

## Characterization of a Root-Knot Nematode Population of *Meloidogyne arenaria* from Tupungato (Mendoza, Argentina)

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**Abstract:** Root-knot nematodes (*Meloidogyne* spp.) are polyphagous plant parasites of global importance. Successful host infection depends on the particular interaction between a specific nematode species and race and a specific plant species and cultivar. Accurate diagnosis of nematode species is relevant to effective agricultural management; and benefits further from understanding the variability within a single nematode species. Here, we described a population of *M. arenaria* race 2 from Mendoza (Argentina). This study represents the first morphometric, morphological, biochemical, reproductive, molecular, and host range characterization of a root-knot nematode species from Argentina. Even after gathering morphological and morphometric data of this population and partially sequencing its rRNA, an unequivocal taxonomic assignment could not be achieved. The most decisive data was provided by esterase phenotyping and molecular methods using SCARs. These results highlight the importance of taking a multidimensional approach for *Meloidogyne* spp. diagnosis. This study contributes to the understanding of the variability of morphological, reproductive and molecular traits of *M. arenaria*, and provides data on the identification of root-knot nematodes on tomato cultivars from Argentina.

**Key words:** *Meloidogyne arenaria*, morphology, root-knot nematode, molecular taxonomy.

Root-knot nematodes belong to the genus *Meloidogyne* Göldi 1887, which are highly-adaptable, obligate, and polyphagous plant parasites. *Meloidogyne* spp. are distributed worldwide and parasitize most flowering plants including economically relevant crops (Moens et al., 2009). Together with other root-knot nematodes, *M. arenaria* is apomictic, and multiplies by mitotic parthenogenesis (Triantaphyllou, 1985; Trudgill and Block, 2001). Nematodes with apomictic reproduction are particularly difficult to control because of their high reproductive rate, short generation time, and wide host range (Trudgill and Block, 2001). The root-knot nematode *M. arenaria* can infect almost every plant family.

Thirteen new species of *Meloidogyne* have been described in the last decade, totaling more than 90 recognized species in the genus (Hunt and Handoo, 2009; Moens et al., 2009). Because distinct species of root-knot nematodes attack different plant cultivars, and resistance is species- or cultivar-specific, and only effective against one or a few nematode species or races, successful control of root-knot nematodes requires accurate identification of the nematodes present in the field (Eisenback, 1982). Diagnosis of species and races of *Meloidogyne* has always been challenging due to highly similar morphology across species, life stages in different habitats, variable host ranges, poorly defined boundaries among species, intraspecific variability, potential hybrid origin, and polyploidy (Blok and Powers, 2009). Species of *Meloidogyne* have been traditionally identified based on morphological features, isozyme patterns, and host

plant response to infection (Esser et al., 1976; Eisenback, 1985; Esbenshade and Triantaphyllou, 1985). Recently, molecular tools have been progressively developed (Powers and Harris, 1993; Powers, 2004; Adam et al., 2007; Blok and Powers, 2009). However, each approach has its limitations. Morphological analyses of several developmental stages of the nematode combined with host plant response tests and molecular analyses should help in the correct identification of a nematode population (Eisenback, 1985; Hunt and Handoo, 2009; Perichi and Crozzoli, 2010).

The root-knot nematodes are among the most relevant group of plant parasitic nematodes in Argentina and are widely distributed throughout the country. At least ten *Meloidogyne* species were reported from this country (Esbenshade and Triantaphyllou, 1985; Doucet and Pinochet, 1992; Chaves, 2001; Hunt and Handoo, 2009; Moens et al., 2009), but the economic impact of root-knot nematodes has not been accurately estimated. Information on the economic losses caused by *Meloidogyne* spp. is fragmented and restricted to particular areas of Argentina (Doucet and Pinochet, 1992; Doucet and Ponce de León, 1995; Doucet and Lax, 2007).

Only few populations of other nematode species from Argentina have been characterized in detail (Doucet, 1989; Lax and Doucet, 2001; Vovlas et al., 2007) and no studies on root-knot nematodes from this country have been undertaken, which constitutes a disadvantage for agricultural management and for understanding *Meloidogyne* species variability around the world. The goals of this study are: i) to characterize a population of root-knot nematodes from a production zone in Tupungato (Mendoza, Argentina); ii) to contribute to the understanding of the species variability using morphological, morphometric, biochemical, molecular, and reproductive traits as well as host range; and iii) to provide data on the identification of root-knot nematodes infecting tomato cultivars from Argentina.

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## MATERIALS AND METHODS

**Nematode population:** A population of root-knot nematodes was collected from a tomato production field in Tupungato, province of Mendoza (Argentina). Second-stage juveniles (J2) obtained from a single egg mass isolate were reared and maintained on susceptible tomato plants (*Solanum lycopersicum* cv. Roma). Tomato plants were cultivated in a greenhouse in pots using autoclaved soil, at 25°C and 16 hr photoperiod.

**Morphometric and morphological characterization:** Female nematodes were dissected from infected tomato roots and maintained in water at 4°C until observation. For morphometric analyses, females were placed on a glass slide with a drop of water and measured under the microscope. To observe the perineal pattern, the posterior end of each female was cut with a razor blade and mounted in clear with lactophenol solution (Franklin and Goodey, 1949). Alternatively, egg-laying females were dissected from infected roots, cleared with lactophenol solution at 75°C for 30 min and observed under the microscope (Robertson et al., 2009). We quantified the number of hatching larvae per egg mass laid by eight females, by placing two egg masses per petri dish with water. Hatched J2s were counted and removed from the petri dishes for morphological characterization (see below), every two days for 60 days. After 55 days, we did not observe any more juveniles hatching. Second-stage juveniles were placed on a slide in a drop of water, immobilized by heat, and covered with a glass coverslip. Males were isolated from the soil taken from infected tomato plants, using the centrifugal-flotation method (Jenkins, 1964). All observations were done under a Nikon microscope (Eclipse E200, USA) at x400 and x1000.

**Biochemical identification:** We analyzed the esterase phenotype of the nematode population, following a protocol by Esbenshade and Triantaphyllou (1985). Young egg-laying females were dissected from roots and macerated in 20 µl of extraction buffer (20% sucrose, 2% Triton X-100) using a small glass pestle. The macerates were frozen immediately and stored at -20°C. Before electrophoresis, the frozen samples were thawed and a drop of Bromophenol blue (1 mg/ml) was added to each tube, which contained 15 to 20 females. The native PAGE was carried out at 5°C in a 7 cm x 8 cm polyacrylamide gel, 0.75-mm thick, in a Bio-Rad (Bio-Rad Labs, Hercules, CA, USA) MiniProtean Tetra System. The separating gel was 7 or 10% polyacrylamide, pH 8.8, but best results were obtained with the highest concentration of polyacrylamide. The stacking gel was 4% polyacrylamide, pH 6.8 and the running buffer was Tris-glycine (3 g/liter Tris, 14.4 g/liter glycine), pH 8.3. Electrophoresis conditions were 80V for 15 min and 130V for 50 min or until the Bromophenol blue migrated to the end of the gel. The gel was rinsed in phosphate buffer pH 6.3 for 15 min at 4°C and stained

for esterase activity. The staining solution (9.5 ml 0.1 M phosphate buffer pH 6.3, 10 mg Fast Garnet GBC, and 10 mg alpha and beta naphthyl acetate dissolved in 0.5 ml acetone) was added to the container with the gel and kept at room temperature for 40 to 60 min in the dark. Females of *Meloidogyne javanica* were included as a reference.

