

Molecular and Morphological Characterization of an Unusual *Meloidogyne arenaria* Population from Traveler's Tree, *Ravenala madagascariensis*

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Abstract: An unusual variant of *Meloidogyne arenaria* was discovered on roots of a traveler's tree (*Ravenala madagascariensis*) intended for display at a public arboretum in Pennsylvania. The population aroused curiosity by the lack of visible galling on the roots of the infected plant, and the female vulval region was typically surrounded by egg sacs. Most morphometrics of the population fit within the ranges reported for *M. arenaria*, with a mosaic of features in common with either *M. platani* or other tropical *Meloidogyne* spp. Molecular characterization included analysis of four loci. The mitochondrial sequence, extending from cytochrome oxidase II (*COII*) to the 16S (*IRNA*) gene, was nearly identical to another *M. arenaria* population and closely related to sequences from *M. morocciensis* and *M. thailandica*. The 28S D2-D3 expansion segment was most similar to those from *M. arenaria*, *M. incognita* and *M. paranaensis*, and the IGS-2 was most related to those from *M. thailandica*, *M. arenaria* and *M. incognita*. Analysis of partial Hsp90 genomic sequences revealed the greatest similarity to *M. arenaria*, *M. thailandica* and an Hsp90 haplotype from *M. floridensis*, and a composite sequence comprised of EST from *M. arenaria*. No morphological or molecular features clearly distinguished this population as a new species, and, when considered as a whole, the evidence points to its identification as *M. arenaria*.

Key words: gall, Hsp90, intergenic spacer, mitochondrial DNA, molecular biology, *Ravenala madagascariensis*, ribosomal DNA, root-knot nematode, taxonomy, traveler's tree, variation.

Root-knot nematodes (*Meloidogyne* spp.) are economically important plant parasites affecting a broad range of host plants, and thus far 95 nominal species have been described. In June 2007, a root-knot nematode (RKN) was discovered on roots of a traveler's tree (*Ravenala madagascariensis* Sonnerat) that was intended for exhibit at a public garden in Pennsylvania. Growing in an artificial soil mixture, the affected palm-like tree was obtained by the arboretum from a grower in Florida. Information regarding the precise origin of the traveler's tree was not available. Diseased root material was sent via a Delaware extension specialist to the USDA Nematology Laboratory in Beltsville, MD, for identification.

The development of molecular methods to identify the four major RKN (*M. incognita* (Kofoid and White, 1919) Chitwood, 1949; *M. arenaria* (Neal, 1889); *M. javanica* (Treub, 1885) Chitwood, 1949; and *M. hapla* Chitwood, 1949) has been the goal of numerous studies. While isozyme analysis continues to be an effective way to discriminate many RKN species (Esbenshade and Triantaphyllou, 1990; Carneiro, 2000), DNA sequencing is straightforward and has the added benefit of providing data useful for phylogenetic analysis. DNA markers that have aided identification of *Meloidogyne* species include the ribosomal DNA small subunit (SSU) 18S (Powers, 2004), large subunit (LSU) 28S D2-D3 expansion segments (Chen et al., 2003; Palomares Ruis et al., 2007), intergenic spacer (IGS) (Blok et al., 1997; Wishart et al., 2002), internal transcribed spacer

(Powers and Harris, 1993) and mitochondrial DNA (Powers and Harris, 1993; Stanton et al., 1997; Blok et al., 2002; Xu et al., 2004; Jeyaprakash et al., 2006). Random amplified polymorphic DNA (RAPD) (Cenis, 1993; Williamson et al., 1997; Dong et al., 2001; Cofcewicz et al., 2004; Adam et al., 2007) and sequence characterized amplified regions (SCAR) markers (Zijlstra et al., 2000; Randig et al., 2002) have also been developed. One study combined IGS PCR, SCAR markers and RAPD analysis into a diagnostic key for discrimination of seven RKN species (Adam et al., 2007). Recently, a partial sequence from the protein-coding gene Hsp90 was used to confirm the identity of an unusually aggressive population of *M. hapla* affecting coffee in Maui, HI (Handoo et al., 2005b). This locus also showed potential for establishing phylogenetic relationships among plant-parasitic nematode species (Skantar and Carta, 2004).

This work describes the morphological features of an unusual population of *M. arenaria* found on traveler's tree, highlighting differences from the values expected for the species and points of overlap with related species. Also, a study of its relationship to other tropical RKN, based upon analysis of mitochondrial sequences (the interval from cytochrome oxidase *COII* to the 16S rRNA gene, *IRNA*), the 28S D2-D3 expansion segment, the ribosomal intergenic spacer regions (IGS) and Hsp90, is presented.

MATERIALS AND METHODS

Morphological Characterization: Soil and root material from the infected traveler's tree were sent to the U.S. Department of Agriculture (USDA) Nematology Laboratory in Beltsville, MD, for species identification. Egg masses were kept in petri dishes at room temperature in a small amount of water. J2 were extracted from soil by sieving and Baermann funnel methods. After fixation overnight in 3% formaldehyde at room temperature, females, males and J2 were later dissected from infected

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roots. J2 and males were fixed in 3% formaldehyde and processed to glycerin by the formalin-glycerin method (Hooper, 1970; Golden, 1990). Procedures used in measuring and preparing specimens were essentially those of Golden and Birchfield (1972), except some fixed females were cut and mounted in clear lactophenol solution. Measurements of all stages were made with an ocular micrometer with measurements in micrometers, unless otherwise stated. Images were taken on a Zeiss Ultraphot III (Carl Zeiss, Inc., Jena, Germany, and Baltimore Instrument Company, Baltimore, MD, USA) with a Toshiba IKTU CCD camera (Toshiba Corp., Japan) or a Zeiss Axiophot 100 Inverted microscope (Zeiss, Inc., Thornwood, NY) with a Q Imaging RTV Micropublisher 5 CCD camera (QImaging, Surrey, BC, Canada). Partial body images were stitched using Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA).

Molecular Characterization: Single J2 of the *Ravenala* population and others (*M. arenaria* from Maryland; *M. floridensis*, from Florida) were mechanically disrupted in 20 μ l of extraction buffer as described by Thomas et al. (1997) then stored in PCR tubes at -80°C until needed. *Meloidogyne thailandica* from Thailand consisted of a pool of nine J2 in 50 μ l, treated as above. Extracts were prepared by incubating the tubes at 60°C for 60 min, followed by 95°C for 15 min to deactivate the proteinase K and were centrifuged briefly prior to use in PCR. Typically, 2.5 μ l of each nematode extract was used per 25 μ l PCR reaction. Primers used in this study are listed in Table 1. Reactions also contained 1 unit Eppendorf MasterTaq (Brinkmann, Westbury, NY) and the buffer supplied by the manufacturer; all other components were added as described in the specific protocols for each gene. Ribosomal PCR products were amplified from the intergenic spacer (IGS-2) according to Blok et al. (2002) and from the 28S D2-D3 expansion segment, as described by De Ley et al. (2005). Partial Hsp90 sequences were amplified as previously described (Skantar and Carta, 2004). Amplification of the mitochondrial region between the *COII* and *IRNA* genes was a modification of Powers and Harris (1993) and Tigano et al. (2005), as follows: 1 cycle at 94°C for 2 min, followed by 45 cycles of 94°C for 30 sec, 48°C for 30 sec and 68°C for 2 min, ending with 1 cycle of 68°C for 5 min.

