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Morphological and Molecular Evaluation of a *Meloidogyne hapla* Population Damaging Coffee (*Coffea arabica*) in Maui, Hawaii

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Abstract: An unusual population of *Meloidogyne hapla,* earlier thought to be an undescribed species, was found causing large galls, without adventitious roots, and substantial damage to coffee in Maui, Hawaii. Only in Brazil had similar damage to coffee been reported by this species. Unlike *M. exigua* from South and Central America, this population reproduced well on coffee cv. Mokka and *M. incognita*-susceptible tomato but poorly on tomato with the *Mi* resistance gene. Characterization included SEM images, esterase isozymes, and five DNA sequences: i) the D3 segment of the large subunit (LSU-D3 or 28S) rDNA, ii) internal transcribed spacer (ITS-1) rDNA, iii) intergenic spacer (IGS) rDNA, iv) the mitochondrial interval from cytochrome oxidase (CO II) to 16S mtDNA, and v) the nuclear gene Hsp90. Sequences for ITS-1, IGS, and COII were similar to other *M. hapla* populations, but within species ITS-1 variability was not less than among species. One LSU-D3 haplotype was similar to a previously analyzed population with two minor haplotypes. Hsp90 exhibited some variation between Maryland and Hawaiian populations distinct from other species. Females were narrow with wide vulval slits, large interphasmidial distances, and more posterior excretory pores; 20% of perineal patterns had atypical perivulval lines. Males had a low b ratio (<12 µm). Juveniles had a short distance between stylet and dorsal gland orifice. Juvenile body length was short (<355 µm) and was different between summer and winter populations.

Key words: climate, *Coffea arabica, Coffea robusta,* detection, identification, India, *Meloidogyne exigua, M. incognita, M. javanica, M konaensis,* molecular diagnostics, nematode, Netherlands, resistance, taxonomy.

A highly damaging population of root-knot nematode was identified in coffee (*Coffea arabica*) roots and from 65% of soil samples on a more than 200-ha farm in Lahaina, northern Maui, in spring 2000. The severity and type of galling damage were similar to that caused by *Meloidogyne exigua* (Goeldi, 1892) Chitwood, 1949, but until now only *M. incognita* (Kofoid and White, 1919) Chitwood, 1949 and *M. konaensis* Eisenback et al., 1994 were reported to parasitize coffee in the Hawaiian islands (Rodríguez et al., 1998). Because initial esterase phenotypes were ambiguous, morphological and molecular characters were used to identify and characterize this unusual, damaging population that was originally suspected of being a new species. This is the first report of *M. hapla* Chitwood, 1949 damaging coffee in the Pacific region.

Materials and Methods

Populations: Meloidogyne sp. populations discussed are listed in Table 1.

Microscopy: Females were dissected from infected roots after fixation in 3% formaldehyde for a minimum of 48 hours. Second-stage juveniles (J2) and males were similarly fixed in 3% formaldehyde and processed to glycerine by the formalin-glycerine method (Golden, 1990; Hooper, 1970). Nematodes were processed for SEM according to the protocol in Handoo et al. (2001) and observed using a Hitachi S-570 SEM at 10 kV. Procedures used in measuring and preparing specimens were those described by Golden and Birchfield (1972), except some fixed females were cut and mounted in clear lactophenol solution. Photomicrographs of perineal patterns, J2, and males were made with an automatic 35-mm camera attached to a compound microscope with differential interference contrast optics. Light microscopic images of fixed nematodes were made using a compound microscope. Morphometrics included body lengths of J2 measured from field samples taken on 29 August and 17 December 2003. Student's *t*-test was selected to compare measurements among populations (Statgraphics Plus, Manugistics, Inc., Rockville, MD).

Esterase tests: Esterase phenotypes of two females from *Coffea arabica* transferred to tomato (*Lycopersicon esculentum* Mill.), six females from *C. arabica* 'Red Catuai,' five females from *C. arabica* 'Mokka,' and six females from the weed Spanish needle (*Bidens pilosa* L.) were compared with a Hawaiian population of *M. javanica* (pop. 32) from coffee grown on tomato. Proteins were separated by the automated PhastSystem (Amersham Biosciences, Inc., Piscataway, NJ) on a Gradient 10–15 gel run and detected with Fast Blue RR (Esbenshade and Triantaphyllou, 1985).

DNA sequencing: Nematode specimens were mechanically disrupted with an eye-knife or sharp forceps tips and then pooled as two J2 in 20 µl or nine J2 in 50 µl of extraction buffer as described in Williams et al. (1992) and stored at <80°C. The pooled samples were thawed and extracts prepared by warming to 60°C for 60 minutes, followed by 95°C for 15 minutes to deactivate the proteinase K. Eleven microliters of the extract were used for each PCR reaction.

PCR reactions: The primers for the four sequences used for this study are listed in Table 2. Except for the

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TABLE 1. List of *Meloidogyne* species and populations used in this study.