**Molecular analyses:** DNA was extracted using Illustra Nucleon Phytopure Genomic DNA Extraction Kits (GE Healthcare, Buckinghamshire, UK), from a pool of 6,000 second-stage juveniles isolated from egg masses incubated in water in petri dishes. Amplification of the internal transcribed spacer (ITS) of the ribosomal DNA was done by PCR using primers rDNA2 (5'TTTCACCTCGCCGTTACTAAGG 3') and 28Sr (5'TTGATTACGTCCCTGCCCTTT 3') following Vrain et al. (1992). PCR conditions included initial denaturing at 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 8 min. Amplification products were sequenced by Sanger sequencing with Applied Biosystems 3730XL. Sequences were deposited in GenBank (JX099853) and aligned using MacClade 4.0 (Maddison and Maddison, 2000). A phylogenetic analysis was performed with Garli 0.951 (Zwickl, 2006) under the General Time Reversible model with parameters for invariable sites and gamma-distributed rate heterogeneity. The substitution model was chosen by using Modeltest (Posada and Crandall, 1998). Ten independent runs were conducted using either the automated stopping criterion or for up to 5,000,000 generations to ensure convergence to a similar topology and likelihood score. A hundred bootstrap replicates were performed.

Additional PCR amplification using specific primers were done following Adam et al. (2007). Primer combinations, 194 (5' TTAAGTTGCCAGATCGGACG3') and 195 (5'TCTAATGAGCCGTACGC3') (Blok et al., 1997); Fjav and Rjav (Zijlstra et al., 2000); Mi-F (5'GTGAGGATTCAGCTCCCCAG3') and Mi-R (5'ACGAGGAACA TACTTCTCCGTCC3') (Meng et al., 2004); and Far (5' TCGGCGATAGAGGTAAATGAC 3') and Rar (5' TCGCGATAGACACTACAAACT3') (Zijlstra et al., 2000), were those included in the molecular diagnostic key developed by Adam et al. (2007). PCR conditions included initial denaturing at 94°C for 2 min; followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min; followed by a final extension at 72°C for 8 min.

**Differential host test:** The host range characterization was done following the Differential Host Test (Sasser and Triantaphyllou, 1977). Each of six standard crop plant cultivars were inoculated with 100 second-stage juveniles: *Nicotiana tabacum* cv. NC 95 (tobacco); *Gossypium hirsutum* cv. Deltapine 16 (cotton); *Capsicum frutescens* cv. California Wonder (pepper); *Citrullus vulgaris* cv. Charleston Grey (watermelon); *Arachis hypogaea* cv. Florunner (peanut), and *Solanum lycopersicum* cv. Rutgers

(tomato). Four replicates of each plant cultivar were included. Infected plants (total: 24 plants) were maintained in a greenhouse at 25°C and 16 hr photoperiod and fertilized as needed. After 60 days, plants were harvested and roots were washed with water and stained with cold yellow eosin solution (0.1 g/L) for 30 min. Stained egg masses and root galls on the root system were counted. Each plant cultivar was classified as susceptible (+) or resistant (-), when the average egg mass count was > 3 or ≤ 3 egg masses per root system, respectively.

**Infection symptoms:** The symptoms of the disease were observed in susceptible tomato plants *Solanum lycopersicum* cv. Roma. The overall plant morphology and the distribution pattern of the root galls were examined.

**Reproductive factor (Rf):** A greenhouse study was conducted to determine the reproduction factor of the nematode population in tomato plants. Eight susceptible tomato plants (*Solanum lycopersicum* var. Río Grande) were cultivated in individual pots containing 270 cc of sterilized loamy soil. Tomato plants with four true leaves were inoculated with 100 second-stage juveniles per pot and maintained in a greenhouse at 25°C and 16 h photoperiod. Plants were fertilized as needed. Sixty days after inoculation, all second-stage juveniles were isolated from the soil of each pot by the centrifugal-flotation method (Jenkins, 1964) and all egg masses

were manually separated from the roots and counted. Eggs were extracted from egg masses placed in 0.1% NaOCl and counted under the microscope. The reproductive factor (Rf= Pf/Pi) was estimated by dividing the average final nematode population (Pf) by the initial nematode population (Pi) (Roberts and May, 1986). The Pf was calculated as the number of eggs on the root system and juveniles in the soil of each pot, while the initial nematode population (Pi) was equal to 100 J2.

RESULTS AND DISCUSSION

Accurate identification of root-knot nematodes is crucial to choose the appropriate management strategy, given that each nematode species has a specific plant host range and biological features. Descriptions of nematode populations from around the world are essential to understand the morphological, biochemical and molecular variability within a species and to recognize stable characters for their diagnosis (Skantar et al., 2008; Hunt and Handoo, 2009). Only few populations of nematodes from Argentina have been well characterized, including the false root-knot nematode *Nacobbus aberrans* (Doucet, 1989; Vovlas et al., 2007), the nematode *Heterodera glycines* (Lax and Doucet, 2001) and *Pratylenchus* spp. (Doucet and Cagnolo, 1998), but no detailed descriptions of root-knot nematodes have

TABLE 1. Comparison of measurements (in µm) of females, eggs, second-stage juveniles, and males of *Meloidogyne arenaria* and *M. incognita*.

	Population under study <sup>a</sup>	<i>M. arenaria</i> Chitwood 1949	<i>M. arenaria</i> Cliff & Hirschmann 1985 <sup>c</sup>	<i>M. incognita</i> Chitwood 1949
<b>FEMALES</b> n = 20				
Body length	672.03 (465.04-1128.95) [210.59]	510-1000		510 - 690
Body width	380.87 (207-529) [11.45]	400-600		300 - 430
Stylet length	16.70 (14.03-19.74) [1.17]	14-16	15.1 (13.1-16.7) [0.05]	15-16
Stylet knob width	4.89 (4.18-5.55) [0.35]	4-5	4.7 (3.8-5.5) [0.03]	4-5
DGO <sup>b</sup> from stylet base	5.09 (4.23-6.24) [0.66]	4-6	4.8 (3.1-6.6) [0.06]	2-4
Distance between phasmids	24.33 (21.01-26.6) [1.61]	28-31		
<b>EGGS</b> n = 30				
Length	98.66 (85.86-106.23) [5.22]	77-105		80 - 98
Width	42.56 (36.21 -50.86) [3.83]	32-44		30 - 38
<b>JUVENILES J2</b> n = 30				
Body length	458.51 (419.62-493.69) [20.48]	450-490	503.6 (391.6-605.2) [4.26]	360 - 393
Body width	15.35 (13.88-16.95) [0.76]		15.3 (12.8-17.8) [0.09]	
Stylet length	14.1 (12.29-15.3) [0.7]	10	11.1 (10.1-11.9) [0.03]	10
Distance from excretor pore to head	86.89 (69.1-98.48) [6.92]		89.8 (75-105.2) [0.56]	
DGO from stylet base	3.61 (2.85-4.1) [0.41]	3	3.7 (2.7-4.7) [0.04]	2 - 2.5
Tail length	56.21 (50.64-63.39) [2.78]		56 (43.6-69.4) [0.53]	
Alpha	29.91 (26.70 -32.92) [1.69]	26-32	33.1 (22.4-40.5) [0.29]	29 - 33
Gamma	8.17 (7.26-8.97) [0.34]	6-7.5	9 (7.5-10.9) [0.05]	8 - 9.4
<b>MALES</b> n = 20				
Body length	1604.00 (721.8-1981.74) [315.68]	1270-2000	1720 (979-2279) [287.22]	1200-2000
Body width	43.15 (28.43-54.25) [7.03]		36 (27-48) [3.89]	
Stylet length	23.06 (21.09-25.16) [1.01]	20-24	23 (20-28) [1.46]	23-26
DGO from stylet base	5.65 (4.76-6.84) [0.50]	4-7	6 (4-8) [0.96]	2,5 (1.75 - 3.5)
Stylet knob width	5.16 (3.98-6.04) [0.49]	4-5	5 (4-6) [0.45]	5.5 - 6.5
Spicule length	35.70 (31.25-40.26) [2.62]	31-34	32 (27-39) [1.98]	34-36

<sup>a</sup> Mean (range) [SD]: Numbers represent the mean values, the range is given in parenthesis and the standard deviation (SD) in brackets.

