1 min at 70 ºC; and final extension of 8 min at 70 ºC. DNA was amplified using a Gene AMP PCR system 9700 thermocycler.

All PCR products were electrophoresed at 100 V for 30 min on 1.5% agarose gels that were stained with ethidium bromide and visualized and photographed under UV light. The PCR products of the 18S and 28S rDNA were purified using a QIA quick PCR purification kit (QIAGEN GmbH, Germany) as recommended by the manufacturer. Five nanograms of the purified PCR product were added to a 1.5 ml Eppendorf tube for each sequencing reaction and the samples were dried

Table II. List of primers used for amplifications of SCAR markers and amplification and sequencing of the partial sequences of 18S and 28S rDNA genes in *Meloidogyne* species.

Primer name	Primer sequence (5'→3')	Target region	Target species
Ex-D15-F	CAT CCG TGC TGT AGC TGC GAG	SCAR markers	*M. exigua*
Ex-D15-R	CTC CGT GGG AAG AAA GAC TG		
inc-K14-F	GGG ATG TGT AAA TGC TCC TG		*M. incognita*
inc-K14-R	CCC GCT ACA CCC TCA ACT TC		
par-C09-F	GCC CGA CTC CAT TTG ACG GA		*M. paranaensis*
par-C09-R	CCG TCC AGA TCC ATC GAA GTC		
MelF	TAC GGA CTG AGA TAA TGG T	18S	*Meloidogyne* spp.
MelR	GGT TCA AGC CAC TGC GA		
D3A	GAC CCG TCT TGA AAC CAC GA	28S	*Meloidogyne* spp.
D3B	TCG GAA GGA ACC AGC TAC TA		

overnight. Fifteen µl of Millipore water was added and the samples were sent to EUROFINS MWG Operon (Ebersberg, Germany) for sequencing with an ABI PRISM-3100 genetic analyzer (Applied Biosystems). Each DNA region was sequenced using both forward and reverse primers. The partial nucleotide sequences of the two ribosomal genes from *M. exigua* and *M. incognita* were submitted to the GenBank. The accession numbers of these sequences are given in Table III. Additional DNA sequences were retrieved from the National Center for Biotechnology Information (NCBI) database for data analysis in combination with the sequences generated in this study.

Sequence alignments and data analyses. Sequences were edited using BIOEDIT sequence alignment editor version 7.0.5 (Hall, 2005) and the sequences were visually inspected using SEQUENCE SCANNER version 1.0 (Applied Biosystems). Sequences were aligned using CLUSTAL X version 1.81 (Thompson *et al*., 1997) followed by manual adjustment. BIOEDIT was used to generate a sequence identity matrix between the sequences generated in this study and those retrieved from the GenBank. Maximum parsimony based phylogenetic analysis was carried out using PAUP* 4.0 Beta 10 (Swofford, 2000) based on the aligned sequences from the present study and from the GenBank. Both strict and bootstrap 50% majority rule consensus trees were constructed and clade support was estimated using bootstrap values (1,000 bootstrap replicates with 100 random additions).

RESULTS

Perineal pattern. Although the perineal patterns were quite variable, detailed observations of 10-20 female nematodes from each isolate indicate that the perineal patterns correspond to *M. exigua* and *M. incognita*. *Meloidogyne exigua* was observed in all eighteen isolates. Typical characteristics are rounded, slightly plane low dorsal arcs, thick and well spaced striae, non-perceptible lateral lines normally demarked by either bent or interrupted striae (Fig. 1a-c). The *M. incognita* perineal pattern was observed in only one isolate from the El Danubio farm in the South Pacific region of Nicaragua. The perineal pattern was roughly oval, dorsal striae closely spaced, wavy to zig-zag, forking to some extent at the lateral lines. The dorsal arch was generally trapezoid in shape, sometimes with a distinct tail whorl and numerous transverse striae at the side of the ventral part (Fig. 1d-f).

SCAR markers. Amplification of DNA from bulk and single females using the *M. exigua* primer-pair resulted in a specific band of expected size, 562 bp in all eighteen isolates (Fig. 2a). There was no amplification with the *M. incognita* primer-pair except in isolate *M-15* (El Danubio farm), which yielded a fragment of 399 bp as expected (Fig. 2b). No amplification was obtained with the *M. paranaensis* primer-pair (Fig 2c).

18S and 28S nuclear rDNA sequences. Amplification of the partial sequence of the 18S nuclear rDNA region

Table III. Sequence identity between sequences generated in this study and sequences retrieved from the GenBank, in *M. exigua* and *M. incognita*.