No.	Isolate name	Geographic location	Gene or subject	Accession #	Reference
Meloidogyne hapla					
1	HI	North Maui, HI	IGS	AY528418	This study
			Mitochondrial	AY539839	This study
			ITS	AY528419	This study
			28S D3	AY528415, AY528420	This study
			Hsp90	AY528416	This study
2	MD	Carmichael, MD	Hsp90	AY528417	This study
3	Q48	Queensland, Australia	IGS	AJ421708	Wishart et al., 2002
4	MH97-1	Yates County, NY	Mitochondrial	No number	Mitkowski and Abawi, 2003
5	MH-97-3	Cayuga County, NY	Mitochondrial	No number	Mitkowski and Abawi, 2003
6	MH-97-14	Genesee County, NY	Mitochondrial	No number	Mitkowski and Abawi, 2003
7	113	Australia	Mitochondrial	L76262	Hugall et al., 1997
8	*M. hapla*	Netherlands	Morphology	No number	APHIS
9	Barossa 1	Australia	ITS	AF516721	Qader and Riley, unpubl.
10	Robe	Australia	ITS	AF516722	Qader and Riley, unpubl.
11	*M. hapla*	Unknown	ITS	AY268108	Lee and Williamson, unpubl.
12	*M. hapla*	Unknown	ITS	U96303	Powers et al., 1997
13	Haplotype 1	Several	28S, D3	AY335815	Chen et al., 2003
14	Haplotype 2	Several	28S, D3	AY335816	Chen et al., 2003
15	86NC Race A	North Carolina	SEM	No number	Eisenback and Hirschmann,
16	48NC Race B	North Carolina	SEM	No number	1978
17	V	Venezuela	Morphology	No number	Dao, 1970
18	N	Netherlands	Morphology	No number	Dao, 1970
19	H. P.	Himchal Pradesh, India	Morphology	No number	Sahoo and Ganguly, 2000
20	*M. hapla* race A	Poland	Morphology	No number	Kornobis, 2001
21	*M. hapla* race B	Poland	Morphology	No number	Kornobis, 2001
22	Type	Bridgehampton, NY	Morphology	No number	Chitwood, 1949
23	*M. hapla*	Tanzania, East Africa	Morphology	No number	Whitehead, 1968
24	*M. hapla*	South India	Host range	No number	Kumar, 1984
25	*M. hapla*	Italy (?)	Host range	No number	Molinari and Miacola, 1997
M. arenaria					
26	26	Portugal	IGS	No number	Blok et al., 1997
M. chitwoodi					
27	*M. hapla*	Unknown	Mitochondrial	AF387089	Sui et al., unpubl.
M. incognita					
28	19	French West Indies	IGS	No number	Blok et al., 1997
M. javanica					
29	23	Burkina Faso	IGS	No number	Blok et al., 1997
30	*M. hapla*	Unknown	Mitochondrial	No number	Hugall et al., 1997
31	*M. hapla*	Unknown	ITS	AF387095	Sui et al., unpubl.
32	Mj	Hailiimale, HI	Esterase	No number	This study

D3 rDNA amplifications, all PCR reactions contained 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 2 U MasterTaq (Eppendorf, Brinkmann, Westbury, NY), plus manufacturer-supplied buffers, in a total reaction volume of 50 µl. The D3 reactions were performed in 25 µl extraction buffer, containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.6 µM of each primer, 1 U MasterTaq, and manufacturer-supplied buffers.

All PCR reactions were performed in a MasterCycler (Eppendorf, Brinkmann, Westbury, NY). The rDNA intergenic spacer (IGS) region was amplified using the 5SF and 18SR primers according to Blok et al. (1997).

TABLE 2. List of primers used for DNA analysis.

Primer	Sequence	Gene	Reference
5SF	TTAACTTGCCAGATCGGACG	rDNA IGS	Blok et al., 1997
18SR	TCTAAGAGCCGTACGC	rDNA IGS	Blok et al., 1997
18S (rDNA2)	TTGATTACGTCCCTGCCCTTT	ITS-1	Vrain et al., 1992
5.8S (RDNA1.58S)	ACGAGCCGAGTGATCCACCG	ITS-1	Cherry et al., 1997
1 RNAF (1108)	TACCTTTGACCAATCACGCT	Mitochondrial COII	Powers and Harris, 1993
COIIR (C2F3)	GGTCAATGTTCAGAAATTTGTGG	Mitochondrial COII	Powers and Harris, 1993
D3A	GACCCGTCTTGAAACACGGA	28S rDNA	Baldwin et al., 1997
D3B	TCGGAAGGAACCAGCTACTA	28S rDNA	Baldwin et al., 1997
U288	GAYACVGGVATYGGNATGACYAA	Hsp90	This study
L1110	TCRCARTTVTCCATGATRAAVAC	Hsp90	This study