<sup>b</sup> DGO: dorsal esophageal gland orifice

been published. In this study, we identified and described a South American population of the root-knot nematode *Meloidogyne arenaria* isolated from a tomato field in Argentina by examining morphological, biochemical, molecular and reproductive features, along with host range of this nematode population.

**Morphometric analyses:** Among the characters commonly used to define nematode species and populations, morphometric characters have a marked significance. The magnitude of these characters is influenced by several factors and can show significant differences not only between species but among populations within a species (Doucet and Cagnolo, 1998; Carneiro et al., 2008; Skantar et al., 2008). Morphometrics of females, eggs, second-stage juveniles and males of the population of *M. arenaria* under study were compared with typical descriptions of this species and *M. incognita* (Table 1). In general, the population of *M. arenaria* under study showed slightly more amplitude in the range of some morphometric values, while averages were generally consistent with previous descriptions (Chitwood, 1949; Esser et al., 1976; Cliff and Hirschmann, 1985); several measures were overlapping with typical *M. incognita* (Table 1).

**Morphological characterization of females:** Species of *Meloidogyne* can be identified based on female adult morphology, including head structures, perineal patterns, and stylet (Eisenback et al., 1980). The perineal pattern is a valuable morphological feature used in the species identification of the genus *Meloidogyne* (Chitwood, 1949; Eisenback et al., 1980; Hirschmann, 1985). However, significant perineal pattern variability observed in females from a single-egg mass population undermines the value of this character for comparing *Meloidogyne* species (Chitwood, 1949; Netscher, 1978; Karssen and van Aelst, 2001; Carneiro et al., 2004).

We analyzed the perineal pattern of 100 females from a *M. arenaria* population and observed moderate morphological variations. Perineal patterns that represent the diversity within the population are shown in Fig. 1. The overall shape was rounded in most cases, but it was oval-shaped in a small proportion (4%). Forty-six percent of females showed low dorsal arch (Fig. 1A, B). In contrast, 38% depicted a high dorsal arch (Fig. 1C), while the rest were intermediate. Lines in the post-anal region were smooth or wavy, continuous or broken, occasionally forming shoulders (18% of the females; Fig. 1D). Wings were generally not observed. Phasmids were visible in 25% of the patterns (Fig. 1E) and the mean distance between them was  $24.33 \pm 1.61 \mu\text{m}$  (Table 1). Only seven out of one hundred females presented perineal patterns with lateral lines weakly demarcated by forked striae (Fig. 1F). Even though the presence of lateral incisures is a strong differential character, weak lateral lines have also been observed in other populations of *M. arenaria* (Cliff and Hirschmann, 1985; Skantar et al., 2008).

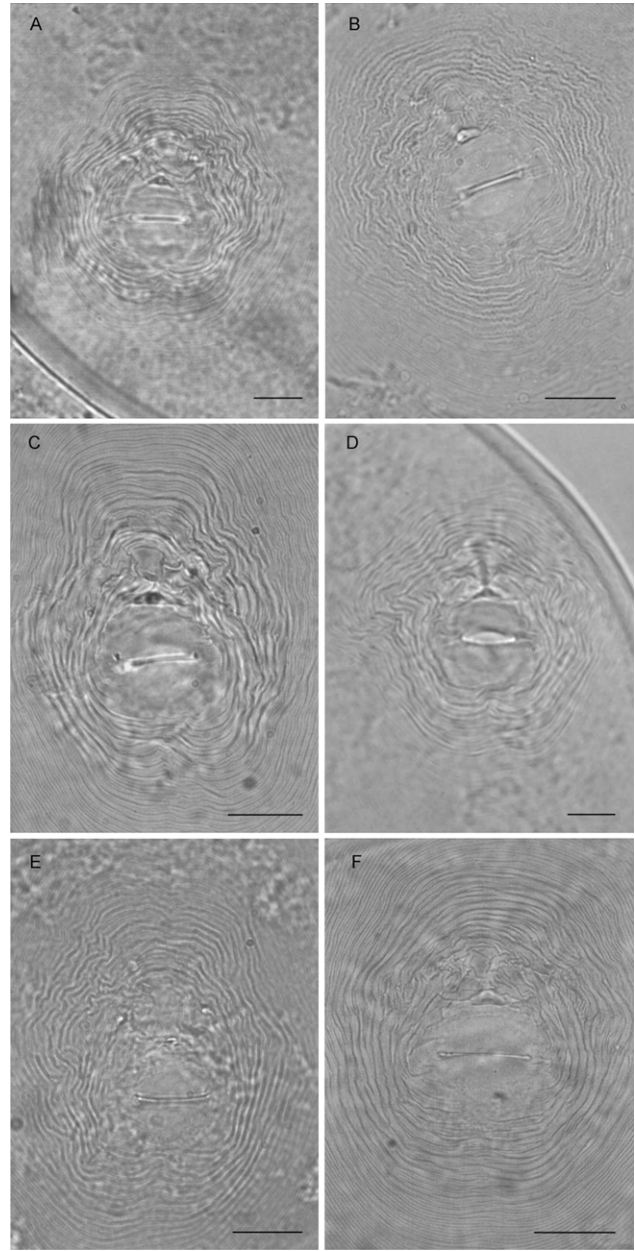


FIG. 1. Micrographs of representative perineal patterns of females of *Meloidogyne arenaria* from Mendoza, Argentina. A-B) Most frequently-observed perineal patterns in the population under study; C) Perineal pattern with high dorsal arch; D) Perineal pattern showing shoulders; E) Perineal pattern with visible phasmids; F) Perineal pattern with weak lateral lines. Scale bar = 20  $\mu\text{m}$ .