PCR products were visualized with UV illumination after ethidium bromide staining. DNA was excised from the gels and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Cleaned PCR products were either sequenced directly or cloned into vector pCR2.1 and transformed into *Escherichia coli* TOP10 cells according to the TOPO TA cloning kit instructions (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared with a Qiagen miniprep kit (Qiagen, Valencia, CA) and digested with Eco RI to confirm the correct insert identity. Sequencing was performed at the University of Maryland Center for Biosystems Research. For each locus, 2 to 4 clones or PCR products representing two or more individuals were analyzed. New sequences were submitted to GenBank under accession numbers EU364878-EU364890 and FJ238508.

DNA sequences were assembled using Sequencher 4.7 (Genecodes, Ann Arbor, MI) and analyzed using the BLASTN megablast program optimized for highly similar sequences (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Similar sequences were downloaded from GenBank, aligned with ClustalW2 with default parameters (Larkin et al., 2007), and, if necessary, alignments were trimmed in GeneDoc (Nicholas et al., 1997). For each locus alignment, distance-based neighbor-joining (NJ) methods were used to infer the phylogenetic relationships among species (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Morphological analysis: The population did not produce any galls on traveler's tree, and female vulval region was typically surrounded by egg sacs. Morphological variation made this population particularly difficult to identify, and it was initially thought to represent a new species closely related to *M. arenaria*, *M. incognita* and *M. javanica*. While this population consisted of a mosaic of morphological similarities to several species, most measurements fit within the ranges previously reported for *M. arenaria*. Perineal patterns in females were quite variable (Fig. 1A–F), with a round to high pattern showing occasional wings or shoulders with a slight lateral line. In several specimens, the central perineum was surrounded by alternating coarse and fine striae, with higher frequency, more interrupted

TABLE 1. List of Primers used for DNA analysis

Locus	Primers	5' to 3' Sequence	Ref. for PCR conditions
Mitochondrial <i>COII</i> to <i>IRNA</i>	C2F3	GGTCAATGTTTCAGAAATTTGTGG	Powers and Harris, 1993 Tigano et al., 2005
	I108	TACCTTTGACCAATCACGCT	
28S D2-D3	D2A	ACAAGTACCGTGAGGGAAAGTT	Ye et al., 2007
	D3B	TCGGAAGGAACCAGCTACTA	
IGS-2	194	TTAACTTGCCAGATTCGGACG	Blok et al., 1997
	195	TCTAATGAGCCGTACGC	
Hsp90	U288	GAYACVGGVATYGGNATGACYAA	Skantar and Carta, 2004
	L1110	TCRCARTTIVTCCATGATRAAVAC	

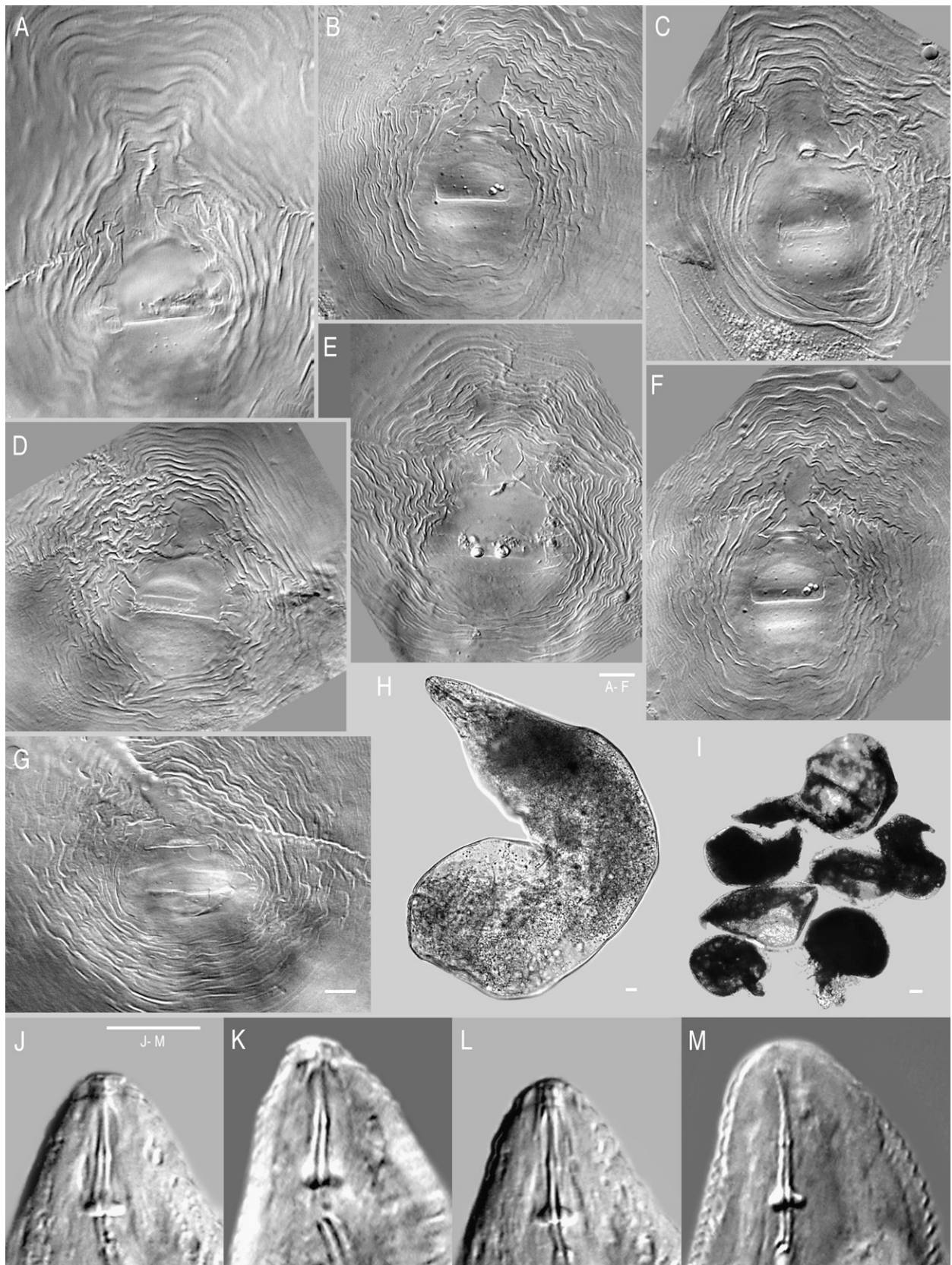


FIG. 1. Light micrographs of *Meloidogyne arenaria* females. (A – F) Perineal patterns from traveler's tree population. (G) Example of typical *Meloidogyne arenaria* perineal pattern, imaged from slide G9957, USDA Nematode Collection. Specimen from Blacksburg, VA; host = tomato. (H – I) Female body, traveler's tree population, high and low power images, respectively. J – M) Traveler's tree population, anterior ends with stylets.