The rDNA internal transcribed spacer region 1 (ITS-1) was amplified using rDNA2 (18S) (Vrain et al., 1992) and rDNA1.58s (5.8S) (Cherry et al., 1997). Mitochondrial sequences were amplified with primers #C2F3 (COII) and #1108 (1rRNA) (Powers and Harris, 1993) as previously described (Blok et al., 2002). The LSU 28S rDNA D3 expansion segment was amplified with primers D3a and D3b (Baldwin et al., 1997; Duncan et al., 1999). Cycling conditions were 94°C for 2 minutes to allow a hot start, followed by 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 90 seconds ending with 72°C for 10 minutes to allow complete extension of PCR products. Hsp90 was amplified using a modification of RAN-PCR (Skantar and Carta, 2000). Hot start reactions were assembled in PCR tubes by first adding dNTPs and primers. This mixture was overlaid with a drop of paraffin wax that was allowed to harden, followed by a top layer containing the DNA template and Taq enzyme mixture. Cycling conditions consisted of a preheat step of 94°C for 10 minutes to allow the layered components to mix followed by 35 cycles of 94°C for 20 seconds, 65°C for 5 seconds, 60°C for 5 seconds, 55°C for 5 seconds, 50°C for 5 seconds, and 68°C for 1 minute. A final step of 68°C for 15 minutes allowed for complete extension of products. PCR products were separated by electrophoresis in Tris-acetate-EDTA (TAE) buffered 1% to 1.5% agarose, depending on the expected product size. Products were visualized with UV illumination after ethidium bromide staining (Sambrook et al., 1989). If Hsp90 reactions yielded faint products, 10% of the PCR reaction was used as the template for a second amplification reaction as described.

Cloning and sequencing: PCR products were excised from agarose gels and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) or by brief centrifugation. For the latter method, a syringe needle was used to pierce a small hole in the bottom of a 0.5-ml microcentrifuge tube. The gel band was placed inside this tube, which was nested inside a 1.5-ml tube. The assembly was spun for 30 seconds in a microcentrifuge at 14,000 × g. Liquid collected in the tube bottom typically contained enough DNA of sufficient purity for cloning. IGS and Hsp90 PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and COII-16S and D3 were cloned into pCR4-TOPO (Invitrogen) according to manufacturer's instructions. Plasmid DNA was prepared with a Wizard Plus miniprep kit (Promega, Madison, WI) and digested with EcoRI to verify the correct insert. Double-stranded DNA was sequenced by cycle sequencing with Big Dye 3.0 Terminator cycle sequencing kit and analyzed with an ABI 310 Gene Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA), or with the CEQ DTCS Quick Start Kit analyzed with a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). For each gene, we analyzed the following: IGS, 2 clones; Hsp90: MD, 3 clones; HI, 3 clones; mitochondrial, 7 clones; D3, 18 clones. Sequence was determined on both strands from all clones using M13 forward and M13 reverse primers. Sequence information was assembled using Sequencher 4.1 (Genecodes Corp. Ann Arbor, MI). Alignments were performed using ClustalW (Thompson et al., 1994). Formatting of alignments was performed with GeneDoc (Nicholas et al., 1997). Sequences have been submitted to GenBank with accession numbers AY528415 (28S haplotype 1, HI), AY528420 (28S haplotype 3, HI), AY528416 (Hsp90, HI), AY528417 (Hsp90, MD), AY528418 (5S-18S IGS rRNA, HI), AY539839 (Mitochondrial), and AY528419 (ITS-18S rRNA, HI).

Tomato host tests: Two to 3-week-old tomato (*Lycopersicon esculentum* 'Rutgers,' and 'Healani') seedlings were planted into 7.6 to 10-cm clay pots with a 1:1 (v/v) soil:silica sand mixture. The seedlings were inoculated on 27 May 2003 with 1,000 root-knot nematode eggs from tomato cultures grown in the greenhouse with a mean daily 27°C temperature. Plants were fertilized with a water-soluble fertilizer approximately once a week and harvested 24 June 2003. After soil was gently removed from roots, shoots were removed and discarded. Eggs were extracted from roots by a 0.4% NaOCl-extraction/sugar flotation-centrifugation method (Jenkins, 1964). Eggs were counted, and wet and dry root weights were taken.

Results

Morphology: Images of females, J2, and males of the atypical species are seen in Figure 1. Twenty percent of perineal patterns had lines perpendicular to the annulations in and around the vulval-anal area (Fig. 1A–C). Most perineal patterns were generally typical for *M. hapla,* (Fig. 1D–F), as were the asymmetrical female lip pattern (Fig. 1G); J2 lips with elevated labial disk, and shallow, acutely angled lateral lips (Fig. 1H); J2 tails (Fig. 1I); and male stylet with cylindrical shaft (Fig. 1J) that dissolved with stylet knobs soon after fixation. The shape of the male tail was characteristic (Fig. 1K), as was the morphology of the spicule. Morphometrics for females, males, and J2 were within the range of other *M. hapla* populations (Sahoo and Ganguly, 2000; Table 1 references) with some exceptions (Tables 3–5). Measurements previously unrecorded for any population are also given for female cuticle thickness, stylet knobs, annules to excretory pore, and head to metacorpus base (Table 3). Whereas J2 body length was relatively short during both seasons (285–322 µm, average (avg.) 301 ± 16 µm, $n = 6$ in August; 321–357 µm, avg. 340 ± 13 µm, $n = 7$ in December), the measurements were significantly lower ($P = 0.0006$) during the summer. Galls were large and ranged in size from 2.5–2.9 mm, avg. = 2.6 ± 0.17 mm standard deviation (SD) by 2.6–3.2 mm, avg. = 2.96 ± 0.23 mm.