Adult females were pyriform and lacking a terminal protuberance in the posterior area of the body (Fig. 2A). The female body lengths ranged between 465  $\mu\text{m}$  and 1,129  $\mu\text{m}$ , slightly increasing the range of previous descriptions of the species (Table 1). The range of the body width was shifted in respect to other descriptions of *M. arenaria* and more similar to *M. incognita* (Table 1). The excretory pore of the females was located closer to the base of the stylet than to the median bulb (Fig. 2B), at approximately 2-stylet lengths, in agreement with a typical *M. arenaria* specimen (Esser et al.,

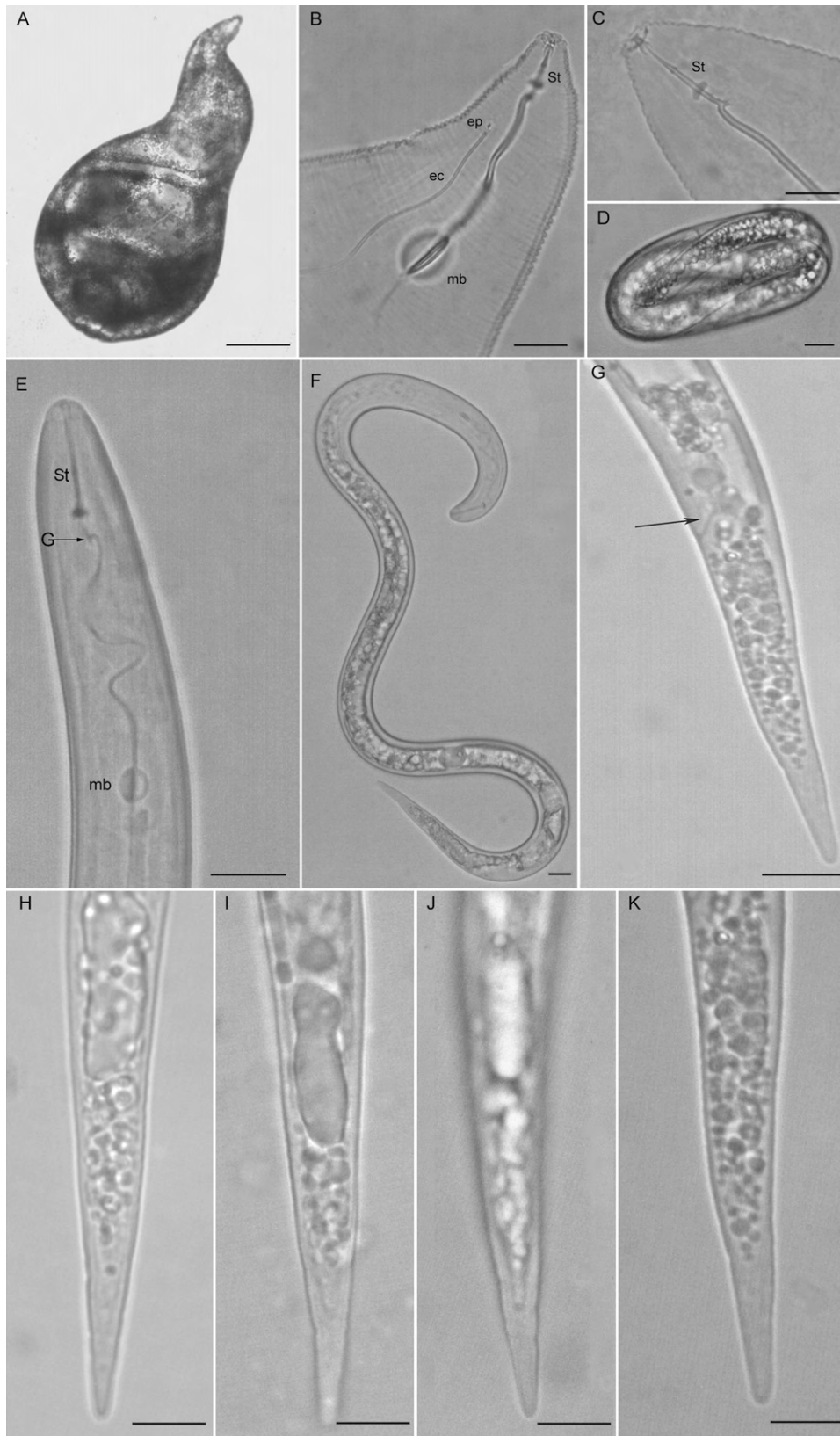


FIG. 2. Micrographs of females (A-C), eggs (D) and second-stage juveniles (E-K) of *Meloidogyne arenaria* from Argentina. A) Adult female; scale bar = 100  $\mu\text{m}$ ; B-C) Anterior end of a female; scale bar = 10  $\mu\text{m}$ ; B) Excretory pore and canal are shown; C) dorsally-curved stylet; D) Egg; scale bar = 10  $\mu\text{m}$ ; E-K) Second-stage juveniles (J2); scale bar = 10  $\mu\text{m}$ . E) Anterior region of a J2; F) Whole body of a J2; G) Posterior region and undilated rectum (arrow); H-K) Tails of J2. Abbreviations, ep: excretory pore, ec: excretory canal, G: dorsal esophageal gland orifice, mb: median bulb, St: stylet.

1976). The stylet was dorsally curved (Fig. 2C) and the mean stylet length was similar to other populations (Table 1). The dimensions of the eggs (Fig. 2D) were comparable to previous measurements of the species (Table 1).

**Morphological characterization of second-stage juveniles:** Second-stage juveniles (Fig. 2E-K) averaged  $458.51 \pm 20.48 \mu\text{m}$  in length and  $15.35 \pm 0.76 \mu\text{m}$  in width and were consistent with typical *M. arenaria* (Table 1). The average stylet length and its range were greater in the population we studied than typical *M. arenaria* and *M. incognita* in Table 1, but similar to other populations of *M. arenaria* (Osman and Dickson, 1985). The rectum was undilated (Fig. 2G) in agreement with Esser et al. (1976), who described juveniles of *M. arenaria* with a dilated or undilated rectum. Tail morphometrics and tail morphology were typical *M. arenaria* (Table 1). Tail length (from anus to posterior end) was  $56.21 \pm 2.78 \mu\text{m}$  and the tail tip was rounded (Fig. 2H-K). The mean length of the hyaline tail terminus was  $14.63 \pm 2.11 \mu\text{m}$  in agreement with previous descriptions (Skantar et al., 2008). The alpha value (body length/maximum body

width) and the gamma value (body length/tail length) were consistent with *M. arenaria* (Table 1).

**Morphological characterization of males:** Nearly all measurements of the males from the population under study were within previous description ranges of *M. arenaria* (Table 1). The mean length of the male body (Fig. 3A) was  $1.6 \pm 0.31 \text{ mm}$ , in agreement with typical *M. arenaria*, but the range was shifted slightly towards lower values (Table 1). The head of the males was flat to concave and showed a moderately elevated labial disc (Fig. 3B). Male stylet length was consistent with typical *M. arenaria* and the stylet knob was laterally elongated, with transversal length of  $5.16 \pm 0.49 \mu\text{m}$  (Fig. 3D). The mean spicule length (Fig. 3C) was greater than other descriptions of *M. arenaria* but within the expected range (Table 1). In addition, the males presented four lateral lines, which were easily observed (Fig. 3E). Lateral lines can vary between 4 to 8 within the genus *Meloidogyne* but *M. arenaria* always has four (Esser et al., 1976).