Gene	Species	Country of origin	Host	Accession number	*M. exigua*[a]	*M. incognita*[a]
18S rDNA	*M. exigua*[a]	Nicaragua	Coffee	HQ709101	-	0.966
	M. exigua[b]	UK	Coffee	AF442200	0.984	0.959
	M. exigua[b]	Brazil	Coffee	AY942627	0.979	0.983
	Mean				0.982	0.969
	M. incognita[a]	Nicaragua	Coffee	HQ709102	0.966	ID
	M. incognita[b]	Brazil	Coffee	AY942624	0.964	0.997
	M. incognita[b]	Netherlands	ng	EU669939	0.963	0.996
	M. incognita[b]	Netherlands	ng	EU669940	0.960	0.993
	M. incognita[b]	Netherlands	ng	AY284621	0.960	0.992
	M. incognita[b]	ng	ng	AY268120	0.960	0.990
	M. incognita[b]	USA	Soybean	AF535868	0.971	0.985
	Mean				0.963	0.992
28S rDNA	*M. exigua*[a]	Nicaragua	Coffee	HQ709103	-	0.929
	M. exigua[b]	Brazil	Coffee	AF435796	0.995	0.933
	M. exigua[b]	Brazil	Rubber tree	AF435795	0.990	0.938
	M. exigua[b]	UK	Coffee	AF435804	0.990	0.929
	Mean				0.992	0.932
	M. incognita[a]	Nicaragua	Coffee	HQ709104	0.929	-
	M. incognita[b]	Korea	Ng	GQ258784, 6-9	0.929	1.000
	M. incognita[b]	USA	Ng	AY355416	0.929	1.000
	M. incognita[b]	USA	Ng	AY355411,3	0.929	1.000
	M. incognita[b]	USA	Soybean	AF435794	0.924	0.995
	M. incognita[b]	USA	Ng	AY355414,7	0.924	0.937
	M. incognita[b]	USA	Ng	AY355412	0.905	0.928
	Mean				0.926	0.983

Ng = not given; [a]sequences generated in this study; [b]sequences retrieved from GenBank

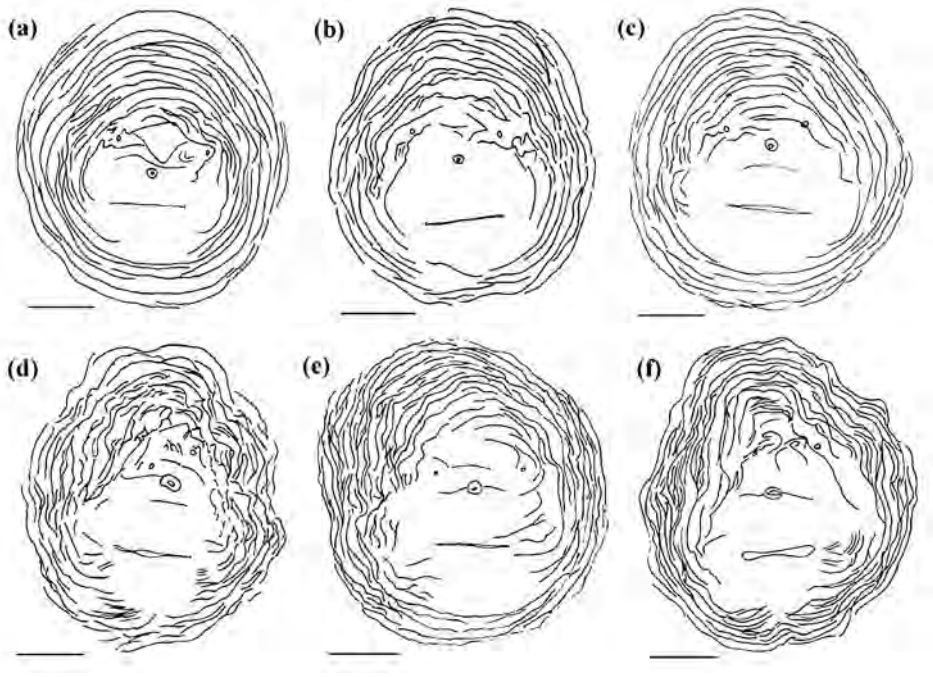

Fig. 1. Variability of perineal patterns of *Meloidogyne exigua* (a, b, c) and *Meloidogyne incognita* (d, e, f) isolates identified from Nicaraguan coffee plantations. Scale bars: 20 µm.

yielded a fragment of 902 bp in both *M. exigua* and *M. incognita*. A total of 34 DNA sequences (two from each isolate) were obtained from the seventeen isolates, excluding isolate *M-15*. The sequences of all the 34 specimens were identical and correspond to *M. exigua*. In the case of the *M-15* isolate, PCR products from twelve specimens were sequenced, of which eight were characteristic for *M. exigua* and four for *M. incognita*. The eight *M. exigua* sequences were identical to the 34 sequences from the other amplified fragments and thus the 18S rDNA sequences of all specimens identified as *M. exigua* through SCAR marker and perineal pattern analyses were identical. The sequences of the four specimens identified as *M. incognita* were also identical.

