Biometrical, Biochemical, and Molecular Diagnosis of Portuguese *Meloidogyne hispanica* Isolates

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Abstract

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Meloidogyne hispanica infects many economically important crops worldwide. The accurate identification of this pathogen is essential for the establishment of efficient and sustainable integrated pest management programs. Portuguese *M. hispanica* isolates were studied by biometrical, biochemical, and molecular characteristics. Biometrical characteristics of *M. hispanica* females, males, and second-stage juveniles were similar to the original description. Biochemical studies revealed a unique enzyme pattern (Hi4) for *M. hispanica* esterases that allowed for species differentiation. Molecular analysis of the mtDNA region from *COII* and 16S rRNA genes resulted in amplification products (1,800 bp) similar to *M. hispanica*, *M. ethiopica*, and *M. javanica*, and the described *Hin*fI was unable to discriminate *M. hispanica* from the other two species. Analysis of the mtDNA sequences revealed altered nucleotides among the isolates that created new restriction sites for *Alu*I and *Dra*III. The resulting restriction patterns successfully discriminated between the three species, providing a new tool for *Meloidogyne* identification. Finally, the phylogenetic relationship between *M. hispanica* and several *Meloidogyne* spp. sequences was analyzed using mtDNA, confirming the divergence between meiotic and mitotic species and revealing the proximity of *M. hispanica* to closely related species. Based on the studies conducted, the application of isozyme or polymerase chain reaction restriction fragment length polymorphism analysis would be a useful and efficient methodology for *M. hispanica* identification.

The "Seville root-knot nematode", isolated from peach rootstock (*Prunus persica* (L.) Batsch) in Spain, was studied for the first time by Dalmasso and Bergé (17) and described later as *Meloidogyne hispanica* Hirschmann, 1986 (27). This species has a worldwide distribution, and has been reported infecting economically important crops in Africa (22,34,48), Asia (21), Australia (21), Europe (4,21,31,49), and North, Central, and South America (10,15,21).

M. hispanica is cytologically similar to the diploid race of *M. arenaria*, and morphologically very close to *M. arenaria*, *M. floridensis*, and *M. incognita* (13,25,27). When tested using the North Carolina differential host test, *M. hispanica* isolates have host responses similar to *M. arenaria* race 2 or *M. javanica* (4,13), *M. arenaria* race 1 or *M. incognita* race 2 (4,27), and *M. incognita* race 3 (4), showing an intraspecific variability among the isolates of this species. The identification of *M. hispanica* only on the basis of morphological characteristics, especially on perineal patterns, or on the pattern of disease reactions induced in the North Carolina differential hosts, is very difficult. One isolate of *M. hispanica* from South Africa was erroneously associated with *M. arenaria thamesi* on the basis of perineal pattern morphology (34). Therefore, biochemical and molecular markers should be used for differentiating this species from the other *Meloidogyne* spp. and to confirm or clarify root-knot nematode (RKN) diagnoses made in the past. The biochemical electrophoretic analysis of nonspecific esterases remains one of the first stages in species identification when adult females are available (4,9,12). Three phenotypes (Hi2, S2-M1=Hi3, and Hi4) have been detected and all the isolates shared two common major bands that have been used to differentiate this species (4,27,28,35).

With the expansion of DNA-based methodologies, new alternatives have been developed and have been shown to be an attractive solution not only for the identification of RKN populations but also to provide important data for phylogenetic analysis. Molecular approaches for distinguishing RKN species have included several techniques, and different regions of the DNA have been studied (8,38,39,42,51). However, isolates of *M. hispanica* have been included in only a few studies (14,35,37,42).

In a study based on restriction fragment length polymorphisms (RFLPs) obtained from amplified mtDNA of *Meloidogyne* isolates digested with *Hin*fI, the patterns of *M. hispanica* consisted of two fragments distinct from those of *M. arenaria*, *M. hapla, M. incognita*, and *M. javanica* (14). The restriction pattern obtained with the probe, designated as pMiK4, showed two bands common to *M. arenaria*, *M. hispanica, M. incognita*, and *M. javanica* (1.6 and 3.0 kb). However, all the species could be distinguished by the presence of a characteristic band for *M. arenaria* at 3.5 kb, *M. incognita* at 2.0 kb, *M. hispanica* at 1.5 kb, and *M. javanica* at 0.7 kb (37). A multiplexed polymerase chain reaction (PCR) test, based on the amplification of *Meloidogyne* mtDNA with primers MORF, MTHIS, TRNAH, and MRH106 and digested with *Hin*fI or *Mnl*I, differentiated *M. hispanica* from *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, and *M. javanica* (42). Recently, in order to support the specific identification of *M. hispanica*, species-specific PCR and phylogenetic analyses of sequences from three rDNA regions (18S, internal transcribed spacer [ITS]1-5.8S-ITS2, and D2-D3 of 28S) were used to characterize three *M. hispanica* isolates from Brazil, Portugal, and Spain (35).

The purposes of this research were to study extensively seven Portuguese isolates of *M. hispanica* by biometrical, biochemical, and molecular characteristics, and develop a new molecular diagnostic method for the identification and differentiation of *M. hispanica* from other RKN species based on the mtDNA region

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between *COII* and 16S rRNA genes. The phylogenetic relationship of *M. hispanica* to other *Meloidogyne* spp. with available mtDNA sequences was also analyzed.

Materials and Methods

Nematode isolates. Seven isolates of *M. hispanica* were used in this study and another eight *Meloidogyne* spp. isolates were included in the biochemical and molecular studies for comparison (Table 1). All the isolates used in this study were originally obtained from a single egg mass and maintained on tomato (*Solanum lycopersicum* L. 'Easypeel') by periodic subculturing in a growth chamber at 25 ± 2°C, in the Nematology Laboratory (IMAR-CMA, Department of Life Sciences, University of Coimbra, Portugal).

Biometrical studies. Morphological and morphometrical studies were conducted on second-stage juveniles (J2) and adult males and females of each of the seven isolates of *M. hispanica* (Table 1). Adult males were recovered from infected roots, J2 hatched from egg masses in moist chambers, and adult females handpicked from infected tomato roots. Freshly hatched J2, adult males and females, and perineal patterns were prepared for light microscope studies as previously described by Abrantes and Santos (2) and measured immediately. Photographs were taken with a Leitz Dialux 20 bright field light microscope.

Freshly hatched J2 and adult males and females were processed for scanning electron microscope studies as described by Abrantes and Santos (2). Perineal patterns of adult females and stylets of J2 and adult males and females were also prepared as described by Abrantes and Santos (1,2) and Eisenback (19). The specimens were mounted on stubs, coated with gold (200Å), viewed, and photographed with a JEOL JSM-35C. At least 50 specimens of each life stage, perineal patterns, and excised stylets of J2 and adult males and females were examined.