**Esterase phenotyping:** Isozyme phenotyping was particularly useful for those populations of *Meloidogyne* with intermediate or aberrant perineal patterns (Eisenback

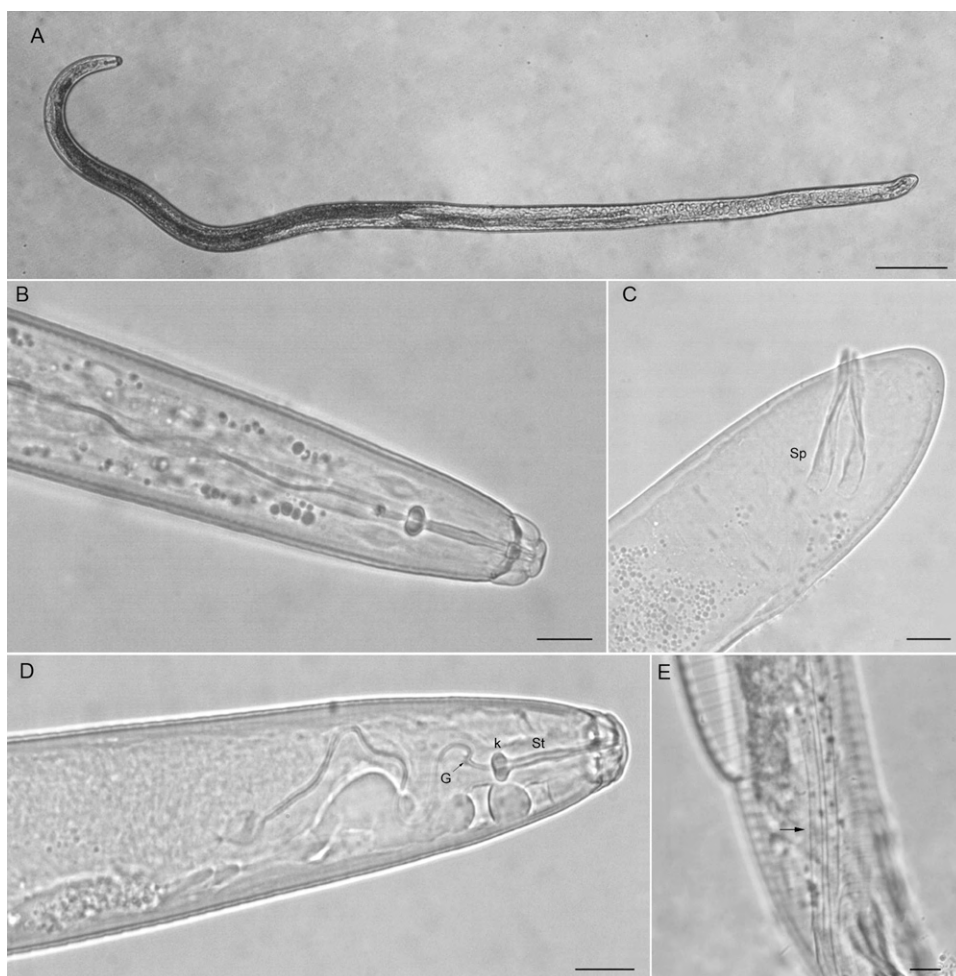


FIG. 3. Micrographs of males of *Meloidogyne arenaria* from Argentina. A) Whole male; scale bar = 100  $\mu\text{m}$ ; B-D) scale bar = 10  $\mu\text{m}$ . B, D) Cephalic region; C) Posterior region showing the spicules; E) Lateral field of a male with four lateral lines marked by arrow. Abbreviations, k: stylet knob, G: dorsal esophageal gland orifice, Sp: spicules, St: stylet.

et al., 1980; Esbenshade and Triantaphyllou, 1985). More than twenty isoenzymes were examined for taxonomic purposes, with esterase being the most effective for identifying *Meloidogyne* species (Dickson et al., 1971; Esbenshade and Triantaphyllou, 1985). Three alternative esterase phenotypes have been recognized for *M. arenaria*, with one (A1), two (A2), or three (A3) bands in specific positions relative *M. javanica* (Esbenshade and Triantaphyllou, 1985).

We examined the esterase pattern of the population under study, including *M. javanica* as a reference. The pattern of *M. javanica* depicted the expected esterase phenotype J3 (Esbenshade and Triantaphyllou, 1985). The esterase phenotype of the nematode population under study showed 3 bands, the slowest of which was a minor band (Fig. 4). The esterase pattern corresponded to the phenotype A3 of *M. arenaria*. However, other *Meloidogyne* spp. also showed the A3 phenotype (Esbenshade and Triantaphyllou, 1985; Carneiro et al., 2008) and thus, we cannot rely on esterase pattern alone to say that the population under study belongs to *M. arenaria*.

**Phylogenetic analysis:** DNA sequences of the internal transcribed spacer (ITS) of rDNA genes have been useful to identify species of nematodes (Zijlstra et al., 1997; Hugall et al., 1999; Zijlstra et al., 2000; Powers, 2004). An approximately 450 bp DNA fragment of the nematode population under study, which included part of the 18S rDNA gene, ITS-1 and part of the 5.8S rDNA gene, was amplified and sequenced. Sequences of the

rDNA were aligned with others from diverse *Meloidogyne* species obtained from GenBank and phylogenetic analyses were performed. The Maximum Likelihood tree (Fig. 5) was highly resolved and showed that the population of *M. arenaria* we studied formed a clade together with sequences from other *M. incognita*, *M. arenaria* and *M. javanica*, with high bootstrap support (BS = 95%). These three species could not be separated based on this molecular marker due to its limited sequence polymorphism. Previous studies also indicated that the parthenogenic *M. incognita*, *M. arenaria* and *M. javanica* are hard to distinguish using nucleotide sequences, including ITS, 18S rDNA and mtDNA, and occasionally showed contradictory results (Blok and Powers, 2009). Problems understanding the relationships among these species are probably caused by their putative hybrid origin. It has been proposed that *M. incognita*, *M. arenaria* and *M. javanica* originated by hybridization between sexual or meiotic parthenogenic taxa (Triantaphyllou, 1985; Castagnone-Sereno, 2006). Here, sequencing the rDNA region of the population we isolated aided our diagnosis but could not discriminate among *M. arenaria*, *M. javanica*, or *M. incognita*.

**Molecular identification:** The RKN Molecular Diagnostic Key (Adam et al., 2007) is useful to distinguish the most common species of *Meloidogyne*, including the economically relevant *M. arenaria*, *M. incognita*, *M. hapla* and *M. javanica*, by performing PCR amplifications with different primer pairs. Using the primers 194 and 195, we amplified the expected 720 bp product common to *M. arenaria*, *M. incognita*, and *M. javanica*. The specific SCAR primers for *M. javanica* (Fjav, Rjav) and *M. incognita* (Mi-F, Mi-R) did not amplify any product, while the SCAR primers for *M. arenaria* (Far, Rar) amplified the specific 420 bp product (Fig. 6). Therefore, the molecular key indicated that the nematode population under study belonged to *M. arenaria*.

**Taxonomic implications:** The perineal pattern of female nematodes alone has been widely used for identification of *Meloidogyne* species. However, the recognition of significant intraspecific variability and the description of new species of root-knot nematodes weaken its use and success (Chitwood, 1949; Netscher, 1978; Karssen and van Aelst, 2001; Carneiro et al., 2004). It has been noted that the variability and similarity of the perineal patterns of some nematodes, such as *M. arenaria*, *M. morocciensis*, *M. paranaensis*, or *M. konaensis*, may result in misidentifications (Netscher, 1978; Cliff and Hirschmann, 1985; Carneiro et al., 2004; Carneiro et al., 2008; Hunt and Handoo, 2009; Robertson et al., 2009). Here, we observed that the perineal patterns of the population under study were frequently intermediate between the prototypes of *M. incognita* and *M. arenaria*, with some perineal patterns typical of one or the other. Characterization of one hundred perineal patterns from the population was not conclusive for species identification.