Molecular: Esterase gels (not shown) showed consis-

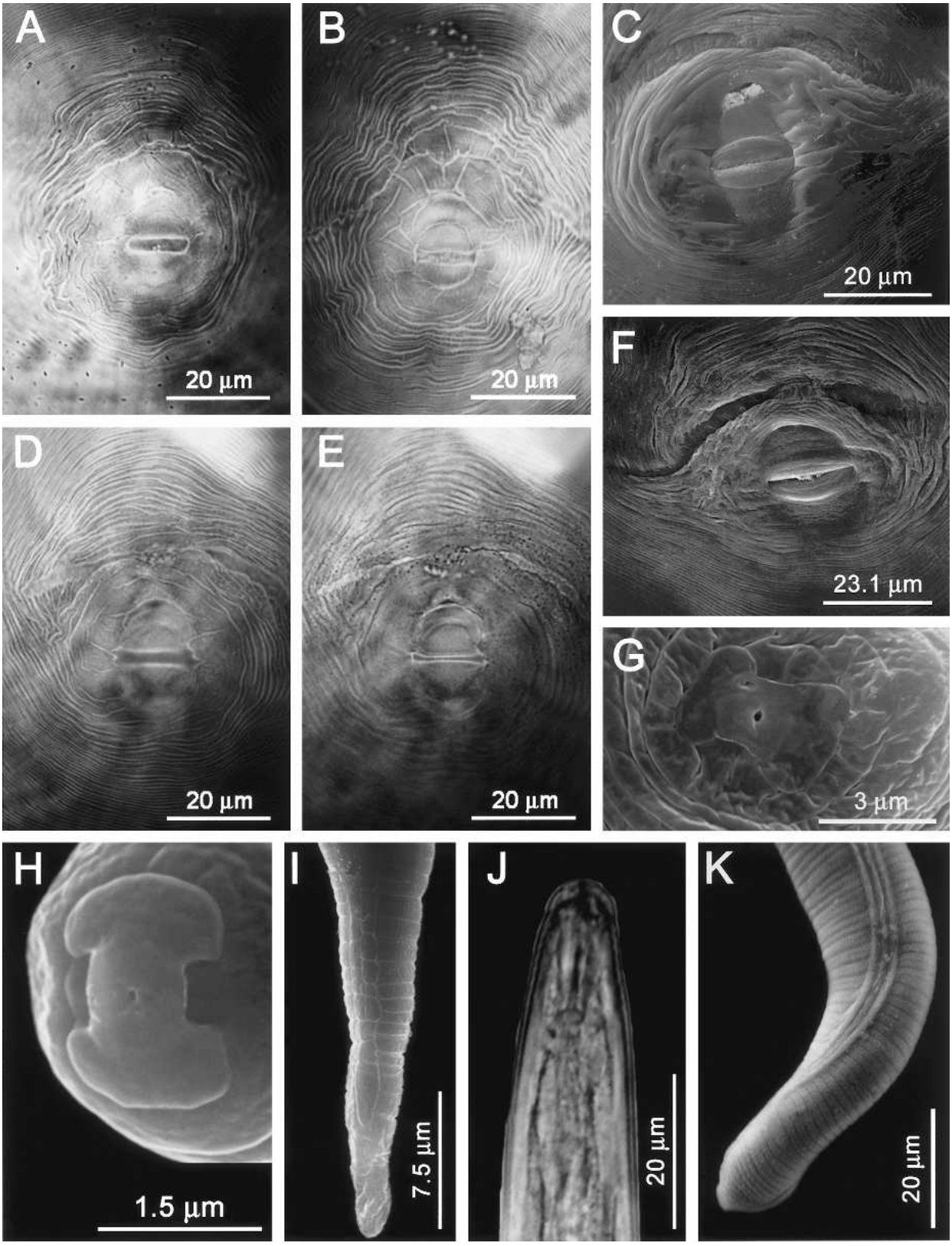

FIG. 1. Female characters of *Meloidogyne hapla,* Population 1. A–C) Perineal patterns unique to population 1. A,B) Viewed by light microscopy (LM). C) Viewed by scanning electron microscopy (SEM). D–F) Typical perineal patterns viewed by LM (D,E) and SEM (F). G) Female face view, SEM. H) Juvenile face view, SEM. I) Juvenile tail, SEM. J) Male head, LM. K) Male tail, lateral field, SEM.

TABLE 3. Measurements of 25 *Meloidogyne hapla* females, population 1.