Biochemical studies. For isozyme analysis, young egg-laying females (5/tube or 10/tube for PtCh) of each *Meloidogyne* sp. and isolate (Table 1) were handpicked from infected tomato roots and transferred to micro-hematocrit tubes with 5 µl of extraction buffer (20% [wt/vol] sucrose and 1% [vol/vol] Triton X-100). The specimens were macerated with a pestle, frozen, and stored at –20°C. Electrophoresis was performed according to Pais et al. (36) in vertical gels in a Mini-Protean Tetra System (Bio-Rad Laboratories, Hercules, CA). The gels were stained for esterase activity with the substrate α-naphthyl acetate. Protein extracts of *M. javanica* young egg-laying females were included in each gel as a reference. Phenotypes were designated with a letter(s) suggesting the nematode species, followed by a number indicating the number of bands (21). Biochemical procedures were repeated every time that the isolates were used in molecular studies.

Molecular studies. *DNA extraction.* Freshly hatched J2 of each isolate (Table 1), obtained from egg masses, were concentrated by centrifugation for 2 min at 594 × *g* and stored at –20°C in Eppendorf tubes. Packed juveniles were placed at –80°C for at least 1 h before genomic DNA extraction and purification according to Randig et al. (40), with modifications. The J2 were mechanically squashed with a pestle, homogenized, and 91.7 µl of lysis buffer (0.1 M Tris-HCl, pH 8.0; 50 mM EDTA; 1% [wt/vol] sodium dodecyl sulfate; and 0.17 M NaCl) and 8.3 µl of proteinase K (6 µg/µl) was added. After the incubation of the homogenates at 60°C for 1 h and 10 min at 95°C, 1 µl of a solution of ribonuclease A (Sigma-Aldrich, St. Louis) at 10 mg/ml was added and the tubes were incubated at 37°C for 15 min. DNA was purified with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) for 1 min, mixed by gentle inversion of the Eppendorf tube, and centrifuged at 14,841 × *g* for 1 min at 4°C. The supernatant was transferred to a new Eppendorf tube, 2.5 volumes of absolute ethanol was added to precipitate the DNA, and the mixture was centrifuged at 14,841 × *g* for 20 min at 4°C. The precipitate was washed with 500 µl of ice-cold 70% ethanol, and dried at 45°C for ±15 min. The DNA was resuspended in 20 µl of sterilized distilled water.

Sequencing. Mitochondrial DNA from isolates PtHi3 of *M. hispanica* and ItE of *M. ethiopica* were sequenced with the primer set C2F3 (5′-GGT CAA TGT TCA GAA ATT TGT GG-3′) and MRH106 (5′-AAT TTC TAA AGA CTT TTC TTA GT-3′) located in the *COII* gene and the 16S rRNA gene (38,42). PCR was performed in a 50-µl volume containing 1× buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.2 µM each primer, 2.5 U of Platinum Taq DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, CA), and 100 ng of nematode DNA. Amplifications were carried using the following conditions: an initial denaturation at 94°C for 4 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 68°C for 2 min; and a final extension for 10 min at 68°C. The amplified products were purified with the High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) and quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE), according to the manufacturer's instructions. Amplified DNA was sequenced in both directions with the same amplification primers and a new intermediate primer due to the high length of the fragment (5′-GAT CGG GGT TTA ATA ATG GG-3′), by standard procedures at Macrogen, Inc. (Seoul, Korea). Chromatograms were checked and corrected manually by use of the freely available online Chromas software (Technelysium Pty Ltd., Brisbane, Australia). The mtDNA sequences of *M. hispanica* and *M. ethiopica* were deposited in GenBank as JN673274 and JN673275, respectively.

Sequences from the PtHi3 and ItE isolates were aligned with ClustalW (45) within BioEdit software (24) with *Meloidogyne* spp. mtDNA sequences between *COII* and the 16S rRNA genes available in the GenBank nucleotide database (National Center of Bio-

Table 1. *Meloidogyne* isolates, hosts, geographic origin, and studies where they have been included in this work

Species (isolate code)[a]	Host plant	Geographic origin	Reference	Studies[b]
Meloidogyne hispanica				
(PtHi1)	*Solanum lycopersicum* L.	Coimbra	...	Mb, B
(PtHi2)	*Ficus carica* L.	Setúbal	4	Mb, B, M
(PtHi3)	*F. carica* L.	Faro	4	Mb, B, M
(PtHi4; selected from PtHi3)	*Capsicum annuum* L.	...	...	Mb, B, M
(PtHi5)	*Dianthus caryophyllus* L.	Aveiro	35	Mb, B, M
(PtHi6)	*S. tuberosum* L.	Santarém	16	Mb, B, M
(PtHi7)	*S. tuberosum* L.	Aveiro	16	Mb, B, M
M. arenaria (PtA)	*Oxalis corniculata* L.	Coimbra	...	B, M
M. chitwoodi (PtCh)	*S. tuberosum* L.	Porto	16	B, M
M. hapla (PtH)	*Alnus glutinosa* (L.) Gaertn.	Viana do Castelo	...	B, M
M. incognita (PtI)	*Cucumis melo* L.	Açores	...	B, M
M. javanica (PtJ)	*S. tuberosum* L.	Guarda	...	B, M
M. mayaguensis (VnM)	*Malpighia glabra* L.	Cabudare	...	B, M
M. megadora (STMe)	*Coffee arabica* L.	S. Tomé	3,5,6	B, M
M. ethiopica (ItE)	*S. lycopersicum* L.	Pontecagnano	...	B, M

[a] Pt = Portugal; Vn = Venezuela; ST = Democratic Republic of S. Tomé and Príncipe; It = Italy.
[b] B = Biochemical studies; M = polymerase chain reaction restriction fragment length polymorphism analysis; Mb = morphobiometrial studies.

technology Information, www.ncbi.nlm.nih.gov). The sequences of PtHi3 and ItE isolates were compared with sequences of *M. ethiopica*, *M. incognita*, and *M. javanica*, because they showed similar products of amplification. We used the freely available online WebCutter 2.0 software to determine restriction enzyme maps based on altered nucleotides between the isolates that created new restriction sites. Enzymes with varied digestion product sizes within *Meloidogyne* spp. were selected to allow for easier species identification during visualization by agarose gel electrophoresis.

PCR-RFLP. PCR amplification was conducted with the primer set C2F3 and MRH106 as already described (38,42). PCR reactions were performed in a 25-µl volume containing 1× buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 2.5 U of Taq DNA polymerase (Bioline, London), and 50 ng of nematode DNA. The amplifications were carried out in a MyCycle Thermal Cycler (Bio-Rad Laboratories) using the following conditions: an initial denaturation at 94°C for 4 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 2 min; and a final extension for 10 min at 72°C. The PCR reaction (5 µl) was analyzed on 1% agarose gel electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer stained with ethidium bromide.

Amplification products with approximately 1,800 bp were digested with 5 U of *Hinf*I (Amersham Biosciences, Barcelona, Spain). If no digestion occurred with *Hinf*I, the amplified products were digested separately with 5 U of *Alu*I (USBiological, Swampscott, MA) and *Dra*III (New England BioLabs Inc., Ipswich, MA). *Dra*III restriction enzyme was applied in 2 µl of PCR products and *Hinf*I and *Alu*I were applied in 3 µl according to the manufacturer's instructions. The digestion was conducted at 37°C for 5 h to *Dra*III and for 8 h to *Hinf*I and *Alu*I restriction enzymes. Restriction fragments were separated on 2% agarose gel electrophoresis in 1× TAE buffer stained with ethidium bromide. Molecular procedures were repeated at least three times to verify the results.