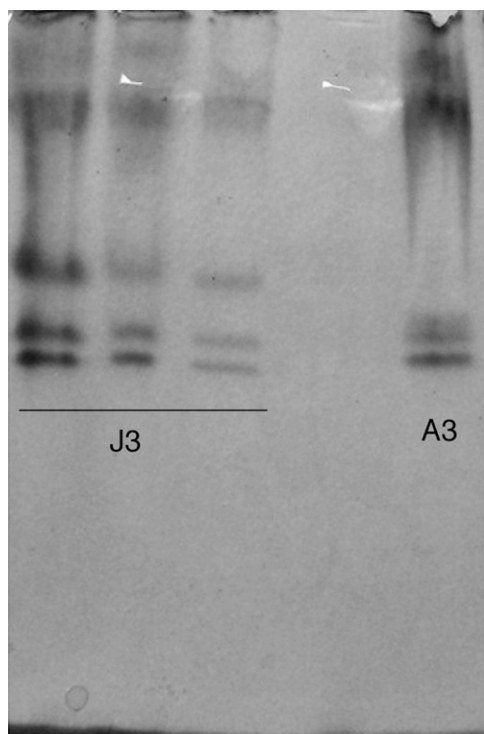


FIG. 4. Esterase phenotype found in the population of *Meloidogyne arenaria* race 2 (A3); *M. javanica* (J3) was included as a reference.

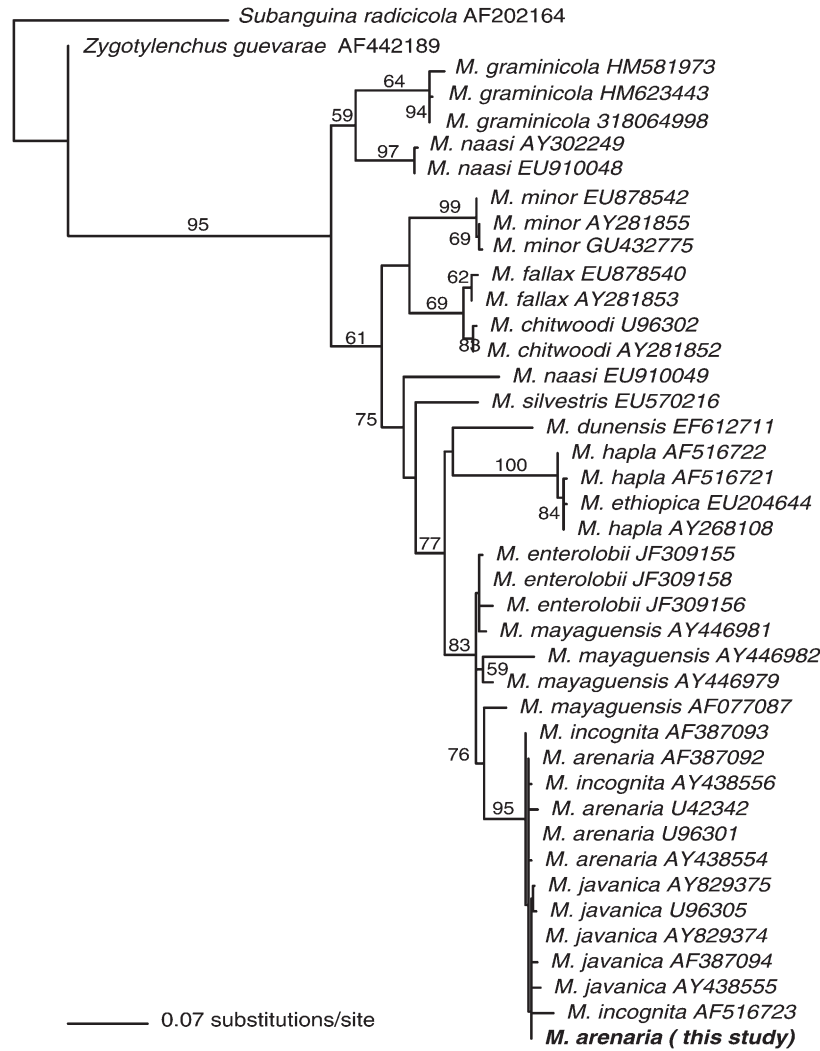


FIG. 5. Phylogenetic tree of the genus *Meloidogyne* based on the ITS of rRNA under ML. Numbers above branches represent bootstrap support values > 50%. GenBank numbers are shown next to the taxon names.

A combination of morphological and morphometric features of females, males and juveniles were suggested as stable characters for diagnosis of *Meloidogyne* species (Chitwood, 1949; Whitehead, 1968; Eisenback et al., 1980; Eisenback, 1982). However, the large variability within a species and the overlap between species make it difficult to identify species from morphological features alone. In this study, we noticed that many of the morphometric characteristics were common to descriptions of *M. incognita* and *M. arenaria*. Even when we sequenced a portion of the rRNA, we could not be sure if the population belonged to *M. arenaria* or *M. incognita*. The most decisive data were provided by esterase phenotyping and molecular methods using SCARs.

Combining the morphometric and morphological description of females, males, second-stage juveniles and eggs, esterase phenotyping, and molecular characterization, indicated that the population under study belonged to *M. arenaria*. Studies of populations of *M. arenaria* from around the globe argue that this the most

variable species of root-knot nematodes in terms of morphology, isoenzymes, and molecular features (Cliff and Hirschmann, 1985; Esbenshade and Triantaphyllou, 1985; Osman and Dickson, 1985; Carneiro et al., 2008). Therefore, identification of *M. arenaria* requires multiple approaches, none of which is sufficient alone for accurate diagnosis.

*Host range characterization:* The differential host test, also known as the “North Carolina differential host test”, is based on the ability of a nematode population to reproduce on a particular plant host (Sasser and Triantaphyllou, 1977). The test consists in infecting six standard plant cultivars and based on the results, it discriminates between nematode races of the most prominent species of the genus *Meloidogyne*: *M. incognita* (six races), *M. arenaria* (three races), and *M. javanica* (four races). Table 2 shows the responses of different hosts to the infection with the population of *M. arenaria* under study. Only three of the cultivars (tobacco, watermelon and tomato) were susceptible to



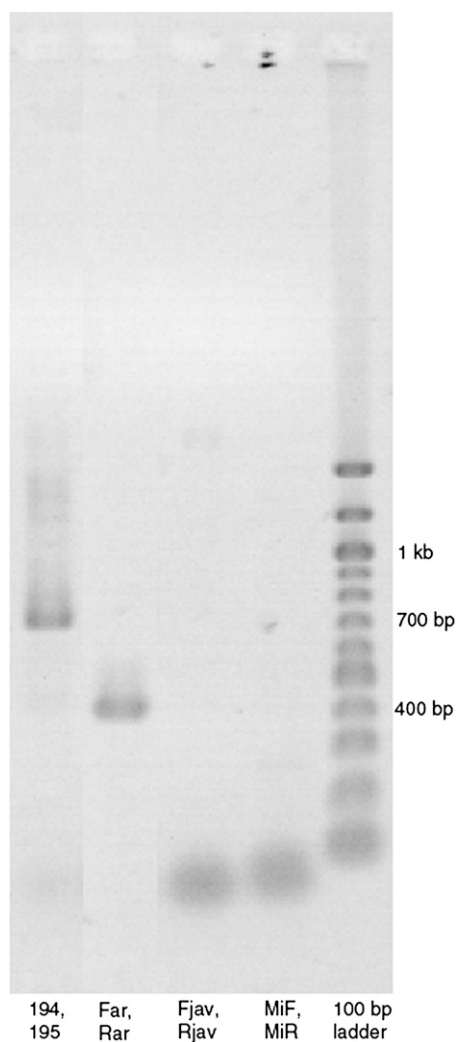


FIG. 6. Amplification products of *Meloidogyne arenaria* race 2, using four primer pairs following the identification key developed by Adam et al 2007. 194,195: primers that amplify the IGS between 5S and 18S rRNA genes; Fjav, Rjav: SCAR primers specific to *M. javanica*; Mi-F, Mi-R: SCAR primers specific to *M. incognita*; Far, Rar: SCAR primers specific to *M. arenaria*.

the infection by the population of *M. arenaria* under study, showing the same host preference to that of *M. arenaria* race 2 originally described by Sasser (1977).