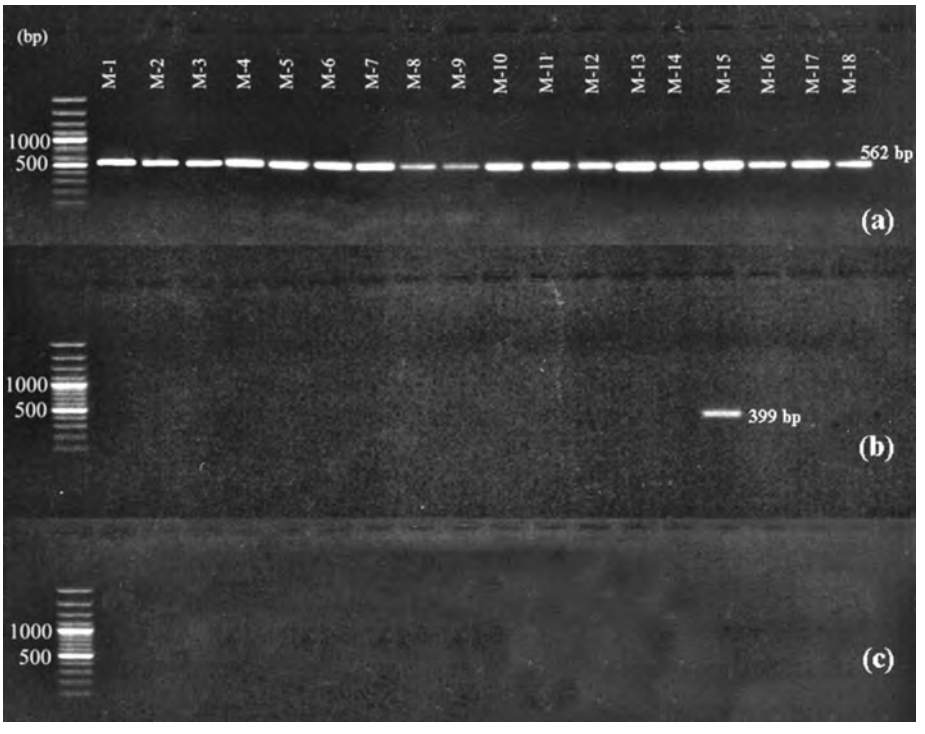

Fig. 2. Gel picture showing the amplification patterns of the 18 *Meloidogyne* isolates using primer-pairs specific to (a) *M. exigua*, (b) *M. incognita* and (c) *M. paranaensis*. *M-1* to *M-18* corresponds to isolate codes given in Table 1.

Table IV. Mean sequence identity matrix between sequences from the two Nicaraguan *Meloidogyne* species (columns) identified in this study and sequences of the same species and other closely related *Meloidogyne* species retrieved from GenBank (rows).

Species sequence from GenBank	*M. exigua* from Nicaragua			*M. incognita* from Nicaragua		
	18S	28s	Mean of 18S and 28S	18S	28s	Mean of 18S and 28S
M. exigua	0.982	0.992	0.987	0.969	0.932	0.951
M. oryzae	0.985	-	-	0.959	-	-
M. graminicola	0.983	0.948	0.966	0.957	0.937	0.947
M. fallax	0.982	0.920	0.951	0.963	0.914	0.939
M. incognita	0.963	0.926	0.945	0.992	0.983	0.988
M. paranaensis	0.966	0.929	0.948	0.993	1.000	0.997
M. javanica	0.963	0.929	0.946	0.995	0.995	0.995
M. hispanica	0.963	0.929	0.946	0.993	0.980	0.987
M. arenaria	0.962	0.918	0.940	0.994	0.982	0.988
M. floridensis	0.964	-	-	0.997	-	-
M. morocciensis	0.964	-	-	0.997	-	-
M. ethiopica	0.965	-	-	0.996	-	-
M. arabicida	0.964	-	-	0.993	-	-
M. chitwoodi	0.980	0.893	0.937	0.961	0.869	0.915
M. konaensis	-	0.929	-	-	0.995	-

These *M. incognita* fragments have a sequence identity of 96.6% to the sequences from *M. exigua* (Table III).

When we compared the *M. exigua* sequences obtained in the present study (HQ709101) with *M. exigua* sequences retrieved from the GenBank (AF442200 and AY942627), the mean sequence identity between them was 98.2% (Tables III and IV), whereas the mean sequence identity between our *M. exigua* (HQ709101) and *M. incognita* sequences from the GenBank was 96.3%. On the other hand, the sequence identity between *M. incognita* from this study (HQ709102) and *M. incognita* from the GenBank was 99.2, whereas our *M. incognita* (HQ709102) and *M. exigua* sequences from the GenBank had a sequence identity of 96.9% (Table III). The sequence of our *M. incognita* (HQ709102) was most similar to that of *M. incognita* isolated from coffee in Brazil (AY942624) with a sequence identity of 99.7% and least similar to *M. incognita* isolated from soybean in USA (AF535868) with a sequence identity of 98.5%.