Phylogenetic analysis. The sequences of *Meloidogyne* spp. from different locations included in the phylogenetic analysis were obtained by searching the GenBank nucleotide database.

Sequences from the PtHi3 and ItE isolates were aligned using Muscle (18) with all sequences or only those of related species with similar amplification products for the mtDNA region studied (*M. arabicida*, *M. ethiopica*, *M. incognita*, and *M. javanica*). Alignment was manually adjusted when necessary. The length of all sequences of *Meloidogyne* spp. was set to 1,673 bp by removing several nucleotides to obtain a common start and end point. The phylogenetic relationship was reconstructed using neighbor-joining (NJ) (30) and maximum likelihood (ML) (41) methods. NJ analyses were performed using the Maximum Composite Likelihood model (43) with complete deletion. One thousand bootstrap replicates were performed to test the support of each node on the trees (23). ML analyses were based on the Jukes-Cantor model (30). All positions containing gaps and missing data were eliminated. Alignment and evolutionary analyses were conducted in MEGA5 (44).

Results

Biometrical studies. Morphometrics of adult females and males and J2 of the seven Portuguese *M. hispanica* isolates are reported in Table 2. Adult females of *M. hispanica* showed a globose to ovoid shape with a distinguished neck (128.3 to 303.3 µm neck) and without tail protuberance. The body was rounded posteriorly (Fig. 1A) and the cephalic region was slightly prominent, not annulated, and very small when compared with body size (Fig. 1A and B). The stylet length ranged from 11.1 to 19.0 µm, with the cone curved dorsally, widening gradually posteriorly, the shaft straight, and the knobs with indented anterior margins (Fig. 1C and D). The distance between the stylet base and the dorsal esophageal gland orifice was short to long (2.8 to 6.8 µm) (Fig. 1B–D). Excretory pore position was variable, 13.2 to 77.9 µm from anterior end. Perineal patterns varied from ovoid to rounded (Fig. 1E–G). Dorsal arch was generally low; however some patterns showed a higher and quadrangular arch. Dorsal striae were relatively uniform, smooth, and straight, sometimes wavy. Ventral pattern area generally showed fine and smooth striae. Lateral lines were distinct; some dorsal and ventral striae connected with each other to originate an angle, while others forked. The perivulval region was not striated. Phasmidial ducts were distinct (Fig. 1E and F).

Adult males lengths ranged from 1,400.0 to 2,357.1 µm, with the anterior end slightly tapered and the posterior rounded (Fig. 1H–J and L). The labial disc was large, elongated, raised, and fused with the medial lips that were narrow and crescent shaped, with smooth outer margins. The head region was smooth (Fig. 1I and J). The stylet was robust and large with a straight cone, pointed and widening gradually to the posterior end (Fig. 1K). Excretory pore position was variable (147.5 to 240.0 µm from anterior end). Spicules were long (28.0 to 41.1 µm) and slightly curved; the gubernaculum was crescent shaped. The tail was short and round, with phasmids generally very close to the level of cloaca (Fig. 1L). The lateral field had four longitudinal incisures.

Body length of J2 varied from 320.0 to 514.4 µm, with a truncate head region and a narrow tail region (Fig. 1M and Q). The labial disc was raised and fused with the crescent shaped medial lips. The head region was usually smooth, occasionally with 1 to 3 incomplete annulations (Fig. 1N). The stylet length was 9.2 to 13.0 µm, with the cone tip slender, widening gradually to the posterior end; the shaft was cylindrical, widening gradually to the posterior end; and the knobs were rounded to ovoid and separated, sloping posteriorly (Fig. 1M and O). The tail had a rounded tip and the hyaline tail terminus was indistinct. The rectal dilation was large (Fig. 1P and Q).

Biochemical studies. The three esterase bands observed in the *M. javanica* isolate (Rm: 0.37, 0.43, and 0.46) were used as a reference isolate to determine the relative position of bands observed in the other *Meloidogyne* isolates (J3; Fig. 2).

In the *M. hispanica* isolates studied, four bands of esterase activity (two major bands and two minor and fainter bands) were detected (Rm: 0.32, 0.35, 0.38, and 0.41), corresponding to the phenotype Hi4 (Fig. 2). The identification of the other eight *Meloidogyne* spp. isolates used for comparison in the molecular studies was confirmed by the esterase phenotypes (Fig. 2; Table 1). In these isolates, 19 bands of esterase activity were observed, comprising eight phenotypes on the basis of single bands or combinations. All of them contained distinct and species-specific phenotypes. The esterase phenotype of *M. hispanica* was clearly distinct from the other *Meloidogyne* spp. used in this study (Fig. 2).

Molecular studies. The mtDNA *COII* and 16S rRNA genes region was selected to carry out the molecular characterization of *M. hispanica* isolates. This region, amplified with the primer set C2F3/MRH106 from purified DNA extracted from J2 from each of the *Meloidogyne* isolates, yielded single fragments of four different sizes (approximately 650, 850, 1,300, and 1,800 bp; Fig. 3). Specifically, the isolates of *M. hapla* and *M. chitwoodi* (with esterase phenotype H1 and Ch1) had a PCR product of approximately 650 bp; the *M. mayaguensis* isolate (M4) produced a fragment of 850 bp; the *M. arenaria* isolate (A2) had a fragment of 1,300 bp; and the isolates of *M. hispanica* (Hi4), *M. ethiopica* (E3), *M. javanica* (J3), and *M. incognita* (I2) gave a fragment of approximately 1,800 bp (Figs. 2 and 3). No amplification occurred with the *M. megadora* isolate (Me3).

When the amplified products of approximately 1,800 bp were digested with the restriction enzyme *Hinf*I, three patterns of digestion were observed but only *M. incognita* was clearly differentiated. Two fragments of approximately 1,300 and 400 bp were produced in the *M. incognita* isolate and two fragments of approximately 1,700 and 100 bp were generated in *M. hispanica* isolates and *M. ethiopica.* No digestion occurred in the *M. javanica* isolate (Fig. 4). Molecular analysis of the mtDNA region, with the described *Hinf*I PCR-RFLP, was unable to discriminate *M. hispanica* from *M. ethiopica* and *M. javanica*. In order to obtain useful information for species discrimination, the PCR products of PtHi3 (*M. hispanica*) and ItE (*M. ethiopica*) isolates for the mtDNA region were sequenced and compared with equivalent sequences of *M. ethiopica* and *M. javanica* available in GenBank. Analysis of the

nucleotide variation observed in the alignment and the restriction enzyme maps produced in WebCutter revealed that altered nucleotides between the isolates created new restriction sites for the enzymes *Alu*I and *Dra*III. Digestion of the approximately 1,800 bp mtDNA PCR product with *Alu*I generated three fragments of approximately 1,000, 580, and 240 bp for *M. hispanica* (six isolates; data shown for only one isolate) and *M. javanica*, while two fragments of approximately 1,240 and 580 bp were observed for *M. ethiopica* (Fig. 5A). On the other hand. *Dra*III could not digest *M. hispanica* but generated two fragments of approximately 1,000 and 800 bp for *M. ethiopica* and *M. javanica* (Fig. 5B).