Character	Range	Mean	Standard deviation
Linear (μm)			
Body width	225–495	342.3	65.2
Neck length	90–195	131.0	32.3
Neck width	50–120	75.0	24.5
Cuticle thickness at neck	2.5–3.5	2.9	0.4
Cuticle thickness at midbody	3.5–10	5.4	1.7
Stylet knob width	2.5–3.5	3.0	0.5
Stylet knob height	2.5–3	2.5	0.1
DGO from base of stylet	5–6.5	5.2	0.5
Excretory pore to anterior end	25–50	36.4	8.0
Body length from anterior end to posterior end of metacorpus	65–115	84.8	14.8
Number of annules from anterior end to excretory pore	16–28	21.8	4.1
Vulval slit length	20–30	23.9	2.7
Vulval slit to anus distance	14–22.5	17.6	2.7
Distance between phasmids	12.5–30	20.9	4.6
Distance from anus to tail terminus	12.5–25	15.4	3.3
a ratio	1.4–2.1	1.7	0.2
Excretory pore/stylet length	1.9–4	2.9	0.7

tent bands among the nematodes from coffee, tomato, and Spanish needle. The three bands of the *M. javanica* control commonly used as a marker allowed determination of the relative position of the single bands in these various samples. The bands for this unusual Hawaiian coffee nematode corresponded more closely to the H1 pattern typical of *M. hapla* than to the similar M1 pattern of *M. incognita* (Esbenshade and Triantaphyllou, 1985).

PCR products were generated and the corresponding DNA sequences determined for three rDNA regions, including the intergenic spacer (IGS), large subunit (LSU) 28S D3 expansion segment, and internal transcribed spacer 1 (ITS-1). For IGS rDNA, a single ~700-bp PCR product was obtained. Two IGS clones were identical and found to be most similar to the *M. hapla* sequence AJ421708, with only two changes at positions 248 and 279 out of 664 total nucleotides. The 28S LSU D3 PCR product from the Hawaiian isolate was 300 bp long and consisted of two different haplotypes, types 1 (AY528415) and type 3 (AY528420). Type 1 was identical to previous *M. hapla* haplotype 1 (AY335815), whereas type 3 shared two changes with type 1: a C to T change at position 202, and T to C at position 214. Type 3 alone had a change from C to T at position 256 in the alignment (not shown). The 426-bp ITS-1 rDNA from the Hawaii isolate was nearly identical to sequences from *M. hapla* populations from South Australia (AF516721, AF516722) and northern California (AY268108).

DNA sequences were also determined for the mitochondrial DNA interval that includes part of the cytochrome oxidase gene, a variable intergenic region, tRNAHis, and part of the 16S rRNA gene. The size of the amplification product from the Hawaiian isolate was ~520 bp and was most similar to the *M. hapla* populations from Australia (L76262), differing at 8 bp with four additional ambiguous positions (R to A; M to A; and two Y to T).

To examine the nuclear single copy Hsp90 gene from the Hawaiian isolate, a single 1078-bp PCR product was generated with the degenerate primers U288 and L1110. The DNA sequence was compared to the corresponding Hsp90 fragments from a Maryland population of *M. hapla* (Fig. 2) and to those from other root-knot nematodes (not shown). The Hsp90 sequences from Hawaii and Maryland *M. hapla* were nearly identical. The differences include changes A to G at position 66; G to A at 842; and ambiguous matches Y to T at positions 75 and 108, and T to Y at 102. The number, length, and position of the three introns in this Hsp90 fragment were identical in the two isolates. Two of the four variable positions were located within an intron. Nucleotide differences observed within coding regions were all third-position changes, and none altered the amino acid encoded.

Host status: Pot cultures of Rutgers tomato were highly susceptible (40 to 2,772 eggs/plant, 4,000 to 70,000 eggs/gram dry root), while Healani tomato was relatively resistant (0 to 34 eggs/plant, 0 to 1,133 eggs/gram dry root). Galls were atypically large and without small lateral roots. In addition to two coffee cultivars, this nematode was also found to para-

TABLE 4. Measurements of nine *Meloidogyne hapla* males, population 1.

Character	Range	Mean	Standard deviation
Linear (μm)			
Stylet knob height	2.5–3	2.7	0.2
b	7.6–11.8	10.1	1.5
b′	4.7–6.3	5.3	0.8
Spicule length	20–25	23.3	2.5
Gubernaculum length	7–7.5	7.2	0.3

TABLE 5. Measurements of 25 *Meloidogyne hapla* second-stage juveniles, population 1.

Character	Range	Mean	Standard deviation
Linear (μm)			
Body length	284–355	323.1	18.4
Body width	10–12	10.8	0.9
Head width	4–5	4.8	0.4
Head height	2–2.5	2.5	0.1
DGO from base of stylet	2.5–2.5	2.5	0.0
Center of median bulb to anterior end	40–50	46.4	2.3
Excretory pore to anterior end	60–88	66.9	7.6
Length from base of esophageal gland lobe to anterior end	80–120	97.9	9.8
Tail length	30–47.5	42.6	4.2
Hyaline tail terminus length	5–15	10.9	2.1
Width of tail at hyaline portion	3–4.5	3.7	0.5
Width of tail 5 μm from tail terminus	2–3	2.4	0.3
Lateral field width	3–4.5	3.4	0.5
a ratio	24.1–35.5	30.2	2.6
b ratio	4.3–5.2	4.7	0.3
b′ ratio	2.7–3.9	3.3	0.3
c ratio	6.8–9.4	7.7	0.6
c′ ratio	4.5–6.0	5.4	0.6
Head width/head height	1.6–2.5	1.9	0.2
Caudal ratio A[a]	1.7–5.0	3.0	0.7
Caudal ratio B[b]	2.4–7.5	4.7	1.3

[a] Hyaline tail length/tail width at hyaline portion.
[b] Hyaline tail length/tail width 5 μm from tail terminus.

sitize the weed *Bidens pilosa* growing in these coffee fields.