When the sequences of *M. exigua*, including the one from the present study, were compared with 43 sequences from thirteen other *Meloidogyne* species from the GenBank, it was revealed that *M. exigua* has a unique variable site within 18S rDNA that corresponds to the

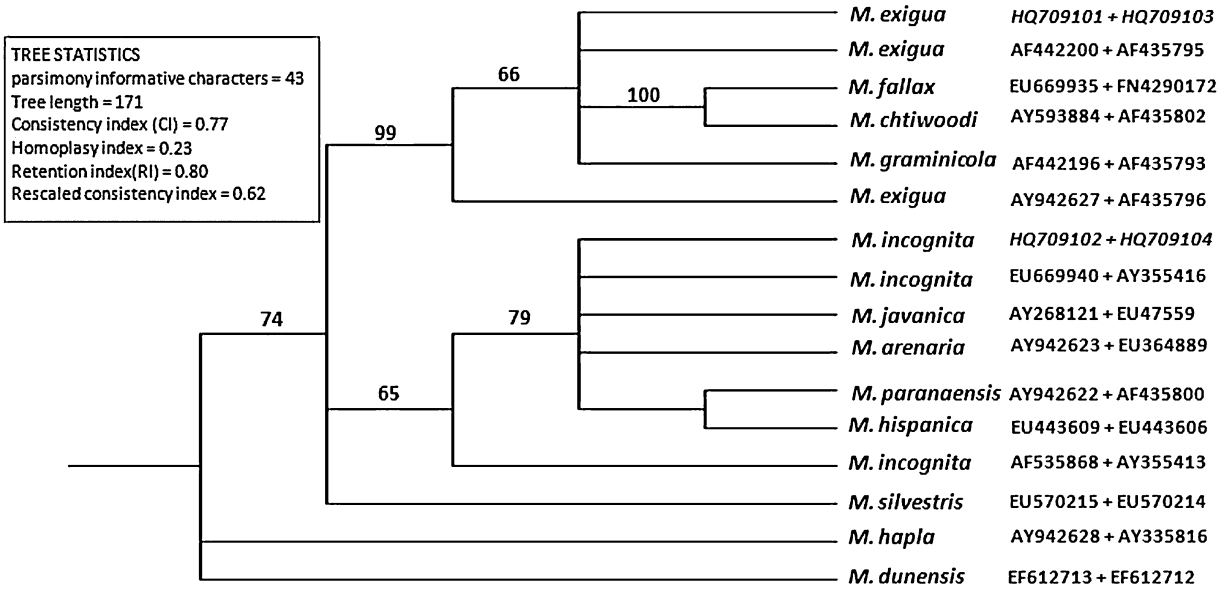

Fig. 3. Strict consensus of 525 trees from the analysis of 18S and 28S rDNA sequences of *Meloidogyne* species. *Meloidogyne* dunensis was used as outgroup. Numbers above the branches are bootstrap values from bootstrap 50% majority rule consensus tree. The texts to the right of the species names refer to the accession numbers of 18S rDNA and 28S rDNA in that order.

196th nucleotide in the HQ709101 sequence (data not shown). At this position, *M. exigua* has a *T* nucleotide instead of *A* or *indel* in the other species included in the analysis. Unlike in *M. exigua*, there was no such unique mutation in *M. incognita*. The 18S rDNA data showed that the sequence similarity between *M. exigua* from the present study and other *M. exigua* sequences is of the same level as the similarity between *M. exigua* and *M. oryzae* Maas, Sanders *et* Dede/*M. graminicola* Golden *et* Birchfirld/*M. fallax* Karssen (Table IV). Similarly, the sequence similarity between *M. incognita* from the present study and other *M. incognita* sequences is of the same level or even lower when compared to the sequence similarity between *M. incognita* and some other *Meloidogyne* species, including *M. paranaensis* and *M. javanica* (Treub) Chitw. (Table IV). These can also be observed from the strict consensus tree constructed from the combined data of the 18S and 28S rDNA genes (Fig. 3).

The amplifications of the 28S rDNA region yielded a fragment of 342 bp for *M. exigua* from all eighteen isolates and a fragment of approximately 334 bp for *M. incognita* from isolate *M-15*. Similarly to the sequences from 18S rDNA, the sequences of all 42 specimens from the eighteen isolates identified as *M. exigua* were identical. The sequences of the four specimens identified as *M. incognita* from isolate *M-15* were also identical. These sequences have a sequence identity of 92.9% to the *M. exigua* sequences generated in this study (Table III).

When we compared the *M. exigua* 28S rDNA sequence obtained in the present study (HQ709103) with *M. exigua* sequences from the GenBank (AF435796, AF435795 and AF435804), the mean sequence identity between them was 99.2% (Tables III and IV), which is significantly higher than the sequence identity between HQ709103 and sequences from other *Meloidogyne* species. The second most similar group of sequences to HQ709103 was from *M. graminicola* with a mean sequence identity of 94.8%. The mean sequence identity between HQ709103 and *M. incognita* sequences retrieved from the GenBank was 92.6%. On the other hand, the mean sequence identity between *M. incognita* from this study (HQ709104) and *M. incognita* sequences from the GenBank was 98.3%. However, HQ709104 had a sequence similarity higher than 98.3% with the GenBank sequences from three other species (*M. paranaensis*, *M. javanica* and *M. konaensis* Eisenbach, Bernard *et* Schmitt) (Table IV). HQ709104 and sequences of *M. exigua* from GenBank had a mean sequence identity of 93.2% (Table III).