Phylogenetic analysis. Mitochondrial DNA sequences (which include part of the *COII*, a variable intergenic region, tRNAHis, and part of the 16S rRNA gene) of PtHi3 (*M. hispanica*) and ItE (*M. ethiopica*) isolates were determined and, although the sequences were not complete, the sequence size of the PtHi3 isolate was 1,680 and 1,684 bp in the ItE isolate. The sequences were compared with the corresponding fragments from closely related species which displayed similar amplification products (*M. arabicida*, *M. ethiopica*, *M. incognita*, and *M. javanica*). The PtHi3 sequence differed by 22 nucleotide positions from *M. ethiopica* (ItE and Me, AY942848), *M. incognita* from Thailand (FY159614), and *M. javanica* (AY635612). The mtDNA sequences of *M. ethiopica* isolates from Italy (ItE) and Brazil (Me) were similar, with only seven differences in alignment. The differences included nucleotide changes at positions 781, 1055, 1148, and 1624; two insertions at 987 and 1340; and, finally, one deletion at position 58 (*data not shown*). Amplified product of *M. arabicida* was slightly longer than those of other species, showing several changes, including two insertions between positions 937 and 951 and 1,068 and 1,128, and one deletion between 864 and 892 (*data not shown*).

Table 2. Morphometric comparison of adult females and males and second-stage juveniles of seven Portuguese *Meloidogyne hispanica* isolates with original *M. hispanica* description (Hirschmann 1986)[a]

	Females		Males		Second-stage juveniles	
Characteristic	**Portuguese isolates**	**Hirschmann 1986**	**Portuguese isolates**	**Hirschmann 1986**	**Portuguese isolates**	**Hirschmann 1986**
Linear (µm)						
Body length	723.3 ± 79.2	830.0 ± 150.4	1,846.2 ± 170.1	1,677.6 ± 168.4	418.4 ± 28.8	392.6 ± 18.7
	(570.0–920.0)	(570.0–1,180.0)	(1,400.0–2,357.1)	(1,340.6–1,990.0)	(320.0–514.4)	(356.4–441.4)
	11.0	18.1	9.2	10.0	6.9	4.8
Body width	503.8 ± 62.6	503.0 ± 108.9	41.6 ± 4.8	41.1 ± 3.5	14.6 ± 1.2	14.5 ± 0.5
	(365.0–660.0)	(330.0–740.0)	(32.5–58.6)	(32.4–47.4)	(13.3–17.8)	(13.4–15.8)
	12.4	21.6	11.7	8.4	8.0	3.7
Neck length	195.4 ± 35.5	232.7 ± 71.7				
	(128.3–303.3)	(140.0–440.0)				
	18.1	30.8	…	…	…	…
Neck width	145.2 ± 24.3	129.8 ± 31.6				
	(93.3–221.7)	(60.0–200.0)				
	16.8	24.4	…	…	…	…
Body width at stylet knobs	…	…	21.7 ± 1.4	20.3 ± 0.7	9.3 ± 0.4	
			(14.3–25.7)	(18.3–21.4)	(8.4–10.5)	
			6.3	3.6	4.5	…
Body width at excretory pore	…	…	34.9 ± 3.0	33.6 ± 2.3	13.5 ± 0.6	13.9 ± 0.4
			(28.5–48.6)	(28.8–38.6)	(11.1–15.3)	(13.1–15.0)
			8.6	6.9	4.5	2.9
Body width at anus	…	…	…	…	10.5 ± 0.6	10.8 ± 0.4
					(9.2–12.0)	(10.2–11.7)
					5.3	3.3
Head region height	…	…	5.1 ± 1.2	7.6 ± 0.3	1.8 ± 0.3	2.7 ± 0.2
			(2.4–9.2)	(7.0–8.1)	(1.1–2.4)	(2.4–3.1)
			23.3	3.9	13.8	6.5
Head region width	…	…	12.6 ± 0.6	12.8 ± 0.5	5.1 ± 0.3	5.4 ± 0.2
			(11.0–14.5)	(11.8–13.7)	(4.5–6.1)	(4.8–5.7)
			5.0	3.8	5.3	2.9
Stylet length	14.7 ± 1.3	14.1 ± 0.3	23.0 ± 1.0	23.5 ± 0.6	10.6 ± 0.8	11.1 ± 0.3
	(11.1–19.0)	(13.6–14.6)	(20.0–24.7)	(21.7–24.3)	(9.2–13.0)	(10.4–11.9)
	8.5	1.9	4.2	2.6	7.2	2.7
Stylet knob height	2.5 ± 0.28	2.5 ± 0.2	3.2 ± 0.3	3.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.1
	(1.8–3.7)	(2.1–2.8)	(2.4–4.0)	(2.9–3.5)	(1.0–1.6)	(1.2–1.6)
	11.1	7.1	9.9	4.8	12.4	7.0
Stylet knob width	4.3 ± 0.3	4.6 ± 0.2	5.1 ± 0.3	5.6 ± 0.3	2.1 ± 0.2	2.6 ± 0.1
	(3.2–5.0)	(4.1–5.1)	(4.2–5.8)	(5.1–6.1)	(1.6–2.9)	(2.3–2.8)
	7.1	5.1	6.6	5.5	9.5	5.0
Dorsal esophageal gland orifice	4.81 ± 0.89	3.2 ± 0.3	3.4 ± 0.8	2.5 ± 0.5	3.6 ± 0.6	2.8 ± 0.3
	(2.8–6.8)	(2.8–4.0)	(2.1–7.9)	(1.4–3.6)	(2.4–5.3)	(2.2–3.4)
	18.6	10.6	23.9	18.2	15.2	9.6
Excretory pore to anterior end	35.2 ± 10.3	30.5 ± 12.3	185.4 ± 18.0	181.5 ± 20.3	79.8 ± 3.8	80.1 ± 2.7
	(13.2–77.9)	(11.9–71.1)	(147.5–240.0)	(148.6–254.1)	(61.6–86.8)	(73.9–86.0)
	29.1	40.3	9.7	11.2	4.7	3.3
Interphasmidial distance	24.2 ± 3.6	22.1 ± 2.8				
	(15.7–35.7)	(16.7–28.1)				
	15.0	12.7	…	…	…	…
Vulva length	25.8 ± 3.1	23.5 ± 1.2				
	(14.3–34.3)	(20.0–25.4)				
	12.0	5.3	…	…	…	…

(*continued on next page*)

[a] All measurements in micrometers with mean ± standard deviation (range) coefficient of variation. Data for Portuguese isolates are means of seven isolates.

M. hispanica displayed sequence divergences ranging from 0.5 to 1.5% when compared with the other species, and *M. ethiopica* (ItE) from 0.1 to 1.5% (Table 3).