waves than seen in typical *M. arenaria* (Fig. 1G). Perineal patterns lacked the laterally elongated perivulval region usually found in *M. arenaria* (Cliff and Hirschmann, 1985; Hirschmann, 1985; Eisenback and Triantaphyllou, 1991), but instead had a round to dorsoventral elongation. Vulval openings in this population exhibited prominent lateral seam-like depressions.

The stylet cone in females was slightly curved (Fig. 1J-M). Female stylet knobs were rounded to broad, sloping posteriorly, and did not consistently have the gradual sloping of the shaft found in typical *M. arenaria* (Eisenback et al., 1980, 1981; Eisenback and Triantaphyllou, 1991), but instead showed a lateral aspect more like that of *M. incognita*. Female stylet length (Table 2) was consistent with *M. arenaria* and distinguished the population from *M. thailandica* Handoo, Skantar, Carta and Erbe, 2005, a species found on ginger (*Zingiber* spp.) from Thailand (Handoo et al., 2005a), and *M. morocciensis* Rammah and Hirschmann, 1990 from peach (*Prunus persica* L. Batsch) in Morocco (Rammah and Hirschmann, 1990). The dorsal gland outlet (DGO), excretory pore position and vulva to anus length were consistent with the ranges for *M. arenaria* (Table 2). Female shapes were typical of *M. arenaria* but many were bent into L-shapes (Fig. 1H,I). Most females exhibited a posterior protuberance (Fig. 1H) that was absent in *M. thailandica*. The ranges for female body length and width were considerably larger than for other *M. arenaria* populations, *M. thailandica* and *M. morocciensis*.

Male stylet length and spicule length were consistent with typical *M. arenaria*, but body length and gubernaculum were shorter (Table 3). Mean male stylet length distinguishes the current population (22 μm) and previously reported *M. arenaria* value (23 μm) from *M. thailandica* (18.7 μm) and *M. morocciensis* (25 μm). Also, mean body length (1,493 μm) was longer than in *M. thailandica* (1,240 μm) and shorter than *M. morocciensis* (1,621 μm). The *Ravenala* population also lacked the bidentate terminus in the spicules (Fig. 2C) found in *M. thailandica*.

Second-stage juvenile stylets (Fig. 3A) were very similar to other *M. arenaria*, with mean length (11.1 μm) distinct from the shorter stylet of *M. thailandica* (10.2 μm) and the longer one of *M. morocciensis* (12.3 μm) (Table 4). Juvenile tails were long, tapering to a rounded to finer point with 1 to 4 refractive bodies consistently noticed in the hyaline portion (Fig. 3B). Juvenile tail length was on the high end of the range of other *M. arenaria* populations and almost as large as that of *M. thailandica* (Table 4). However, the *Ravenala* population was easily distinguished by a much shorter hyaline tail length (14 μm) than reported for *M. thailandica* (18.3 μm).

Considering characters previously shown to exemplify *M. arenaria*, the *Ravenala* population instead shared some qualitative characters with *M. platani*

TABLE 2. Morphometric comparison^a of females from the *Ravenala* isolate compared with other *M. arenaria* populations, *M. platani*, *M. thailandica* and *M. morocciensis*

Character (μm)	<i>M. arenaria</i> (<i>Ravenala</i> isolate) n = 6-12		<i>M. arenaria</i>		<i>M. platani</i> ^d n = 30		<i>M. thailandica</i> ^e n = 25		<i>M. morocciensis</i> ^f n = 35	
	Body length with neck	893 ± 258	(718 - 1424) n = 11	741 ± 115 ^b	(601 - 985)	678 ± 80	(540 - 860)	762 ± 115.8	(570 - 955)	755 ± 90.3
Body width	566 ± 179	(290 - 906) n = 11	448 ± 89 ^b	(334 - 626)	453 ± 71	(320 - 585)	459 ± 109.1	(272 - 690)	490 ± 72.8	(219 - 608)
Stylet length	15.5 ± 0.8	(15 - 17.5) n = 12	15.1 ± 0.05 ^c	(13 - 17) n = 150	16.5 ± 0.5	(15.8 - 17.3)	13.8 ± 1.2	(12.0 - 15.5)	16.5 ± 0.8	(15 - 18)
Stylet knob height	2.5 ± 0.4	(2 - 2.9) n = 6	2.5 ± 0.2 ^e	(2.2 - 3.1) n = 150	2.5 ± 0.2	(2.2 - 3.1)	2.4 ± 0.5	(1.5 - 3)	2.3 ± 0.2	(2 - 2.6)
DGO from stylet base	4.5 ± 0.05	(4 - 5) n = 12	4.8 ± 0.06 ^c	(3.1 - 6.6) n = 150	3.7 ± 0.46	(2.8 - 4.6)	4.0 ± 0.9	(3 - 5)	3.9 ± 0.6	(2.4 - 5.2)
Excretory pore from anterior end	37.3 ± 10.5	(17.5 - 50) n = 12	42.2 ± 0.94 ^c	(18 - 80) n = 150	26.8 ± 6.7	(16 - 40)	30.6 ± 8.4	(15 - 47)	60.3 ± 21.7	(24 - 126)
Vulval slit length	25.8 ± 2.9	(20 - 29) n = 8	29.3 ± 4.1	(24 - 37) n = 9 ^b	27 ± 2.9	(22 - 33)	29.1 ± 2.6	(25.0 - 35.1)	25.2 ± 2.2	(22 - 30)
Vulva - anus length	22.5 ± 1.7	(21 - 24) n = 8	20.7 ± 2.6	(18 - 21) n = 9 ^b	16.4 ± 2.6	(10 - 23)	18.0 ± 2.4	(15 - 22)	19.5 ± 2.7	(15 - 27)

^a Mean ± standard deviation (range); n = number of specimens.

^b Jepson (1987); author composite of multiple populations; no n given unless noted.

^c Cliff and Hirschmann (1985); author composite of multiple populations.