Comparison of the *M. exigua* 28S rDNA sequences revealed that our sequence (HQ709103) was more similar to the sequence of an isolate from coffee in Brazil (AF435796) than to the sequence of isolates from rubber trees in Brazil (AF435795) and coffee in the UK (AF435804; Table III). The 28S rDNA sequence of *M. incognita* from this study (HQ709104) was identical to the sequences of three accessions from the USA and five accessions from Korea, but had a sequence identity of only 92.8% with another accession from the USA (AY355412; Table III). Similarly, comparison of the 28S rDNA sequences of *M. exigua*, including that of the present study, with those of the species listed in Table IV (excluding *M. oryzae, M. floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar *et* Higgins, *M. morocciensis* Rammah *et* Hirschmann, *M. ethiopica* Whitehead and *M. arabicida*) revealed that *M. exigua* has three unique variable sites at positions that correspond to the 26th, 31st and 55th nucleotides of the HQ709103 sequence. These sites are *T vs G/A*, *T vs C* and *T vs G/indel*, respectively. Unlike in *M. exigua*, there was no such unique mutation in *M. incognita*. We also made comparisons of sequences based on the combined sequences of the 18S and 28S rDNA regions. The sequence identity between *M. exigua* from the present study and from the GenBank was 98.7%, which is significantly higher than that between sequences of *M. exigua* and other *Meloidogyne* species. For *M. incognita*, the identity between sequences from this study and from the GenBank was 98.8% (Table IV).

DISCUSSION

The present study used a combination of tools (perineal pattern, SCAR markers and DNA sequence data) to identify and characterize the RKNs in Nicaraguan coffee plantations. Although analysis of perineal patterns of RKN is a difficult task that demands considerable skills because of significant intra-specific variation, it has been used as a diagnostic tool to provide support for species identification (Carneiro *et al.*, 1996a; Hernández *et al.*, 2004). In this study, considerable variation in perineal patterns was observed both within and between the eighteen isolates. However, after analyzing a large number of egg-laying females from each isolate, as suggested by Carneiro and Cofcewicz (2008), and comparing with previously published RKN perineal patterns, it was possible to group them into two distinct types in which *M. exigua* and *M. incognita* were considered as the most likely species. Although *M. incognita* was identified in only one of the eighteen isolates, its perineal pattern was more variable than that of *M. exigu*a.

The use of SCAR markers, originally developed by Randig *et al.* (2002) to discriminate the three major RKN species parasitizing coffee (*M. exigua*, *M. incognita* and *M. paranaensis*), on both single and bulk nematode extracts of all eighteen isolates successfully identified the nematodes as *M. exigua* and *M. incognita*. The results were in complete agreement with the identification based on perineal patterns. The RKN in coffee plantations are often found as mixed populations of more than one species in different proportions (Carneiro *et al.*, 1996a; Campos and Villain, 2005), as exemplified with isolate *M-15* in this study. In this isolate, the frequency of *M. exigua* was significantly higher than that of *M. incognita*. The application of a single nematode

based SCAR marker analysis on specimens that represent the perineal pattern variants identified the specimens either as *M. exigua* or *M. incognita* and thus suggests the absence of other RKN species in the eighteen coffee plantations sampled.

The analysis of the partial sequences of 18S and 28S rDNA genes revealed that both *M. exigua* and *M. incognita* from this study comprised single haplotypes regardless of the variation in perineal pattern within species. For 28S rDNA, the *M. incognita* haplotype from this study is the same as a haplotype previously reported by Chen *et al.* (2003) from the USA and Oh *et al.* (2009) from Korea (see Table IV). In the case of 18S rDNA, the haplotypes of both species are different from those retrieved from the GenBank. The same was observed for 28S rDNA from *M. exigua*.

The phylogenetic analysis by De Ley *et al.* (2002) on RKN showed that *M. incognita is* closely related to *M. javanica* and *M. arenaria* whereas *M. exigua* is closely related to *M. graminicola* and *M. chitwoodi* Golden, O'Bannon, Santo *et* Finley, which is in line with the maximum parsimony based phylogenetic analysis from the combined sequences of 18S and 28S rDNA in the present study (Fig. 3). *Meloidogyne incognita* shares obligatory mitotic parthenogenesis with its closely related species, *M. javanica*, *M. arenaria* and *M. paranaensis* (Chen *et al.*, 2003; Oh *et al.*, 2009) whereas *M. exigua* shares meiotic parthenogenesis with *M. oryzae, M. graminicola* and *M. fallax* (Tigano *et al.*, 2005). Both sequence identity and phylogenetic analyses suggest that the use of only 18S and 28S rDNA genes for species identification based on sequence identity is not a reliable approach, as it may lead to misclassification of the species. However, it seems that the 28S rDNA region can be used to identify *M. exigua*, as the 99.2% sequence identity between *M. exigua* haplotypes was significantly higher than their sequence identity with any other *Meloidogyne* species, which was also supported by the results of phylogenetic analysis. A combination of several fast-evolving nuclear and mitochondrial DNA regions should be used in order to resolve the phylogenetic relationship between the closely related RKN species discussed above.