Both NJ and ML trees obtained were similar; therefore, only the NJ tree is exhibited with bootstrap values (Figs. 6 and 7). The five *Meloidogyne* spp. with similar amplification products clustered separately. *M. ethiopica* and *M. javanica* were sister taxa to *M. hispanica* (PtHi3) but with lower bootstrap support (50%; Fig. 6). The isolate ItE formed a clade with *M. ethiopica* with 94% bootstrap. *M. incognita* and *M. arabicida* were the most divergent species (Fig. 6; Table 3).

The tree obtained from NJ analysis of mtDNA sequences of several *Meloidogyne* spp. available in GenBank showed that all *Meloidogyne* spp. formed two well-supported clades (72 and 95%) with the exclusion of *M. graminis* (Fig. 7). First, *M. chitwoodi* and *M. fallax* were clustered together with high bootstrap support (100%) and as a sister taxon to *M. graminicola* (95%). Second, *M. hispanica* formed a clade well supported (99%) with *M. thailandica*, *M. arenaria*, *M. morocciensis*, *M. javanica*, *M. floridensis*, *M. incognita*, *M. ethiopica*, *M. paranaensis*, and *M. arabicida*; however, the relationship within this clade was poor. This clade was a sister to *M. haplanaria* and *M. mayaguensis*. Another clade was formed by *M. partityla* and *M. hapla* (90%; Fig. 7).

Discussion

Effective *M. hispanica* management programs require accurate and rapid identification tools for this nematode species. However, identification of *Meloidogyne* spp. is difficult due to morphological similarity between species, intraspecific variability, and the number of described *Meloidogyne* spp. (33).

Range values of the morphometric characteristics for adult females and males and J2 of the seven isolates overlapped or were within the expected range according to Hirschmann (27). The morphology of the different development stages of the *M. hispanica* isolates was similar to the original description (27). *M. hispanica* has several morphobiometric characteristics similar to other *Meloi-*

Table 2. (*continued from preceding page*)

	Females		Males		Second-stage juveniles	
Characteristic	**Portuguese isolates**	**Hirschmann 1986**	**Portuguese isolates**	**Hirschmann 1986**	**Portuguese isolates**	**Hirschmann 1986**
Vulva-anus distance	20.5 ± 2.2 (12.1–25.7) 11.0	19.0 ± 1.3 (17.2–22.6) 6.7				
			…	…	…	…
Tail length	…	…	10.8 ± 1.9 (5.8–16.6) 17.7	13.3 ± 1.7 (10.7–16.2) 13.1	48.1 ± 3.8 (33.2–56.8) 7.8	46.4 ± 2.8 (41.1–53.4) 6.1
Phasmids to tail end	…	…	13.1 ± 2.9 (7.1–20.8) 22.0	14.4 ± 2.3 (10.3–19.4) 16.1	…	…
Spicule length	…	…	34.7 ± 2.1 (28.0–41.1) 6.0	32.1 ± 0.8 (31.1–33.7) 2.4	…	…
Gubernacule length	…	…	8.1 ± 0.8 (5.8–11.8) 10.4	8.3 ± 0.5 (7.4–9.3) 5.8	…	…
Testis length	…	…	949.3 ± 180.1 (471.4–1,357.1) 19.0	808.4 ± 113.6 (650.0–1,150.0) 14.0	…	…
Ratios						
a	1.5 ± 0.2 (1.2–2.0) 10.1	1.7 ± 0.3 (0.9–2.2) 17.0	44.7 ± 4.3 (36.5–55.4) 9.6	41.2 ± 6.1 (31.4–61.4) 14.8	28.7 ± 2.5 (22.4–35.7) 8.6	27.1 ± 1.2 (24.6–30.9) 4.5
c	…	…	176.7 ± 36.9 (104.7–350.4) 20.9	128.1 ± 19.0 (98.2–172.6) 14.8	8.7 ± 0.9 (5.8–12.8) 10.3	8.5 ± 0.4 (7.7–9.4) 4.3
d	…	…	…	…	4.6 ± 0.4 (3.3–5.6) 9.0	4.3 ± 0.3 (3.8–5.0) 6.5
Body length/neck length	3.8 ± 0.6 (2.4–5.3) 15.0	3.7 ± 0.8 (2.5–5.9) 21.0	…	…	…	…
Head region width/height	…	…	2.6 ± 0.7 (1.3–5.1) 24.9	1.7 ± 0.1 (1.6–1.8) 2.9	2.8 ± 0.4 (2.0–4.8) 14.9	2.0 ± 0.1 (1.7–2.3) 6.9
Stylet knob width/height	1.7 ± 0.2 (1.1–2.4) 13.2	1.9 ± 0.2 (1.6–2.2) 7.7	1.6 ± 0.2 (1.2–2.2) 11.0	1.7 ± 0.1 (1.5–2.0) 5.9	1.7 ± 0.3 (1.2–2.8) 15.0	1.8 ± 0.1 (1.6–2.2) 7.1
Stylet length/body width at stylet knobs	…	…	1.1 ± 0.1 (0.8–1.5) 8.0	…	…	…
Excretory pore from anterior end/stylet length	2.4 ± 0.7 (0.8–4.8) 30.0	…	…	…	…	…
Percentages						
(Excretory pore to head end/body length) × 100	…	…	10.1 ± 1.0 (7.1–13.3) 10.0	10.9 ± 1.7 (8.2–18.1) 15.1	19.1 ± 1.2 (14.0–25.8) 6.5	20.4 ± 0.6 (19.0–21.7) 3.1
T	…	…	51.3 ± 8.0 (28.3–69.7) 15.6	48.7 ± 8.2 (32.7–62.2) 16.8	…	…

dogyne spp., including *M. arenaria*, *M. floridensis*, and *M. incognita*, which has resulted in taxonomic confusion (25,27,34). Therefore, the biometrical diagnostic characteristics need to be supported by other characteristics resulting from biochemical and molecular studies.

The esterase phenotypes of the seven *M. hispanica* isolates were similar (Hi4). The two major bands were used to characterize the isolates of this species because the two fainter bands can vary with the amount of protein and time of staining. This pattern is unique in *M. hispanica* and is very useful to differentiate this species from other *Meloidogyne* spp. (3,4,10–12,21,22,35).

The mtDNA-PCR-RFLP analysis was also an efficient methodology to discriminate *M. hispanica* from the other RKN species. The amplified product of *M. hispanica* (approximately 1,800 bp), using primers C2F3 and MRH106, has been reported in this study for the first time. These primers had been used to identify the major RKN species occurring in China (50). Taking into consideration that the amplified products with primers C2F3/MRH106 were approximately 130 bp larger than those obtained with primers C2F3/1108, our results for *M. hapla* (approximately 650 bp), *M. chitwoodi* (approximately 650 bp), *M. mayaguensis* (approximately 850 bp), *M. javanica* (approximately