^d Hirschmann (1982).

^e Handoo et al. (2005b).

^f Rammah and Hirschmann (1990).

TABLE 3. Morphometric comparison^a of males from the *Ravenala* isolate with other *M. arenaria* populations, *M. platani*, *M. thailandica* and *M. morocciensis*

Character (μm)	<i>M. arenaria</i> (<i>Ravenala</i> isolate) n = 6	<i>M. arenaria</i> ^b n = 150	<i>M. platani</i> ^c n = 30	<i>M. thailandica</i> ^d n = 25	<i>M. morocciensis</i> ^e n = 35
Body length	1,493 \pm 442 (1,100 - 1,900)	1,720 \pm 287 (979 - 2,279)	1,355 \pm 276 (960 - 2,010)	1,240 \pm 205.2 (950 - 1,510)	1,621 \pm 155 (1,296 - 1,863)
Stylet length	22 \pm 1.5 (20 - 23.5)	23 \pm 1.46 (22 - 28)	22 \pm 1.1 (19.4 - 24.3)	18.7 \pm 1.1 (17 - 20)	25 \pm 0.7 (23 - 26)
Stylet knob height	2.6 \pm 0.4 n = 6 (2.2 - 3.1)	4 \pm 0.4 (3 - 5) n = 100	3.3 \pm 0.3 (2.8 - 3.8)	2.6 \pm 0.2 (2.5 - 3.0)	3.1 \pm 0.2 (2.5 - 3.6)
DGO from stylet	4.2 \pm 0.8 (3.7 - 5.0) n = 3	6 \pm 1 (4 - 8)	3.7 \pm 0.6 (2.3 - 4.5)	3.7 \pm 0.9 (2.5 - 5.0)	4.7 \pm 0.7 (3.5 - 6.2)
Spicule length	31.4 \pm 0.9 (30 - 35)	32 \pm 2 (27 - 39) n = 66	27.5 \pm 1.41 (25 - 32)	31.2 \pm 3.8 (25 - 38)	34.8 \pm 2.1 (31 - 39)
Gubernaculum	8.1 \pm 1.2 (7.5 - 10)	9 \pm 0.8 (7 - 10) n = 25	8.5 \pm 0.6 (8 - 10)	8.9 \pm 1.2 (7.5 - 11.0)	9.4 \pm 0.8 (8.1 - 10.7)

^a Mean \pm standard deviation (range); n = number of specimens.

^b Cliff and Hirschmann (1985); author composite of multiple populations.

^c Hirschmann (1982).

^d Handoo et al. (2005b).

^e Rammah and Hirschmann (1990).

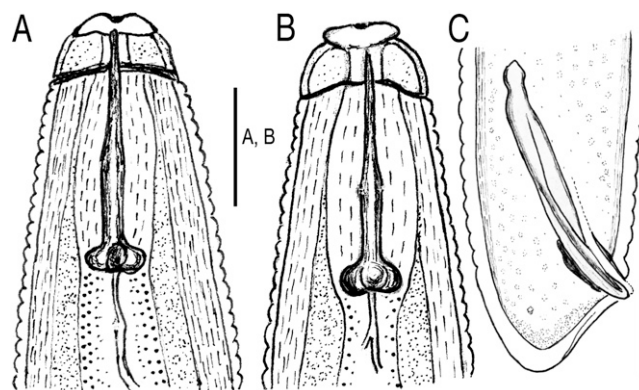


FIG. 2. Drawings of *Meloidogyne arenaria* males from traveler's tree. (A) Lateral view of the anterior end. (B) Dorso-ventral view, anterior end. (C) Tail with spicule and gubernaculum.

Hirschmann, 1982 (Rammah and Hirschmann, 1990). Most notably among them were a wavy female perineal pattern, less sloping male and female stylet knob shape, and shorter female knob height. However, the *Ravenala* population differs markedly from *M. platani* in having longer female body and longer vulval-anal distance (Table 2); the perineal pattern lines are not as uniformly fine, and lateral field and tail tip are generally distinct. While stylet knob heights in both *M. platani* and the *Ravenala* population were lower than the range reported by Cliff and Hirschmann (1985), they were consistent with measurements (range 2-4 μm ; average 2.8 μm) from other triploid populations (Rammah and Hirschmann, 1990).

The male DGO was also found to be useful for characterizing *M. arenaria* populations (Rammah and Hirschmann, 1990). The *M. arenaria* population from *Ravenala* had an intermediate range and average DGO (Table 3), falling between *M. platani* (Hirschmann, 1982) and the values reported for multiple *M. arenaria* populations. However, the *Ravenala* population and *M. platani* both lacked bifid spicule termini and areolation in the male lateral field and had an unusually high head region below the similarly shaped cap (Fig. 2A,B). The shape of the male tail was relatively acute in both *M. platani* and the *Ravenala* population (Fig. 2C) as compared to typical *M. arenaria* (Jepson, 1987). The hyaline tail length was indistinct in typical *M. arenaria* (Cliff and Hirschmann, 1985), but was apparent in the *M. arenaria* population and in *M. platani*. Although the *Ravenala* population showed features intermediate between typical *M. arenaria* and *M. platani*, the latter induced large galls on sycamore, a non-host of *M. arenaria*, and failed to reproduce on peanut, corn or sweet potato (Hirschmann, 1982).

Molecular analysis: Results of the molecular studies mirrored the morphological analysis, revealing a mosaic of molecular similarities to several RKN species. Unfortunately, no DNA sequences were available for