Meloidogyne exigua has unique variable sites within both the 18S and 28S rDNA regions when compared to several other *Meloidogyne* species, including those sharing a similar reproductive mechanism. These variable sites can be used to develop new species-specific SCAR markers for the identification of this species. The suggestion by Page and Holmes (1998) to consider the 18S rDNA has been very useful in RKN phylogenetic studies but there are groups of species that are not well resolved. The comparison of the levels of sequence identity between haplotypes in terms of host similarity and geographic proximity did not show consistent patterns, suggesting that these species are less specialized in host preference and also may have less geographic differentiation.

Meloidogyne exigua is probably the most widespread species parasitizing coffee in America causing significant yield losses (Garcia *et al.*, 2009; Muniz *et al.*, 2009; Barbosa *et al.*, 2010). Thus, the presence of this species in Nicaraguan coffee plantations is not surprising. However, the fact that *M. exigua*, unlike *M. incognita*, is widely distributed in Nicaragua is important to consider. Many factors, such as soil type, the distribution of annual rainfall and the presence of other hosts, such as weeds and shade trees among others, could affect the distribution of *Meloidogyne* species in coffee plantations. For example Villain *et al.* (2008) indicated an association between soil types and the distribution of *Meloidogyne* species in coffee plantations in Central America. Barbosa *et al.* (2004) also reported a similar association for *M. paranaensis* and *M. incognita* in Brazil, which were restricted to certain localities.

Meloidogyne incognita is among the most economically important nematode pests, causing serious damage to a variety of crops throughout the world (Oh *et al.*, 2009), as it has a wide range of crop and weed hosts (Trudgill, 1997; Rich *et al.*, 2008). The effect of *M. incognita* on coffee plantations was reported to be most serious in Brazil (Campos and Villain, 2005). This species seems to be restricted in distribution in Nicaragua and thus an effective pest management strategy should be applied in order to prevent its spread to new areas. In areas where it already exists, the use of the recently identified resistant *C. arabica* genotype 'UFV 408-28' (Albuquerque *et al.*, 2010) and/or other resistant genotypes that might exist in the Nicaraguan coffee gene pool is recommended.

In summary our results demonstrated clearly the presence of *M. exigua* and *M. incognita* in the eighteen isolates collected from the two coffee growing regions of Nicaragua. As the levels of damage caused by nematodes depend greatly on the nematode species, our findings are of prime importance for the implementation of appropriate integrated pest management strategies. In this context the use of resistant coffee genotypes represents the most promising option for the control of *Meloidogyne* species (Noir *et al.*, 2003; Albuquerque *et al.*, 2010; Villain *et al.*, 2010).

ACKNOWLEDGMENTS

The authors wish to thank the Swedish International Development Agency (Sida) for financing this work. We are grateful to Marie-Louise Måtegen, Ann-Charlotte Strömdahl and Maimoun Hassoun for their assistance in the laboratory work. We would also like to thank Ledis Navarrete and Elvin Navarrete for their help in collecting nematode isolates from coffee plantations.

LITERATURE CITED

Albuquerque S.E.V., Carneiro R.M.D.G., Costa P., Gomes A.C.M.M., Santos M. and Grossi-de-Sa M.F., 2010. Resis-

tance to *Meloidogyne incognita* expressed a hypersensitive-like response in *Coffea arabica*. *European Journal Plant Pathology, 127*: 365-373.

Barbosa D.H.S.G., Viera H.D., Souza R.M., Viana A.P. and Silva C.P., 2004. Field estimate of coffee yield losses and damage threshold by *Meloidogyne exigua*. *Nematologia Brasileira*, *28*: 49-54.

Barbosa D.H.S.G., Souza R.M. and Viera H.D., 2010. Field assessment of coffee (*Coffea arabica* L.) cultivars in *Meloidogyne exigua* infested or free fields in Río de Janeiro State, Brazil. *Crop Protection, 29*: 175-177.

Bertrand B., Aguilar G., Bompard E., Rafinon A. and Anthony F., 1997. Comportement agronomique et résistance aux principaux déprédateurs des lignées de Sarchimor et Catimor (*Coffea arabica* L.) au Costa Rica. *Plantations, Recherche, Développpment, 4*: 312-321.

Blok V., 2005. Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Canadian Journal Plant Pathology*, *27*: 176-185.