Discussion

Morphology: Various quantitative features characterize this Hawaiian (1) population of *M. hapla* relative to others (see population [pop.] citations Table 1, Tables 3–5). Many of the characters used here have not been consistently measured across referenced populations. In comparison to some other female populations, there is a long vulval slit length (avg. 24 vs. 19 μm for pop. 17, or 15 μm for pop. 19) and anus-to-tail terminus distance (13–25 vs. 9–11 μm for pop. 19, or 13–22 μm for pop. 8). The interphasmidial distance range is extended on the high end (30 vs. 23 μm for pop. 19, or 21 μm for pop. 22) and low end (12.5 vs. 18 μm for pop. 8). There is a longer excretory pore distance from anterior (25–50 vs. 17–19 μm for pop. 19), vulval slit to anus distance (14–22 vs. 13–14 μm for pop. 19), and 'a' ratio (avg. 1.7 ± 0.2 μm vs. 1.4 ± 0.1 μm). The range extends considerably on the low end for body width (225 vs. 311 μm for pop. 12), neck length (90 vs. 102 μm for pop. 19), and neck width (50 vs. 75 μm for pop. 19). In males (Table 4) there was a discretely smaller b ratio (7.6–11.8 vs. 12–15 or 13–18 μm) compared to populations 22 and 23. Compared to population 23 there were also taller stylet knobs (2.5–3 vs. 1.7–2 μm for pop. 23) and slightly shorter values for spicule (20–25 vs. 22–28 μm) and gubernaculum lengths (7–7.5 vs. 7.2–9.4 μm). Juveniles (Table 5) had a very short, invariant 2.5-μm DGO position behind the stylet (vs. 3–4 μm in pop. 22, 4–6 μm in pop. 19). The juvenile 'b' value range (2.7–3.9 vs. 2.3–3 μm) was higher, and median valve height shorter (40–50 vs. 53–56 μm), with smaller J2 tail (30 vs. 38 μm) and head-to-excretory-pore lengths (60 vs. 72 μm) than in population 19 (Sahoo and Ganguly, 2000).

Various qualitative morphological characteristics have also been found to distinguish *M. hapla* populations. In J2, relative size of lateral lip sectors and prominence of the labial disk (Eisenback and Hirschmann, 1979) and shape of the male stylet shaft (Eisenback, 1993) were found in cytological races A and B. In this population juvenile lateral lip sectors (Fig. 3A–F) were recessed as in population 86NC race A (pop. 15) or race B populations (pop. 16) (Eisenback and Hirschmann, 1979), the labial disk was somewhat elevated, and the junction of the medial and lateral lips was perpendicular to the lateral edge of the labial disk as in race B (Eisenback and Hirschmann, 1979). The appearance was also very similar to the face pattern for *M. exigua* (Fig. 4.83; Jepson, 1987), the coffee root-knot nematode. Because recent molecular phylogenies of the 18S rDNA (De Ley et al., 2002), ITS, and 28S LSU D3 rDNA (Castillo et al., 2003) did not support *M. hapla* as a close relative of *M. exigua* and its relatives, this morphology must be convergent. The males of this population had a cylindrical stylet base that was typical of race B populations rather than a tapered base as in race A (Eisenback, 1993).

Selected features of this *M. hapla* population (1) were compared with other *M. hapla* populations in the literature. While some *M. hapla* populations had 5 lateral lines on the J2 lateral field (Jepson, 1987), the Hawaiian population (1) had 4, as did *M. exigua*. The average