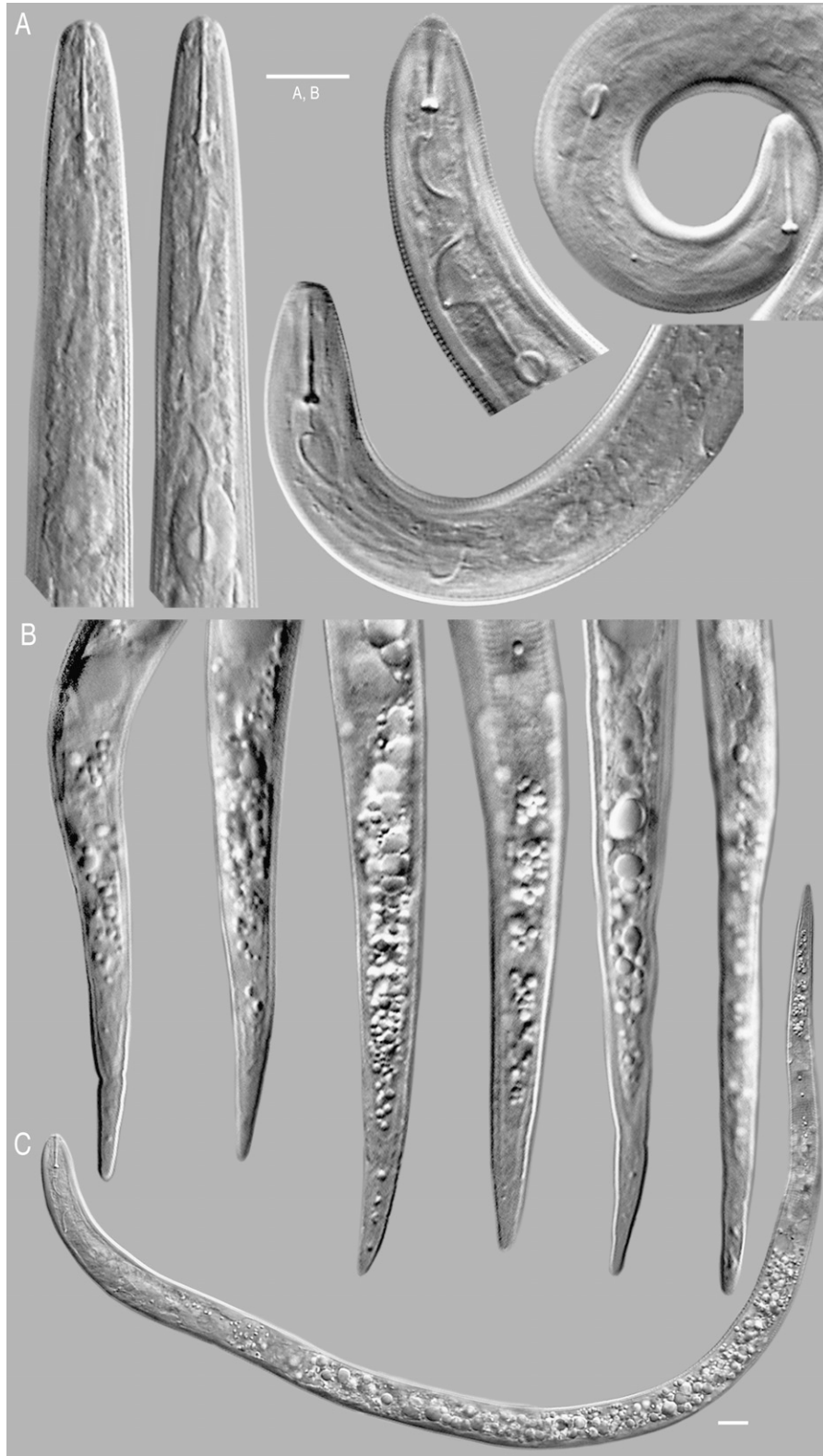


FIG. 3. Light micrographs of *Meloidogyne arenaria* second-stage juveniles from traveler's tree. (A) Anterior ends with stylet and pharynx. (B) Tails. (C) Whole specimen.

M. platani, so it was not possible to complement those morphological comparisons with molecular data.

The 1.1 kb size of the mitochondrial *COII-12S* PCR product from the *Ravenala* population is shared in

common with race 1 and race 2 of *M. arenaria* populations from Georgia, North and South Carolina, and Florida (Powers and Harris, 1993), populations from Ivory Coast, Portugal and the French West Indies (Blok

TABLE 4. Morphometric comparison^a of second-stage juveniles from the *Ravenala* isolate with other *M. arenaria* populations, *M. thailandica* and *M. morocciensis*

Character (μm)	<i>M. arenaria</i> (<i>Ravenala</i> isolate) n = 17	<i>M. arenaria</i> ^b n = 150	<i>M. platani</i> ^c n = 50	<i>M. thailandica</i> ^d n = 25	<i>M. morocciensis</i> ^e n = 35
Body length	475 ± 54 (390 – 570)	504 ± 4.3 (392 – 605)	443 ± 22.5 (395 – 497)	484 ± 25.5 (450 – 540)	400.8 ± 21.7 (374 – 454)
Body width	14.1 ± 1.4 (12.7 – 16) n = 5	15.3 ± 0.1 (13 – 18)	17 ± 1.2 (15 – 20)	14.3 ± 0.6 (13 – 15)	15.2 ± 0.5 (15 – 16)
Stylet length	11.1 ± 0.8 (10 – 12.5)	11.1 ± 0.03 (10 – 12)	12.2 ± 0.04 (11.6 – 12.6)	10.2 ± 0.3 (10 – 11)	12.3 ± 0.5 (11 – 13)
DGO from stylet	3.7 ± 0.2 (3.6 – 4.1) n = 5	3.7 ± 0.04 (3 – 5)	3.5 ± 0.04 (2.7 – 4)	2.9 ± 0.3 (2.5 – 3.5)	3.8 ± 0.4 (3 – 4.4)
Tail length	59 ± 6.6 (50 – 67)	56.0 ± 0.43 (44 – 59)	54.2 ± 0.47 (50 – 65)	61.2 ± 3.0 (55 – 65)	52.6 ± 2.7 (47 – 58)
Hyaline tail length	14 ± 3.3 (10 – 21)	–	12.4 ± 0.2 (9.3 – 15.3)	18.3 ± 1.9 (15 – 20)	–

^a Mean ± standard deviation (range); n = number of specimens.
^b Cliff and Hirschmann (1985); author composite of multiple populations.
^c Hirschmann (1982).
^d Handoo et al. (2005b).
^e Rammah and Hirschmann (1990).

et al., 2002), and one from Brazil (Tigano et al., 2005). Other species that presented similarly sized products include *M. morocciensis* (1,113 bp), and *M. floridensis* (1,110 bp) (Tigano et al., 2005), isolated from peach (*Prunus persicus* L.) in Morocco and Florida, respectively; *M. thailandica* (1,113 bp) from ginger (*Zingiber* spp.) in Thailand (Handoo et al., 2005a); and *M. paranaensis* (1,255 bp) from coffee (*Coffea arabica* L.) (Tigano et al., 2005). Mitochondrial *COII-IRNA* PCR products from *M. javanica* and *M. incognita* are larger (Powers and Harris, 1993; Tigano et al., 2005), thus distinguishing these species from the *Ravenala* population. Products of 1.7 kb have been reported for some *M. arenaria* populations (Powers and Harris, 1993). These most likely correspond to previously described haplotype C/H, which can be distinguished from the smaller type A by virtue of a 529 bp deletion in the intergenic region (Hugall et al., 1994; Stanton et al., 1997). The mitochondrial *COII-IRNA* sequence (EU364879) was identical to *M. arenaria* (Table 5), with one ambiguous position (A/G) found in the *Ravenala* population. Neighbor-joining (NJ) analysis (not shown) grouped the *Ravenala* isolate with another *M. arenaria* population (AY635610) and with *M. morocciensis* (AY942849), in agreement with previous results (Tigano et al., 2005), and with *M. thailandica*

(EU364879). Despite the similarly sized amplification product, the *M. floridensis* mitochondrial sequence contained large indels relative to the *M. arenaria* sequence and was more similar to one from *M. incognita* (Tigano et al., 2005).