Blok V.C., Phillips M.S. and Fargette M., 1997. Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root-knot nematodes. *Journal of Nematology, 29*: 16-22.

Blok V.C., Wishart J., Fargette M., Berthier K. and Phillips M., 2002. Mitochondrial DNA differences distinguish *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology, 4*: 773-781.

Campos V.P. and Villain L., 2005. Nematode parasites of coffee and cocoa. Pp. 529-579. *In*: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture – Second Edition (Luc M., Sikora R.A. and Bridge J., eds). CABI Publishing, Wallingford, UK.

Carneiro R.M.G.D. and Cofcewicz E.T., 2008. Taxonomy of coffee-parasitic root-knot nematodes, *Meloidogyne* spp. Pp: 87-122. *In*: Plant-parasitic Nematodes of Coffee (Souza R.M., ed.). Springer Science + Busines Media B. V., Dordrecht, Netherlands.

Carneiro R.M.G.D., Almeida R.M.A. and Carneiro R.G., 1996a. Enzyme phenotypes of Brazilian population of *Meloidogyne* spp. *Fundamental and Applied Nematology, 19*: 555-560.

Carneiro R.M.G.D., Carneiro R.G., Abrantes I.M.O., Santos M.S.N.A. and Almeida M.R.A., 1996b. *Meloidogyne paranaensis*. n. sp. (Nematoda: Meloidogynidae) a root-knot nematode parasitizing coffee in Brazil. *Journal of Nematology, 28*: 177-189.

Chen P., Roberts P.A., Metcalf A.E. and Hyman B.C., 2003. Nucleotide substitution patterning within the *Meloidogyne* rDNA D3 region and its evolutionary implications. *Journal of Nematology, 35*: 404-410.

De Ley I.T., De Ley P., Vierstraete A., Karssen G., Moens M. and Vanfleteren J., 2002. Phylogenetic analysis of *Meloidogyne* small subunit rDNA. *Journal of Nematology, 34*: 319-327.

Garcia A.L.A., Campos V.V. P. and Padilha L., 2009. Cultivars evaluation of *Coffea arabica* to the parasitism of *Meloidogyne exigua*. *In*: 22nd International Conference on Coffee Science, ASIC 2008, Campinas, SP, Brazil, 14-19 September, 2009. pp 1484-1486 [Abstr.]

Geleta M., Bryngelsson T., Bekele E. and Dagne K., 2007. Comparative analysis of genetic relationship and diagnostic markers of several taxa of *Guizotia* Cass. (Asteraceae) as relvealed by AFLP and RAPDs. *Plant Systematics and Evolution, 265*: 221-239.

Hall T., 2005. BioEdit v. 7.0.5. Biological sequence alignment editor for Windows. Ibis Terapeutics a division of Isis Pharmaceuticals. http://www.mbio.ncsu.edu/BioEdit/bioedit.html Accessed 01 Feb 2011.

Hartman K.M. and Sasser J.N., 1985. Identification of *Meloidogyne* species on the basis of differential host test and perineal-pattern morphology. Pp. 69-77. *In*: An Advanced Treatise on *Meloidogyne*. Vol. 2, Methodology (Barker K.R., Carter C.C. and Sasser J.N., eds). North Carolina State University Press, Raleigh, USA.

Hernández A., 1997. Étude de la variabilité intra et interspécifique des nématodes du genre *Meloidogyne* parasites des caféiers en America Central. These de Doctorat, Academie de Montepellier II Science et Techniques du Languedoc, Montpellier, France, 98 p.

Hernández M.G.R., Sánchez L., Arocha Y., Peteira B., Solórzano E. and Rowe J., 2001. Identification and characterization of *Meloidogyne mayaguensis* from Cuba. 33rd *Annual Meeting of Organization of Nematologists of Tropical America*, Cuba, Varadero, June 11-15 2001, pp 6-7, [Abstr.]

Hernández M.G.R., Fargette M. and Sarah J.L., 2004. Characterization of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) from coffee plantations in Central America and Brazil. *Nematology*, *6*: 193-204.

Hirschmann H., 1986. *Meloidogyne hispanica* n. sp. (Nematoda: Meloidogynidae) the "Seville root-knot nematode". *Journal of Nematology, 18*: 520-532.

Hugall A., Moritz C., Stanton J. and Wolstenholmes D.R., 1994. Low but strongly structured mitochondrial DNA diversity in root knot nematodes (*Meloidogyne*). *Genetics*, *136*: 903-912.

Jepson S.B., 1987. *Identification of root-knot nematodes*. CAB International, Wallingford, UK, 265 pp.

Landa B.B., Palomares R.J.E., Vovlas N., Carneiro R.M.D.G., Maleita C.M.N., Abrantes I.M de O. and Castillo P., 2008. Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. *Plant Disease, 92*: 1104 -1110.

Mulholland V., Carde L., O' Donnell K.J., Fleming C.C. and Powers T.O., 1996. Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. Pp. 247-252. *In*: Proceedings of Diagnostics in Crop Protection Symposium (Marshall G. ed.). *British Crop Production Council*, Farnham, UK.