Despite its frequent use, the differential host test has several disadvantages: i) it does not take into account or distinguish species described after the four original *Meloidogyne* species were reported; ii) it is limited to

a small host sample precluding the possibility of broader host variability (Moens et al., 2009); iii) it prevents characterization of virulent populations, which would infect resistant varieties (Robertson et al., 2009); and iv) it forces nematode populations into groups, which may not reflect actual variability (Stanton and O'Donnell, 1998). In fact, a few populations of *M. arenaria* and *M. incognita* did not belong to any of the described races, and new races had to be established (Stanton and O'Donnell, 1998; Robertson et al., 2006; Robertson et al., 2009). We agree with Moens et al (2009) that the test should be discontinued for taxonomic purposes; however, it may still be useful for host range characterization, which includes infection of major cultivars.

*Reproductive characterization and symptoms:* Infection of susceptible plants of tomato *Solanum lycopersicum* cv. Roma or Río Grande by the population of *M. arenaria* under study caused swelling and root galls. Galls were irregularly shaped, variable in size, and were located at some distance from the root tip, but never at the root tip. Eggs formed gelatinous masses on the surface of the galls and were clearly observed in red when stained with yellow eosin. Newly-laid egg masses were hyaline and turned brown with age. Above-ground symptoms were not apparent.

The average number of second-stage juveniles that hatched from each egg mass was 550 juveniles per egg mass (SD = 175.79), ranging from 436 to 858 J2 per egg mass. Hatching occurred sporadically for a maximum period of 55 days. An estimation of the mean number of eggs per egg mass was 768.72 (SD = 476.90), and had a range of 265 to 1474 eggs per egg mass.

The reproductive factor (Rf) of a nematode population is used to measure its reproductive capacity. The reproductive factor was calculated as the final nematode population/initial nematode population ( $Rf = Pf/Pi$ ) per tomato plant, estimated 60 days after inoculation. The mean Pf consisted of 11,518 juveniles in the soil (3.7%) and eggs on the root system (96.3%). The average Rf of *M. arenaria* race 2 infecting tomato plants was 115.19 (SD=55.37), which is comparable with other populations of this species growing on tomato. Previous studies on reproduction of *M. arenaria* race 1 in tomato plants indicated that the average Rf was 91.86, 55 days after inoculation (Hadisoeganda and Sasser, 1981), and

TABLE 2. Differential host test for classifying races of *Meloidogyne arenaria* populations.

Races of <i>Meloidogyne arenaria</i>	<i>Nicotiana tabacum</i> cv. NC 95 (tobacco)	<i>Gossypium hirsutum</i> cv. Deltapine 16 (cotton)	<i>Capsicum frutescens</i> cv. California Wonder (pepper)	<i>Citrullus vulgaris</i> cv. Charleston Grey (watermelon)	<i>Arachis hypogaea</i> cv. Florunner (peanut)	<i>Solanum lycopersicum</i> cv. Rutgers (tomato)
Race 1	+	-	+	+	+	+
Race 2	+	-	-	+	-	+
Race 3	+	-	+	+	-	+
This study	+	-	-	+	-	+

+ = host; - = non-host.

the Rf ranged from 40.9 to 79.9, 45 days after inoculation, only counting the final population of eggs (Noe, 1992).

In conclusion, the results presented here represent the first morphometric, morphological, reproductive, biochemical, molecular, and host range characterization of a root-knot nematode from Argentina. Understanding the reproductive features of nematode populations as well as their correct identification should aid effective management programs. Here, we provided a thorough description of a South American population of *M. arenaria* and employed diverse approaches to reach an accurate identification. This study highlights the importance of taking a multidimensional approach for *Meloidogyne* spp. diagnosis, and that great care should be taken when observing perineal patterns.