The D2-D3 expansion segment of large subunit (LSU) 28S rDNA was 754 bp for the *Ravenala* population (EU364889). An alignment with six of the most closely related sequences from GenBank revealed 13 variable nucleotide positions (Table 6). The most similar sequence (AF435803) was from an *M. arenaria* race 2 population from soybean (Tenente et al., 2004), which differed at 4 bp, including one ambiguous position. Other highly similar sequences included *M. incognita* (AF435794) also from soybean, *M. konaensis* (AF435797) from coffee in Hawaii, and Brazilian *M. paranaensis* populations Mp61 (AF435798), Mp62 (AF435799) and Mp71 (AF435800), also isolated from coffee. Because some previously reported 28S D2-D3 sequences (Tenente et al., 2004) contained ambiguous bases (most likely due to direct sequencing of PCR products, not clones), it is unclear how many separate haplotypes occur within these species. A separate comparison of D3 sequences including several more from GenBank (not shown) provides some clues. Previous D3 analyses showed that *M. arenaria*, *M. incognita*

TABLE 5. Variable nucleotide positions in mitochondrial DNA

Species name & accession number	Variable nucleotide position ^a							# Variable positions
	199	405	696	706	722	845	1,083	
<i>Meloidogyne arenaria</i> ^b	A	:	A/G	G	T	A	A	-
<i>M. arenaria</i> (AY635610)			A					1
<i>M. morocciensis</i> (AY942849)			A		C		G	3
<i>M. thailandica</i> ^b	G	T	A	A/G		G		5

^aNucleotides are numbered according to the sequence alignment, starting with the first base in the 5' primer sequence.
^bThis study.

TABLE 6. Variable nucleotide positions in 28S rDNA D2-D3 expansion segment of various root-knot nematodes

Species name & accession number	Variable nucleotide position ^a													# Variable positions
	1	15	95	96	103	178	302	312	393	507	545	751	754	
<i>Meloidogyne arenaria</i> ^b	A	C	A	T	G	A	T	A	C	A	T	C	A	-
<i>M. arenaria</i> (AF435803)		T		G/T			C						G	4
<i>M. incognita</i> (AF435794)		T		G/T			C/T			A/G			G	5
<i>M. paranaensis</i> Mp61 (AF435798)		T	T	G		T			T				G	6
<i>M. konaensis</i> (AF435797)		T		G/T	G/T		C	A/T			C/T		G	7
<i>M. paranaensis</i> Mp62 (AF435799)		T	T	G		T	C/T		T				G	7
<i>M. paranaensis</i> Mp71 (AF435800)	T	T	T	G		T			T			G	G	8

^aNucleotides are numbered according to the sequence alignment, starting with the first base in the 5' primer sequence.

^bThis study.

and *M. hapla* contained seven haplotypes among them, with two (haplotypes 5 and 7) found in all three species (Chen et al., 2003), and examination of *M. thailandica* cloned D3 PCR products revealed five haplotypes (A – E) (Handoo et al., 2005a). Here we found that the *M. arenaria* population from *Ravenala* shared the D3 haplotype 5 in common with populations *M. arenaria* MA, *M. incognita* COA and MUL and *M. javanica* JNC, J811 and VW4 (Chen et al., 2003), which was also identical to the B haplotype of *M. thailandica* (Handoo et al., 2005a). Since most of the distinguishing polymorphisms in our comparisons were located in D2 (Table 6), it appears this region may provide better phylogenetic resolution within the apomictic group, particularly when multiple clones from a population are examined.

The amplified IGS-2 region, between the 5S and 18S rDNA, was 716 bp for the *Ravenala* population (EU364878), consistent with the size previously reported for the tropical RKN species and distinct from larger products found in *M. mayaguensis* and smaller in *M. hapla* (Blok et al., 1997). Out of 717 bp aligned, 17 positions were variable among the RKN compared (Table 7). The most similar sequences were from *M. thailandica*, *M. arenaria*, *M. incognita*, *M. javanica* and *M. floridensis*. No polymorphisms set *M. arenaria* clearly apart, and, except for the distinctive *M. floridensis* sequence, nucleotide ambiguities in this marker obscured the boundaries between species.

Hsp90 PCR products of ~1 kb were sequenced for the *Ravenala* population (EU364880), *M. arenaria* from Maryland (FJ238508) and several other RKN species,

including *M. thailandica* (EU364882) from Thailand (Handoo et al., 2005a), *M. floridensis* (EU364884 - EU364888) from Florida and Georgia (Handoo et al., 2004), *M. javanica* (AF201338) from Maryland and *M. incognita* (EU364881). All partial Hsp90 sequences consisted of five exons and four introns. The three Florida and one Georgia *M. floridensis* populations were represented by two distinct Hsp90 haplotypes. The rest of the *Meloidogyne* populations yielded single sequences, although a few polymorphic positions were detected among them (Table 8). The alignment was trimmed to 972 bases to eliminate the degenerate primer end sequences, which were assumed to be unreliable. Among the sequences compared, there were 20 polymorphic positions, with eight base changes within introns, two changes at first codon positions, five at second positions and four at third positions. *Meloidogyne arenaria* from Maryland was nearly identical to the *Ravenala* population, with one C-T transition and one single base insertion. Sequence divergences for *M. thailandica*, *M. floridensis* haplotypes A and B, *M. javanica* and *M. incognita* were <1%, similar to what was found for the other loci examined. In contrast to the low level of divergence among the apomictic species of RKN, two *M. hapla* sequences determined previously (Handoo et al., 2005b) were significantly different (17%) from the *Ravenala* population. This separation of *M. hapla* from the clade of the apomictic species is consistent with findings involving other loci (Hugall et al., 1994, 1999; Chen et al., 2003; Tigano et al., 2005; Castagnone-Sereno, 2006).

TABLE 7. Variable nucleotide positions in the ribosomal intergenic space region IGS-2 of various root-knot nematodes

Species name & accession number	Variable nucleotide position ^a																# Variable positions	
	14	32	67	89	117	118	144	182	211	250	274	379	442	487	534	635		694
<i>Meloidogyne arenaria</i> ^b	T	:	T	A	C	T	T/C	C	A/C	A/T	T	T	G	A/G	A	A	G	-
<i>M. thailandica</i> (AY858796)	:						T		A	T				A				5
<i>M. arenaria</i> (Blok et al., 1997)	:						T	A/C	A	T				A			C	7
<i>M. incognita</i> (Blok et al., 1997)	:			A			T	A/C	A	T				A				7
<i>M. javanica</i> (Blok et al., 1997)	:						T	A/C	A	T		C/T		A	A/T	AG	C	10
<i>M. floridensis</i> (AY194853)	:	C	G	C		A	T		A	T	G		:	A				11

^aNucleotides are numbered according to the sequence alignment, starting with the first base in the 5' primer sequence.