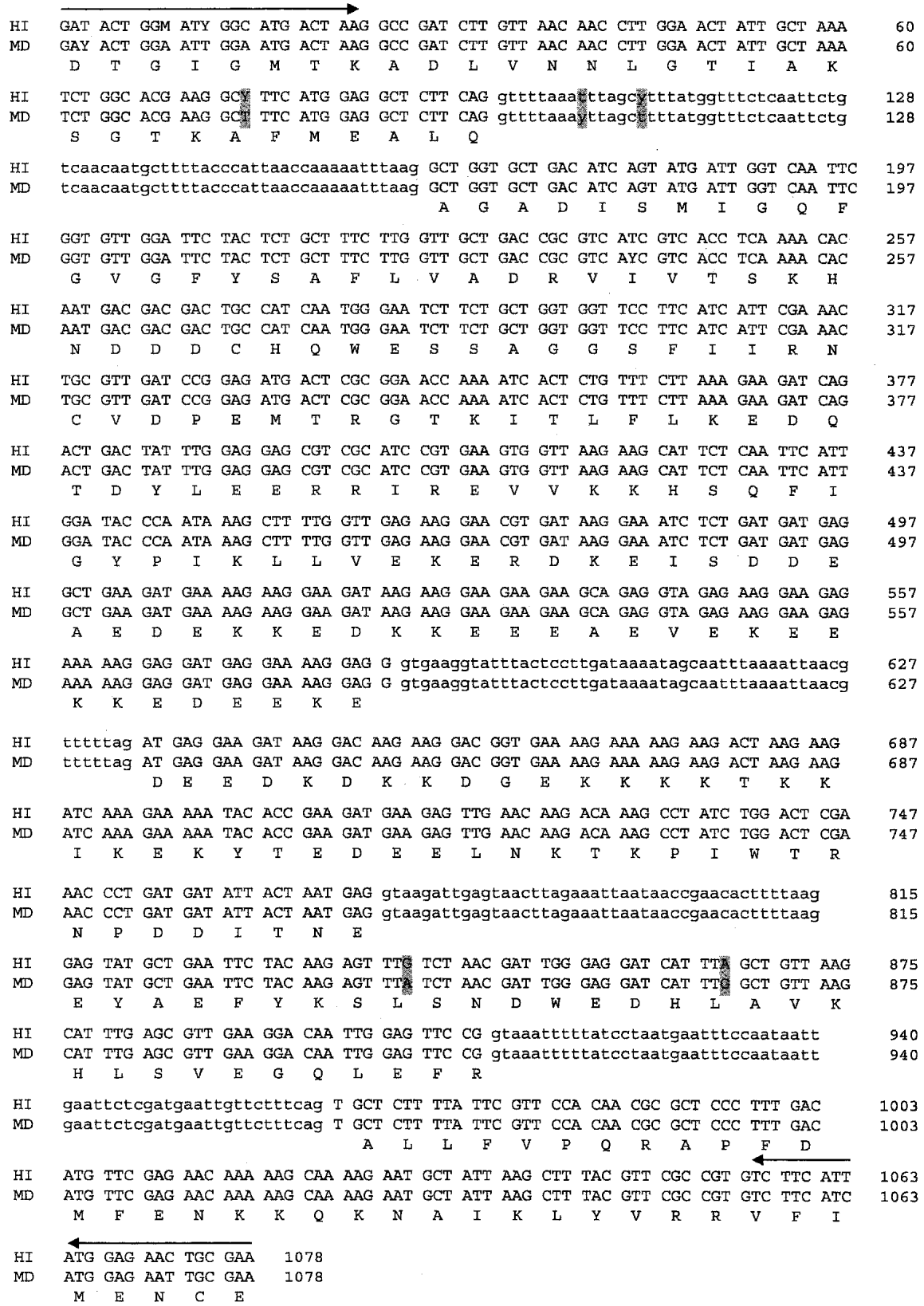

FIG. 2. Alignment of partial genomic sequence for the Hsp90 gene obtained from *Meloidogyne hapla* isolates from Maryland (MD) (population 2) or Hawaii (HI) (population 1). Coding sequences are shown as triplet codons in capital letters with the corresponding amino acids below the aligned DNA sequences. Intron sequences are depicted in lower case. Arrows indicate position of primer sequences U288 and L1100. Nucleotides that differed between the two isolates are shaded in grey.

for vulval slit width was considerably higher (24 µm) (Table 3) than for other populations (pop. 17: avg. 19 µm, pop. 18: avg. 18.6 µm, pop. 19: avg. 15 µm), with the exception of population 8 from the Netherlands. The Hawaiian population had one of the lower interphasmidial distance (IPD) means (avg. = 21 µm), similar to populations from India (avg. = 20 µm) (Sahoo and Ganguly, 2000) and a recent USDA-APHIS NY port interception from the Netherlands (8) (avg. = 22 µm), but much lower than other populations (avg. = 28 and 24 µm) from the Netherlands (18) and Venezuela (17), respectively (Dao, 1970). Anus-to-tail-terminal distance (ATT) was fairly high in the newer Netherlands population (8) (avg. = 17 µm), intermediate in the Hawaiian (1) (avg. = 15 µm), and Venezuelan (17) (avg. = 14 µm) populations, and low in another Netherlands (18) (avg. = 11 µm) (Dao, 1970) and Indian (19) population (avg. = 10 µm) (Sahoo and Ganguly, 2000). Because these IPD and ATT do not have a consistent relative ranking among these populations and are fairly invariant characters (Sahoo and Ganguly, 2000), together they might be useful in characterizing populations. In this study, a moderate number of female perineal patterns had unusual intermittent perpendicular lines to the annulations, independent of season. Other atypical patterns are not uncommon within other populations of root-knot nematodes such as *M. incognita* (Triantaphyllou and Sasser, 1960).