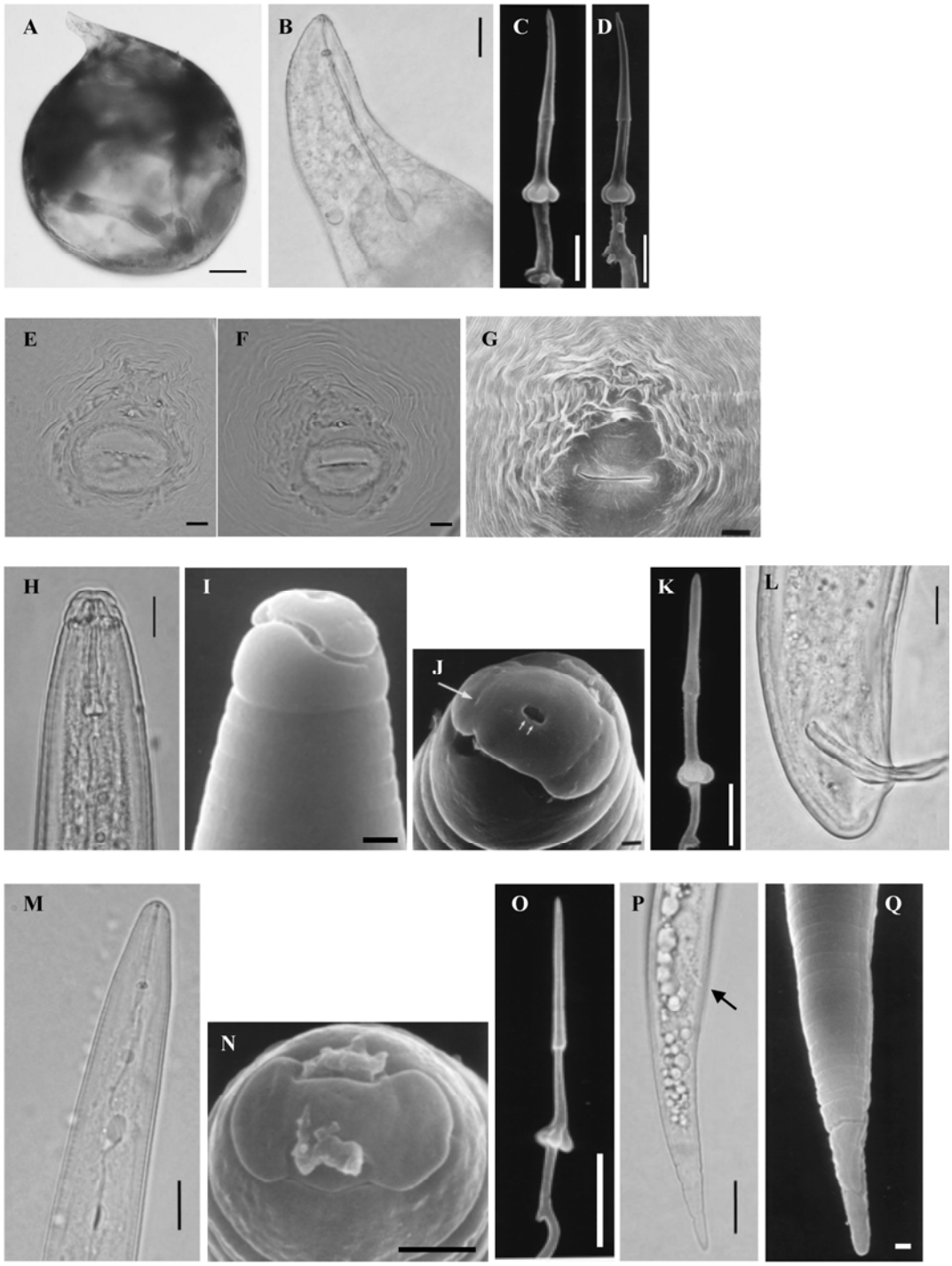

Fig. 1. A, **B**, **E**, **F**, **H**, **L**, **M**, and **P,** Light and **C**, **D**, **G**, **I**, **J**, **K**, **N**, **O**, and **Q,** scanning electron microscope photographs of *Meloidogyne hispanica*. Females: **A,** whole specimen; **B,** anterior end and esophageal region; **C** and **D,** excised stylets; and **E–G,** perineal patterns. Males: **H** and **I,** anterior region in lateral view; **J,** head region showing cephalic sensilla (arrow) and inner labial sensilla (double arrow); **K,** excised stylet; and **L,** posterior region in lateral view. Second-stage juveniles: **M,** anterior region; **N,** head region; **O,** excised stylet; and **P** and **Q,** tail region with **P,** anus and inflated rectum (arrow). Scale bars: 100 µm (A), 20 µm (B), 10 µm (E–H, L, M, and P), 5 µm (C ,D, K, and O), 2 µm (I), and 1 µm (J, N, and Q).

1,800 bp), and *M. ethiopica* (approximately 1,800 bp) agree with previous reports (8,26,29,38,46,50). The *M. arenaria* isolate produced a fragment of approximately 1,300 bp, which agrees with other studies, except with the populations from the French West Indies which have produced a PCR product of approximately 1,700 bp (8,29,38,46,50). Although *M. incognita* has been reported as having populations that produced two sizes of PCR products (approximately 1,500 and 1,700 bp) with C2F3/1108 primers, the Portuguese isolate produced a product with approximately 1,800 bp (8,29,38,46).

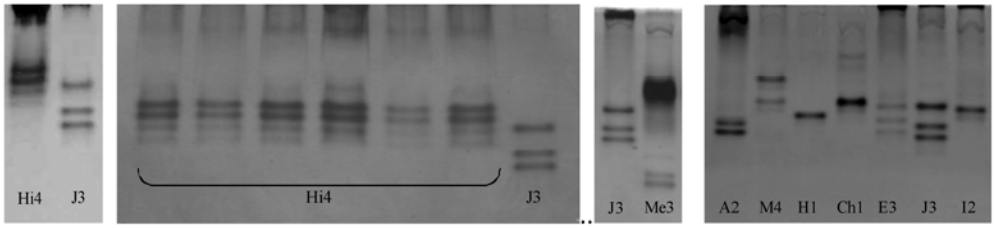

Fig. 2. Esterase phenotypes of protein homogenates from five egg-laying females of *Meloidogyne* spp. isolates included in this study. Hi4, *Meloidogyne hispanica*; J3, *M. javanica* (reference isolate); Me3, *M. megadora*; A2, *M. arenaria*; M4, *M. mayaguensis*; H1, *M. hapla*; Ch1, *M. chitwoodi* (10 egg-laying females); E3, *M. ethiopica*; and I2, *M. incognita*.

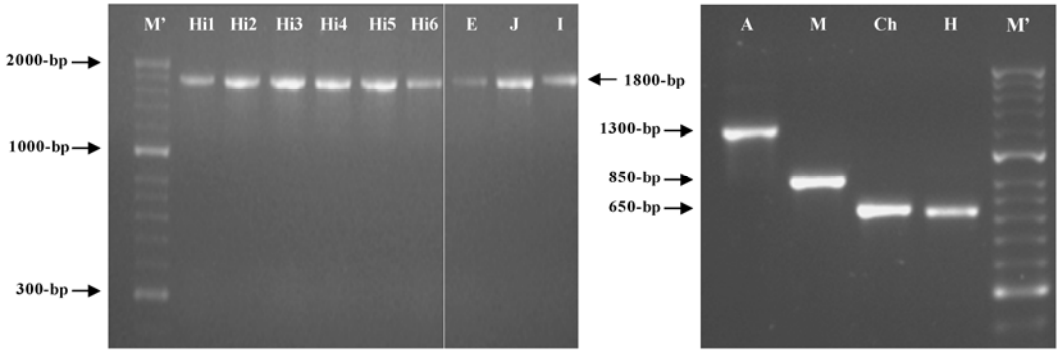

Fig. 3. DNA amplification products obtained from 13 isolates of eight *Meloidogyne* spp. using C2F3 and MRH106 primers. Hi2–Hi7, *Meloidogyne hispanica*; E, *M. ethiopica*; J, *M. javanica*; I, *M. incognita*; A, *M. arenaria*; M, *M. mayaguensis*; Ch, *M. chitwoodi*; H, *M. hapla*; and M', DNA marker (HyperLadder II; Bioline).