#### LITERATURE CITED

- Adam, M. A. M., Phillips, M. S., and Blok, B. C. 2007. Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathol.* 56:190–197.
- Blok, V., Phillips, M., and Fargette, M. 1997. Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root-knot nematodes. *J. Nematol.* 29:16–22.
- Blok, V. C., and Powers, T. O. 2009. Biochemical and molecular identification. Pp. 98–112 in R. N. Perry, M. Moens and J. Starr, eds. *Root-knot nematodes*. Lincoln: CABI.
- Carneiro, R. M. D. G., Dos Santos, M. F. A., Almeida, M. R. A., Mota, F. C., Gomes, A. C. M. M., and Tigano, M. S. 2008. Diversity of *Meloidogyne arenaria* using morphological, cytological and molecular approaches. *Nematology* 10:819–834.
- Carneiro, R. M. D. G., Tigano, M. S., Randig, O., Almeida, M. R. A., and Sarah, J. L. 2004. Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) on coffee from Brazil, Central America and Hawaii. *Nematology* 6:287–298.
- Castagnone-Sereno, P. 2006. Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. *Heredity* 96:282–289.
- Chaves, E. 2001. Nematodos parásitos de la papa en regiones productoras de papa semilla en Argentina. *Revista de la Facultad de Agronomía, Universidad Nacional de Buenos Aires* 21:245–259.
- Chitwood, B. G. 1949. Root-knot nematodes I. A revision of the genus *Meloidogyne* Goeldi, 1887. *Proc. Helminth. Soc.* 16:90–104.
- Cliff, G., and Hirschmann, H. 1985. Evaluation of morphological variability in *Meloidogyne arenaria*. *J. Nematol.* 17:445–459.
- Dickson, D., Huisingh, D., and Sasser, J. 1971. Dehydrogenases, acid and alkaline phosphatases and esterases for chemotaxonomy of selected *Meloidogyne*, *Ditylenchus*, *Heterodera* and *Aphelenchus* spp. *J. Nematol.* 1:1–16.
- Doucet, M. E. 1989. The genus *Nacobbus* Thorne and Allen, 1944 in Argentina. 1. Study of a population of *N. aberrans* (Thorne, 1935) Thorne and Allen, 1944 on *Chenopodium album* L. from Rio Cuarto, Province of Córdoba. *Revue de Nematologie* 12:17–26.
- Doucet, M. E., and Cagnolo, S. 1998. Variabilidad intra e inter-específica de caracteres morfométricos en poblaciones del orden Tylenchida (Nematoda) provenientes de Argentina. *Nematol. Medit.* 26:231–236.
- Doucet, M. E., and Lax, P. 2007. El género *Meloidogyne* y su situación con respecto a la agricultura en Argentina. *Anales Acad. Nac. Agr. Vet.* 61:31–50.
- Doucet, M. E., and Pinochet, J. 1992. Occurrence of *Meloidogyne* spp. in Argentina. *J. Nematol.* 24:765–770.
- Doucet, M. E., and Ponce de León, E. L. 1995. *Meloidogyne* spp. (Nemata): una seria amenaza para la papa (*Solanum tuberosum* L.). *Revista Investig. Agropec.* 26:45–51.
- Eisenback, J. D. 1982. Morphological comparison of head shape and stylet morphology of second-stage juveniles of *Meloidogyne* species. *J. Nematol.* 14:339–343.
- Eisenback, J. D. 1985. Diagnostic characters useful in the identification of the four most common species of root-knot nematodes (*Meloidogyne* spp.). Pp. 95–112 in J. N. Sasser and C. C. Carter, eds. *An advanced treatise on Meloidogyne*, 1 Biology and control. Raleigh, USA: North Carolina State University.
- Eisenback, J. D., Hirschmann, H., and Triantaphyllou, A. 1980. Morphological comparison of *Meloidogyne* female head structures, perineal patterns, and stylets. *J. Nematol.* 12:300–313.
- Esbenshade, P., and Triantaphyllou, A. 1985. Use of enzyme phenotypes for identification of *Meloidogyne* species. *J. Nematol.* 17:6–20.
- Esser, R. P., Perry, V. G., and Taylor, A. L. 1976. A diagnostic compendium of the genus *Meloidogyne* (Nematoda: Heteroderidae). *Proc. Helminthol. Soc. Wash* 43:138–150.
- Franklin, M., and Goodey, J. 1949. A cotton blue-lactophenol technique for mounting plant-parasitic nematodes. *J. Helminthol.* 23:175–178.
- Hadisoeganda, W., and Sasser, J. N. 1981. Resistance of tomato, bean, southern pea, and garden pea cultivars to root-knot nematodes based on host suitability. *Plant Disease* 66:145–150.
- Hirschmann, H. 1985. The genus *Meloidogyne* and morphological characters differentiating its species. Pp. 79–93 in J. N. Sasser and C. C. Carter, eds. *An advanced treatise on Meloidogyne* Volume I: Biology and control. Raleigh.: North Carolina State University.
- Hugall, H., Stanton, J., and Moritz, C. 1999. Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Mol. Biol. Evol.* 16:157–164.
- Hunt, D., and Handoo, Z. 2009. Taxonomy, identification and principal species. Pp. 55–88 in R. N. Perry, M. Moens and J. L. Starr, eds. *Root-knot nematodes*, 1. London: CABI.
- Jenkins, W. R. 1964. A rapid centrifugal flotation technical for separating nematodes from soil. *Plant. Dis. Rep.* 48:692.
- Karsen, G., and van Aelst, A. 2001. Root-knot nematode perineal pattern development: a reconsideration. *Nematology* 3:95–111.
- Lax, P., and Doucet, M. E. 2001. *Heterodera glycines* Ichinohe, 1952 (Nematoda: Tylenchida) from Argentina. 1. Morphological and Morphometrical characterisation of second-stage juveniles. *Nematology* 3:165–170.
- Maddison, W., and Maddison, P. 2000. *MacClade* version 4: analysis of phylogeny and character evolution. Sunderland, MA, Sinauer Associates.
- Meng, Q., Long, H., and Xu, J. 2004. PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. *Acta Phytopathol. Sinica* 34: 204–210.
- Moens, M., Perry, R., and Starr, J. 2009. *Meloidogyne* species—a diverse group of novel and important plant parasites. Pp. 483 in R. N. Perry, M. Moens and J. L. Starr, eds. *Root-knot nematodes*, 1. Wallingford, UK.
- Netscher, C. 1978. Morphological and physiological variability of species of *Meloidogyne* in West Africa and implications for their control. *Meded. LandbHoegesch. Wageningen* 3:1–46.
- Noe, J. 1992. Variability among populations of *Meloidogyne arenaria*. *J. Nematol.* 24:404–414.
- Osman, H., and Dickson, D. 1985. Morphological comparisons of host races 1 and 2 of *Meloidogyne arenaria* from Florida. *J. Nematol.* 17:279–285.
- Perichi, G., and Crozzoli, R. 2010. Morfología, morfometría y hospedantes diferenciales de poblaciones de *Meloidogyne* de los estados Aragua y Zulia, Venezuela. *Fitopatol. Venez.* 23:5–15.

- Posada, D., and Crandall, K. 1998. ModelTest: testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Powers, T., and Harris, T. 1993. A polymerase chain reaction method for the identification of five major *Meloidogyne* species. *J. Nematol.* 25:1–6.
- Powers, T. O. 2004. Nematode molecular diagnostics from bands to barcodes. *Annu. Rev. Phytopathol.* 42:367–383.
- Roberts, P. A., and May, D. M. 1986. *Meloidogyne incognita* resistance characteristics in tomato genotypes developed for processing. *J. Nematol.* 18:353–359.
- Robertson, L., Díez- Rojo, M. A., Lopez-Pérez, J. A., Piedrabuena, A., Escuer, M., Lopez-Cepero, J., Martínez, C., and Bello, A. 2009. New host races of *Meloidogyne arenaria*, *M. incognita* and *M. javanica* from horticultural region of Spain. *Plant Disease* 93:180–184.
- Robertson, L., J.A., L.-P., Bello, A., Díez- Rojo, M. A., Escuer, M., Piedrabuena, A., Ros, C., and Martínez, C. 2006. Characterization of *Meloidogyne incognita*, *M. hapla* and *M. arenaria* population from Spain and Uruguay parasitizing pepper (*Capsicum annuum* L.). *Crop Protection* 25:440–445.
- Sasser, J. N., and Triantaphyllou, A. C. 1977. Identification of *Meloidogyne* species and races. *J. Nematol.* 9:283.
- Skantar, A. M., Carta, L. K., and Handoo, Z. A. 2008. Molecular and morphological characterization of an unusual *Meloidogyne arenaria* population from Traveler's tree *Ravenala madagascariensis*. *J. Nematol.* 40:179–189.
- Stanton, J. M., and O'Donnell, W. E. 1998. Assessment of the North Carolina differential host test for identification of Australian populations of root-knot nematodes (*Meloidogyne* spp.). *Australasian Plant Pathol.* 27:104–111.
- Triantaphyllou, A. C. 1985. Cytogenetics, cytotaxonomy and phylogeny of root- knot nematodes. Pp. 113–126 in S. J. N. and C. C. Carter, eds. An advanced treatise on *Meloidogyne*, 1. Biology and Control. Raleigh: North Carolina State University.
- Trudgill, D. L., and Block, V. C. 2001. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol.* 39:53–77.
- Vovlas, N., Nico, A. I., De Luca, F., De Giorgi, C., and Castillo, P. 2007. Diagnosis and molecular variability of an Argentinian population of *Nacobbus aberrans* with some observations on histopathology in tomato. *J. Nematol.* 39:17–26.
- Whitehead, A. G. 1968. Taxonomy of *Meloidogyne* (Nematodea: Heteroderidae) with descriptions of four new species. *Trans. Zool. Soc. Lond* 31:263–401.
- Zijlstra, C., Donkers-Venne, D., and Fargette, M. 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology* 2:847–853.
- Zijlstra, C. M., Uenk, B. J., and van Silfhout, C. H. 1997. A reliable, precise method to differentiate species of root- knot nematodes in mixtures on the basis of ITS- RFLPs. *Fund. Appl. Nematol.* 20: 59–63.
- Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, University of Texas.