^bThis study.

TABLE 8. Variable nucleotide positions in an alignment of partial Hsp90 genomic sequences of various root-knot nematodes

Species name	Variable nucleotide position ^a																		# Variable positions			
	11	(42)	(53)	(89)	108	110	150	256	288	320	361	369	443	452	(728)	(738)	819	(846)		(861)	(869)	
<i>Meloidogyne arenaria</i>	T	a	a	c	C/T	A	T	G	A	A	T	T	A	A	t	c	T	:	a	t	-	
<i>M. arenaria</i> (MD)				t	T														t		2	
<i>M. thailandica</i>				t	C/T				A/C											a/t	a/t	4
<i>M. floridensis</i> haplotype A				t	C												t		a		4	
<i>M. javanica</i>				t	T		C	T											C	t		4
<i>M. incognita</i>	:	:		t	C								T		:				a		:	6
<i>M. floridensis</i> haplotype B	C			t	T	C					G	A	A		G				a			7

^a Nucleotide positions are numbered according to the sequence alignment, trimmed to remove degenerate primer ends. The alignment starts with the second codon in the first K within the amino acid sequence "KSGTKAF." Positions in parentheses with nucleotides in lower case correspond to changes within introns.

An existing sequence dataset from an *M. arenaria* cDNA library contained several overlapping EST corresponding to Hsp90, so a composite sequence was constructed from GenBank accessions BI747178, BI747494 and BI745855. The EST assembly sequence contained a number of ambiguous bases and differed

	1	10	20	30	40	50	60	70	
Ma_PA	AATCTGGAAC	TAAGGCTTTC	ATGGAGGCTC	TTCAGGTTTT	TAATTTTGGT	TAAATGAGT	ATGTTCTCCA	70	
Ma_MD	AATCTGGAAC	TAAGGCTTTC	ATGGAGGCTC	TTCAGGTTTT	TAATTTTGGT	TAAATGAGT	ATGTTCTCCA	70	
Ma_ESTs	-----	-----	-----	-----	-----	-----	-----	-----	
Ma_PA	TCTGAAAAG	CTTAAATACT	AAAATTTAGG	CTGGTGCYGA	TATTAGTATG	ATTGGTCAAT	TCGGTGTGG	140	
Ma_MD	TCTGAAAAG	CTTAAATACT	AAAATTTAGG	CTGGTGCYGA	TATTAGTATG	ATTGGTCAAT	TCGGTGTGG	140	
Ma_ESTs	-----	-----	-----	-----GCCGA	TATTAGTATG	ATTGGTCAAT	TCGGTGTGG	35	
Ma_PA	TTTCTACTCT	GCCTTCTTGG	TTGCTGACCG	CGTCACTGTC	ACTTCTAAAC	ATAATGATGA	TGACTGTCTAT	210	
Ma_MD	TTTCTACTCT	GCCTTCTTGG	TTGCTGACCG	CGTCACTGTC	ACTTCTAAAC	ATAATGATGA	TGACTGTCTAT	210	
Ma_ESTs	TTTCTACTCT	GCCTTCTTGG	TTGCTGACCG	CGTCACTGTC	ACTTCTAAAC	ATAATGATGA	TGACTGTCTAT	105	
Ma_PA	CAATGGGAGT	CTTCCGCTGG	CGGTTCAATC	ATCATCCGTA	ACTGCGTTGA	TCCTGAGATG	ACTCGCGGAA	280	
Ma_MD	CAATGGGAGT	CTTCCGCTGG	CGGTTCAATC	ATCATCCGTA	ACTGCGTTGA	TCCTGAGATG	ACTCGCGGAA	280	
Ma_ESTs	CAATGGGAGT	CTTCCGCTGG	CGGTTCAATC	ATCATCCGTA	ACTGCGTTGA	TCCTGAGATG	ACTCGCGGAA	175	
Ma_PA	CGAAGATAAC	TCTTTACCTT	AAAGAGGATC	AGACTGACTA	TTTGAAGAG	CGTCGTATTC	GTGAAGTGGT	350	
Ma_MD	CGAAGATAAC	TCTTTACCTT	AAAGAGGATC	AGACTGACTA	TTTGAAGAG	CGTCGTATTC	GTGAAGTGGT	0350	
Ma_ESTs	CGAAGATAAC	TCTTTACCTT	AAAGAGGATC	AGACTGACTA	TTTGAAGAG	CGTCGTATTC	GTGAAGTGGT	245	
Ma_PA	TAAGAAGCAC	TCCCAATTTA	TTGGATATCC	GATAAAGCTC	TTGGTTGAGA	AGGAACGTGA	TAAGGAGATT	420	
Ma_MD	TAAGAAGCAC	TCCCAATTTA	TTGGATATCC	GATAAAGCTC	TTGGTTGAGA	AGGAACGTGA	TAAGGAGATT	420	
Ma_ESTs	TAAGAAGCAC	TCCCAATTTA	TTGGATATCC	GATAAAGCTC	TTGGTTGAGA	AGGAACGTGA	TAAGGAGATT	315	
Ma_PA	TCTGATGATG	AGGCTGAGGA	TGAGAAAAAG	GATGTAAGA	AGGAAGAGGA	AAAGGAGGAA	GAGAAGGAGA	490	
Ma_MD	TCTGATGATG	AGGCTGAGGA	TGAGAAAAAG	GATGTAAGA	AGGAAGAGGA	AAAGGAGGAA	GAGAAGGAGA	490	
Ma_ESTs	TCTGATGATG	AGGCTGAGGA	TGAGAAAAAG	GATGTAAGA	AGGAAGAGGA	AAAGGAGGAA	GAGAAGGAGA	385	
Ma_PA	TTAAAAAGGA	AGAGGGTGAA	GACAAGGAGG	GAGAAGgtat	ttttctttga	tcgtaattgt	ttaattaatc	560	
Ma_MD	TTAAAAAGGA	AGAGGGTGAA	GACAAGGAGG	GAGAAGgtat	ttttctttga	tcgtaattgt	ttaattaatc	560	
Ma_ESTs	TTAAAAAGGA	AGAGGGTGAA	GACAAGGAGG	GAGAAG:::	:::~::~:	:::~::~:	:::~::~:	421	
Ma_PA	ttattagATG	AAGATAAGGA	CAAGAAGGAC	GGTGAGAGA	AAAAGAAGAC	CAAGAAGATC	AAAGAGAAAT	630	
Ma_MD	ttattagATG	AAGATAAGGA	CAAGAAGGAC	GGTGAGAGA	AAAAGAAGAC	CAAGAAGATC	AAAGAGAAAT	630	
Ma_ESTs	:::~::~:ATG	AAGATAAGGA	CAAGAAGGAS	GGTGAGMAGA	AAAAGAAGAC	CAAGAAGATC	AAAGAGAAAT	484	
Ma_PA	ACACCGAAGA	TGAAGAGTTG	AACAAGACAA	AGCCCATTTG	GACACGAAAC	CCCGATGACA	TTACTAATGA	700	
Ma_MD	ACACCGAAGA	TGAAGAGTTG	AACAAGACAA	AGCCCATTTG	GACACGAAAC	CCCGATGACA	TTACTAATGA	700	
Ma_ESTs	ACACCGAAGA	TGAAGAGTTG	AACAAGACAA	AGCCCATTTG	GACACGAAAC	CCCGATGACA	TTACTAATGA	554	
Ma_PA	Ggtaaactg	atacatgtat	aaattagtaa	ctgttctcca	cttttaagga	ATATGCTGAA	TTCTACAAGA	770	
Ma_MD	Ggtaaactg	atacatgtat	aaattagtaa	ctgttctcca	cttttaagga	ataatgctgaa	TTCTACAAGA	770	
Ma_ESTs	G:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:GA	ATATGCTGAA	577	
Ma_PA	GCTTGTCTAA	CGATTGGGAG	GACCAATTTG	CTGTTAAGCA	TTTGAGCGTT	GAAGGACAAAT	TGGAATTCCG	840	
Ma_MD	GCTTGTCTAA	CGATTGGGAG	GACCAATTTG	CTGTTAAGCA	TTTGAGCGTT	GAAGGACAAAT	TGGAATTCCG	840	
Ma_ESTs	GCTTGTCTAA	CGATTGGGAG	GACCAATTTG	CTGTTAAGCA	TTTGAGCGTT	GAAGGACAAAT	TGGAATTCCG	647	
Ma_PA	gtaaa~tttc	aattccttct	actacttgta	ttagtgtaatt	ttggttaaat	gtttcatttt	taaagTGCC	909	
Ma_MD	gtaaa~tttc	aattccttct	actacttgta	ttagtgtaatt	ttggttaaat	gtttcatttt	taaagTGCC	910	
Ma_ESTs	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:~TGVC	652	
Ma_PA	TTTTGTTCGT	TCCACAACGT	GCTCCCTTTG	ACATGTTCGA	GAATAAGAAG	CAAAAGAATG	CTATCAAGCT	979	
Ma_MD	TTTTGTTCGT	TCCACAACGT	GCTCCCTTTG	ACATGTTCGA	GAATAAGAAG	CAAAAGAATG	CTATCAAGCT	980	
Ma_ESTs	TTTTGTTCGT	TCCACAACGT	GCTCCCTTTG	ACATGTTCGA	GAATAAGAAA	CAAAAGAATG	CTATCAARCT	717	
Ma_PA	TTATGTTCGA	CGTGT	994						
Ma_MD	TTATGTTCGA	CGTGT	995						
Ma_ESTs	TTATGTTCGA	CGTGT	732						