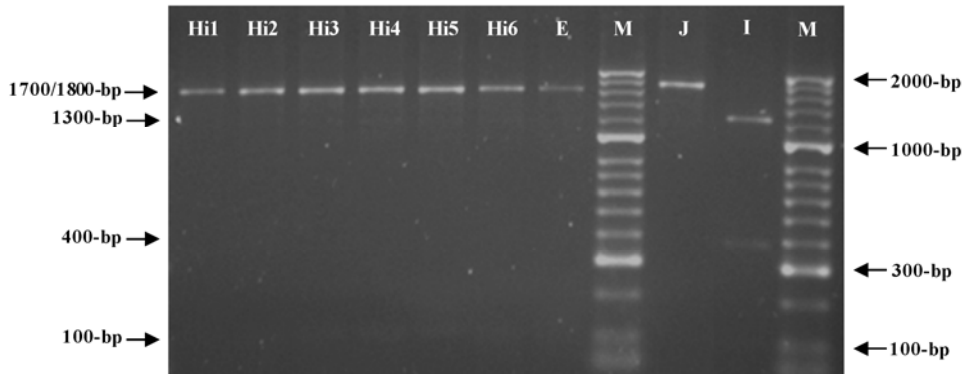

Fig. 4. *Hin*fI digestion patterns of the approximately 1,800-bp amplification products from *Meloidogyne* spp. after 8 h of digestion. Hi2–Hi7, *Meloidogyne hispanica*; E, *M. ethiopica*; J, *M. javanica*; I, *M. incognita*; and M, DNA marker (HyperLadder II; Bioline).

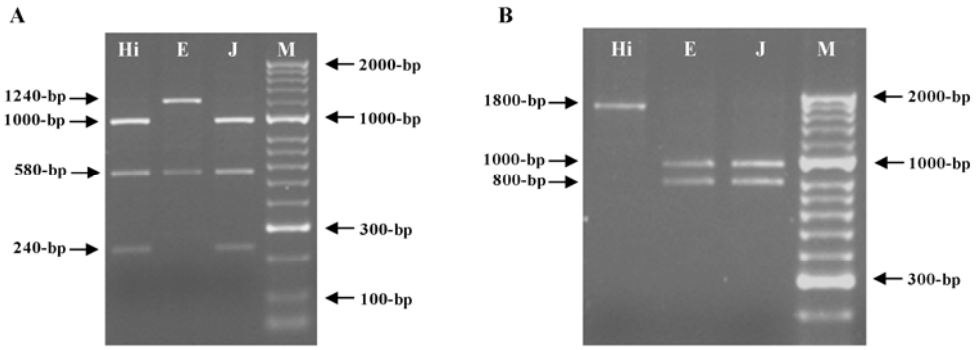

Fig. 5. A, *Alu*I and **B,** *Dra*III digestion patterns of the approximately 1,800-bp amplification products from *Meloidogyne* spp. after 8 and 5 h of digestion. Hi, *Meloidogyne hispanica* (similar for all *M. hispanica* isolates); E, *M. ethiopica*; J, *M. javanica*; and M, DNA marker (HyperLadder II; Bioline).

Table 3. Pairwise sequence divergences between *Meloidogyne hispanica* (PtHi3), *M. arabicida* (Mar, AY942852), *M. ethiopica* (ItE and Me, AY942848), *M. incognita* (Mi, AY635611; Mi1, FY159614; and Mi2, FJ159616), and *M. javanica* (Mj, AY635612 and Mj1, FJ159612) sequences of mtDNA using MEGA5[a]

Species	PtHi3	ItE	Mar	Me	Mi	Mi1	Mi2	Mj	Mj1
PtHi3	…	…	…	…	…	…	…	…	…
ItE	0.007	…	…	…	…	…	…	…	…
Mar	0.015	0.015	…	…	…	…	…	…	…
Me	0.005	0.001	0.013	…	…	…	…	…	…
Mi	0.008	0.007	0.016	0.006	…	…	…	…	…
Mi1	0.006	0.006	0.014	0.005	0.003	…	…	…	…
Mi2	0.006	0.006	0.014	0.005	0.003	0.001	…	…	…
Mj	0.006	0.005	0.014	0.003	0.007	0.005	0.005	…	…
Mj1	0.005	0.004	0.013	0.003	0.007	0.005	0.005	0.000	…

[a] Analyses were conducted using the Maximum Composite Likelihood model. All positions containing gaps and missing data were eliminated.

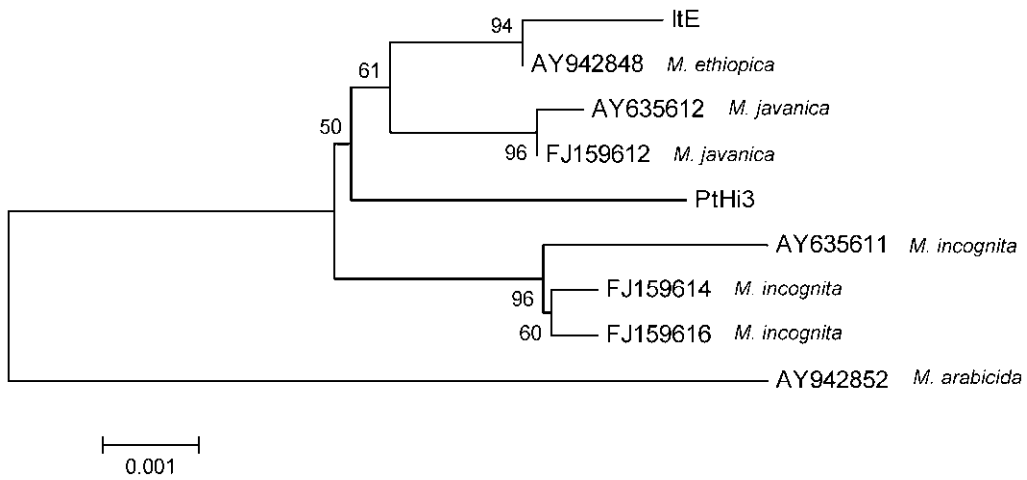

Fig. 6. Neighbor-joining tree based on mtDNA sequences of *Meloidogyne* spp. with approximate amplification product sizes to *Meloidogyne hispanica* (PtHi3) and *M. ethiopica* (ItE). The percentage of replicate trees in which the associated *Meloidogyne* spp. clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Evolutionary distances were computed using the Maximum Composite Likelihood method and all positions containing gaps and missing data were eliminated.