Muniz M.F., Campos V., Moita A., Almeida M.R., Rodríguez F. and Carneiro R.M.D.G., 2009. Reaction of coffee genotypes to different populations of *Meloidogyne* spp.: Detection of a naturally virulent *M. exigua* population. *Tropical Plant Pathology*, *34*: 370-378.

Noir S., Anthony F., Bertrand B., Combes M.C. and Lashermes P., 2003. Identification of major gene (Mex-1) from *Coffea canephora* conferring resistance to *Meloidogyne exigua* in *Coffea arabica*. *Plant Pathology, 52*: 97-103.

Oh H.K., Bae C., Kim M., Wan X., Oh S., Han Y., Lee H. and Kim I., 2009. Molecular biological diagnosis of *Meloidogyne* species occurring in Korea. *Plant Pathology Journal, 25*: 247-255.

Ou S., Peng D., Liu X. and Moens M., 2008. Identification of *Heterodera glycines* using PCR with sequence characterized amplified region (SCAR) primers. *Nematology, 10*: 397-403.

Page R.D.M. and Holmes E.C., 1998. *Molecular evolution: A phylogenetic approach*. Blackwell Science Ltd., Oxford, UK, 352 pp.

Paran I. and Michelmore R.W., 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics, 85*: 985-993.

Randig O., Bongiovanni M., Carneiro R.M.D.G. and 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.

Reineke A., Karlovsky P. and Zebitz C.P.W., 1998. Preparation and purification of DNA from insects for AFLP analysis. *Insect Molecular Biology, 7*: 95-99.

Rich J.R., Brito J.A., Kaur R. and Ferrell J.A., 2008. Weed species as hosts of *Meloidogyne*: A review. *Nematropica, 39*: 157-185.

Rodríguez M.G., Rodríguez I. and Sánchez L., 1995. Especies del género *Meloidogyne* en Cuba. Distribución geográfica y sintomatología. *Revista de Protección Vegetal, 10*: 123-128.

Rosales-Mercado J.A. and Reyes-García P.A., 1991. Estudio de dinámica poblacional de nematodos en dos zonas cafetaleras de las regiones III y IV de Nicaragua. XIV Simposio sobre Caficultura Latinoamericana, 20-24 de mayo 1991, Ciudad Panamá, Panamá, pp. 291-304. [Abstr.]

Sequeira F., 1977. Muestreo de nematodos fitoparásitos en dos zonas cafetaleras de Nicaragua. Ministerio de Agricultura y Ganadería, Managua, Nicaragua, 13 pp.

Subbotin S.A., Peng D.L. and Moens M., 2001. A rapid method for the identification of soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology, 3*: 365-371.

Swofford D.L., 2000. PAUP*: Phylogenetic analysis using parsimony, version 4.0, beta. Sinauer Associates Inc., Sunderland, Massachusetts, USA.

Taylor D.P. and Netscher C., 1974. An improved technique for preparing perineal patterns of *Meloidogyne* spp. *Nematologica*, *20*: 268-269.

Thompson J.D., Gibson T.J. and Plewniak F., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*: 4876-4882.

Tigano M., Carneiro R.M.G.D., Jeyaprakash A., Dickson D. and Adams B., 2005. Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. *Nematology, 7*: 851-862.

Trudgill D.L., 1997. Parthenogenetic root-knot nematodes (*Meloidogyne* spp.); how can these biotrophic endoparasites have such an enormous host range? *Plant Pathology, 46*: 26-32.

Villain L., Anzueto F., Hernández A. and Sarah J.L., 1999. Los nematodos parásitos del cafeto. Pp. 327-368. *In*: Desafíos de la Caficultura en Centroamérica (Bertrand B. and Rapidel B., eds). IICA-PROMECAFE, San José, Costa Rica.

Villain L., Sarah J.L., Hernandez A., Charmetant P., Bertrand B., Anthony F., Topart P., Lashermes P., Anzueto F. and Carneiro R.M.D.G., 2007. Biodiversity of root-knot nematodes, *Meloidogyne* spp., on coffee in Central América. 21st International Conference on Coffee Science, Montpellier, France, 11-15 September 2006, pp. 1321-1324.

Villain L., Hernández A. and Anzueto F., 2008. Central America. Pp. 261-275. *In*: Plant Parasitic Nematodes of Coffee (Souza R., ed). Springer Science + Busines Media B.V., Dordrecht, The Netherlands.

Villain L., Aribi J., Réversat G. and Anthony F., 2010. A high-throughput method for early screening of coffee (*Coffea* spp.) genotypes for resistance to root-knot nematodes (*Meloidogyne* spp.). *European Journal of Plant Pathology, 128*: 451-458.

Zijlstra C., Donkers-Venne D.T.H.M. and Fargette M., 2000. Idenfication of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based PCR assays. *Nematology, 2*: 847-853.

Accepted for publication on 10 June 2011.