FIG. 4. Alignment of partial Hsp90 genomic sequence from variant *M. arenaria* population from traveler's tree compared with sequence from morphologically typical *M. arenaria* from MD and composite cDNA sequence assembled from *M. arenaria* EST. The genomic sequence was trimmed of degenerate primer end bases, and the 3' end of the EST composite extending beyond the genomic amplicons was trimmed. Genomic intron sequences from the *M. arenaria* population are in lower case. Abbreviations: R = A or G; Y = C or T; W = A or T; M = A or C; S = C or G; dash (-) indicates missing data; colon (:~) indicates intron gaps not present in EST. Positions that vary in at least one of the sequences are indicated by underline.

from the *Ravenala* population coding sequence at six positions (Fig. 4). Some of the ambiguities appeared towards the ends of the individual EST (not shown) and so may be due to unreliable base calls from the single-pass reads or they may represent genuine Hsp90 sequence variants. Examination of Hsp90 clones from multiple populations of *M. arenaria* will be necessary to establish the extent of intraspecific variability within the gene, but it appears that Hsp90 is neither better nor worse at resolving these species than the mitochondrial or ribosomal markers examined.

While most populations of *M. arenaria* induce robust galls, some cause smaller, bead-like galls (Eisenback and Triantaphyllou, 1991). However, none have been reported to fail to induce galls as this one did. The plant order Zingiberales contains eight families, including Strelitziaceae, Zingiberaceae and Heliconiaceae. Strelitziaceae (Quénéhervé et al., 1997) contains one species within the genus *Ravenala* (including traveler's tree, *R. madagascariensis*), one in *Phenakospermum* and five within *Strelitzia* (including bird-of-paradise). Very little has been reported on *Meloidogyne* species incidence specifically within *Ravenala*. However, all three major parthenogenetic species attack ginger (*Zingiber*, within the Zingiberaceae) (Baptista dos Santos and Lazaro Lozano, 1993), and *M. thailandica* (Handoo et al., 2005a) also attacks *Heliconia* spp. (false bird-of-paradise), a genus within Heliconiaceae. Host preferences represent convergent phenotypes, since members of the same host race show substantial genetic variability (Baum et al., 1994). The paradoxical genetic diversity of the four major parthenogenetic RKN species (Castagnone-Sereno, 2006) is most prominent in *M. arenaria* populations (Powers, 2004). Perhaps then, it is not surprising that such a rare host response phenotype should accompany such morphological and molecular variation.

Specimens of the appropriate stage or quantity desirable for replicates of isozyme analysis (Esbenshade and Triantaphyllou, 1990), RAPD (Cenis, 1993; Randig et al., 2002) or host range tests were lacking and unfortunately could not be included in the diagnosis. SCAR PCR assays using species-specific primers (Zijlstra et al., 2000) were not performed, as the choice of molecular markers examined for limited specimens was influenced by future utility of the sequences for broader phylogenetic studies. These assays would likely have affirmed the morphological and molecular results presented here. Positive scoring for *M. arenaria* through isozymes, RAPD or SCAR markers would have added further support for the diagnosis; however, confusing, new or negative results with these tests would have affirmed our conclusion that this population was an atypical variant of *M. arenaria*.

This study highlights the fact that the lines for species demarcation seem to be fading as new *Meloidogyne* species and populations are characterized. Accurate

diagnosis requires a wide range of morphological and molecular characters such as those presented here. The degree of intraspecific variation relative to interspecific variation observed for the tropical RKN further highlights the need for additional characters and molecular markers that will clearly discriminate these species as well as inform their evolutionary relationships.

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