*Hinf*I restriction patterns of the approximately 1,800 bp amplified products discriminated *M. incognita* from the other species but no digestion occurred in *M. javanica*, which is in agreement with the results reported by Xu et al. (50). *M. hispanica* was distinguished from *M. hapla* and *M. chitwoodi*, *M. mayaguensis*, and *M. arenaria* by the size of the PCR products but showed the same size product of amplification as that of *M. ethiopica, M. incognita*, and *M. javanica*. *M. hispanica* could be differentiated from *M. javanica* and *M. ethiopica* by the size of amplified products after digestion with *Dra*III and *Alu*I, as predicted by the restriction enzyme maps. The sequence of the Italian *M. ethiopica* isolate was identical to previous reports for this species, which confirmed the results obtained by isozyme analysis (E3). The RFLP strategy implemented required only one to four steps for quick identification of eight important and damaging *Meloidogyne* spp. based on variable mtDNA amplification products and respective nucleotide variations. This methodology could be adapted to single specimens for use in routine examination of soil samples where J2 are found.

Mitochondrial DNA has been used for molecular diagnostics at the species level and construction of phylogenies of RKN (7). The mtDNA region from *COII* and 16s rRNA genes varies in length and encompasses an AT-rich noncoding sequence with different sizes, as a result of deletions and insertions (7,29). The RKN species *M. arabicida*, *M. arenaria*, *M. ethiopica*, *M. floridensis*, *M. hispanica*, *M. incognita*, *M. javanica*, *M. morocciensis*, and *M. paranaensis* formed a well-supported clade, with the exclusion of *M. mayaguensis* and *M. hapla.* This group includes the mitotic parthenogenetic RKNs that possess the AT-rich region; except for *M. floridensis*, which reproduces by facultative meiotic parthenogenesis, and *M. thailandica*, for which the mode of reproduction has not yet been studied (25,32). *M. mayaguensis* formed a second group with *M. haplanaria* (57% of bootstrap support) and showed an amplified product for the *COII*/16s rRNA region different from all studied species (8). According to Eisenback et al. (20), *M. haplanaria* was distinct from other species, such as *M. chitwoodi*, *M. fallax*, and *M. graminis* (meiotic parthenogenetic pathogens); *M. hapla* (reproduction by facultative meiotic or mitotic parthenogenesis); and *M. arenaria*, *M. incognita*, and *M. javanica* (reproduction by mitotic parthenogenesis), which grouped with *M. mayaguensis*. On the other hand, *M. partityla*, which reproduces by obligatory mitotic parthenogenesis, was included together with species that reproduce by meiotic parthenogenesis and lack the AT-rich region in the amplified product (7,29). Although the mode of reproduction of *M. hapla* isolates used for mtDNA sequencing is not mentioned in the GenBank database, in general, the results support the hypothesis that the ancestral state of the genus is characterized by the absence of this AT-rich region, and the evolution of *Meloidogyne* spp. is related to the mode of reproduction, with amphimixis being the ancestral reproductive state of the genus (7,47).

Considering the main clades, our results also agree with those obtained by Tigano et al. (46) for the 18S rRNA gene. Relationships between RKN species were not clarified for mtDNA, which limits the confidence of this molecule for species discrimination. Both NJ analyses showed that mtDNA sequences were not enough to differentiate *M. hispanica* and *M. ethiopica* from other species with morphological similarities, such as *M. arenaria*, *M. floridensis*, and *M. incognita* (25,27). Thus, the examination of more than one molecular characteristic is very important for identification and evolution studies of a particular species. The MP analysis of the D2-D3 region of 28S rDNA and ITS revealed that *M. hispanica* formed a clade with high bootstrap support and distinct from *M. arenaria*, *M. incognita*, *M. konaensis*, and *M. paranaensis*. However, *M. hispanica* has been reported to have an 18S rDNA sequence identical to *M. ethiopica* (35).

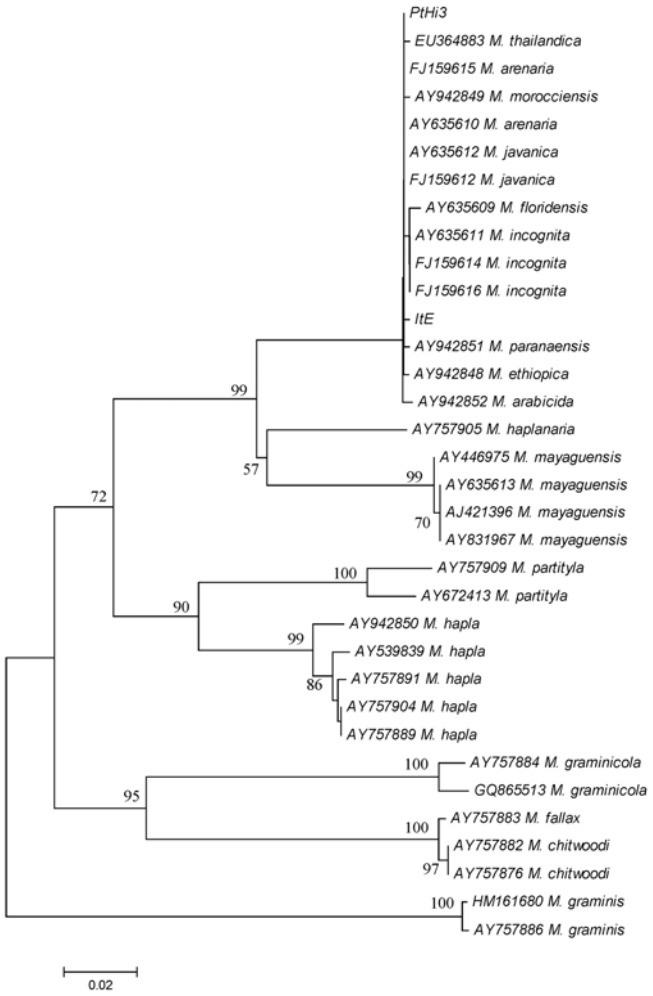

Fig. 7. Neighbor-joining tree based on analysis of alignment and adjusting the length of sequences of mtDNA region of *Meloidogyne hispanica* (PtHi3) and *M. ethiopica* (ItE) with available sequences of other *Meloidogyne* spp. Evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were eliminated.

The reliable and rapid identification of *M. hispanica*, a potentially economically important plant pathogen, is of great importance for the diagnosis of this species and can be useful to monitor its distribution and spread. Isozyme analysis remains an effective methodology for precise identification and differentiation of *M. hispanica* (Hi4) when females are available in field root samples. Variability in mtDNA sequences among *Meloidogyne* spp. allowed the discrimination of *M. hispanica* and *M. ethiopica* from six other species studied by PCR-RFLP, providing a new tool for *Meloidogyne* spp. identification based on J2, the most common stage found in the soil, eliminating the need to use females from roots of field samples or to establish a culture in the laboratory. However, it will be necessary to analyze a broad range of *M. hispanica* isolates to validate the obtained results and strengthen the potential applicability of the mtDNA-PCR-RFLP analysis in diagnosis.

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