The significant reduction in J2 length during summer compared to winter in this Hawaiian population is not unexpected given the experimentally induced decrease in J2 body length with increasing temperature in *Pratylenchus vulnus* (Doucet et al., 2001). It appears that on a broader climate scale, larger J2 size generally correlates with a cooler climate in most *M. hapla* populations for which morphometrics are available. Variations in J2 length of other *M. hapla* populations include native Polish populations with significantly different J2 body lengths (357–467 µm, avg. = 413 µm, race A; 410–517 µm, avg. = 474 µm, race B) (Kornobis, 2001). A more southerly population (19) from Simla, Himachal Pradesh, India, had a larger body size and stylet (379–390 µm, avg. = 382 µm) (Sahoo and Ganguly, 2000) than the original New York type (22) (395–466 µm) (Chitwood, 1949) and a very short population from east Africa (23) (312–355 µm, avg. = 337 µm) (Whitehead, 1968). Because *M. hapla* has been confused and associated with *M. chitwoodi* (Golden et al., 1980) and *M. fallax* (Nobbs et al., 2001), we were initially uncertain about the species identity of this African population because of its very short J2 body and tail lengths, and different female parameters (Whitehead, 1968). This African population also had atypical values of the reliable character of excretory pore/stylet length (EP/ST) based on reported averages (Taylor, 1987) that are considerably smaller (similar to 1.8 of *M. chitwoodi*) than the 3.0 value measured for *M. hapla*. This Hawaiian population now has the shortest J2 body lengths yet recorded (284 vs. 312 µm for pop. 23). It also has a tail/hyaline tail length like the African population (23) rather than the type population (22). The following molecular work supports the Hawaiian population (1), with morphology similar to the African population (23), to be conspecific with other populations (e.g., 4 to 6) possessing greater similarity to the type (22).

Molecular: The esterase pattern was consistent with that for *M. hapla,* but minor variation from the pattern of *M. incognita* made supplemental confirmation important. A variety of DNA sequences have utility for confirming *M. hapla* species diagnosis, and slight differences in populations also may be evident. Molecular diagnosis of the Hawaiian population based upon the IGS supported *M. hapla.* Length polymorphisms in the IGS, resulting from differing numbers of repeats between the 5S and 18S genes, have been shown to discriminate *M. hapla* from *M. fallax* and *M. chitwoodi,* and intraspecific variability for *M. hapla* was also reported (Wishart et al., 2002). The Hawaiian population IGS sequence exhibited no such variation in the size of the IGS PCR product or in the sequences of different clones, although slight variation from previously reported *M. hapla* IGS sequences was observed. Compared to the low level of intraspecific variation, the Hawaiian isolate IGS sequence was ~21% different from *M. javanica* and other major root-knot nematode species.

The results from the LSU D3 region of the rDNA also indicate a diagnosis of *M. hapla.* The Hawaiian isolate showed one haplotype that was identical to a previous GenBank accession (AY335815), with another haplotype appearing so far unique to this population. Because our amplification was performed on DNA obtained from a pool of nematodes, it is not possible to say whether an individual nematode contains only a single haplotype. However, Chen et al. (2003) have shown that individual *M. hapla* contained both haplotypes 1 and 2. Although the D3 expansion segment showed relatively low (~4%) sequence variation compared to *M. javanica* and the other major root-knot nematode species, a 3-bp insertion present in the Hawaiian population is unique to *M. hapla* (Chen et al., 2003). Although the ITS-1 rDNA sequence was consistent with those from other *M. hapla* populations, there was only minor variation in these sequences compared to *M. javanica* (not shown). Thus, ITS-1 sequences appear not to be reliable for discriminating this population from other root-knot nematodes.

Compared to four previously reported *M. hapla* populations (Mitkowski and Abawi, 2003), the mitochondrial sequence of the Hawaiian population had two unique character differences in alignment positions 3 and 12 (not shown), and was most similar to sequences from a population from Australia (Hugall et al., 1997). The Hawaiian mitochondrial sequence dif-

fered from *M. javanica* by 29% and was distinct from others previously reported for other *Meloidogyne* species (Hugall et al., 1997; Powers and Harris, 1993).

Hsp90 is a relatively new molecular tool for root-knot nematode diagnostics and phylogeny (Skantar and Carta, 2000, 2005). The Hsp90 sequence of the Hawaiian isolate was nearly identical to one from an *M. hapla* population from Maryland (Fig. 3). In contrast, this sequence was quite distinct from Hsp90 from *M. javanica* (AF201338) and other common root-knot nematodes (not shown), exhibiting a difference of 18% over 1026 bp. Each molecule we examined shows strengths and weaknesses for the diagnosis of *M. hapla* and for population studies. These results indicate that Hsp90 performed at least as well as IGS, 28S D3, and mitochondrial sequences, and better than ITS-1 for molecular diagnosis of the Hawaiian population.

Biogeography: In India, *M. hapla* (24) was found in coffee field soil, but *C. robusta* and *C. arabica* coffee varieties were immune to this population when tested in the greenhouse (Kumar, 1984). *Meloidogyne hapla* has been found occasionally on coffee roots (Luc et al., 1990) in Brazil (Lordello, 1982), Kenya, Tanzania, and Zaire (Whitehead, 1969). In Tanzania, *M. hapla* may cause small galls on coffee (Bridge, 1984) and do little to no damage. Larger galls, similar to *M. exigua,* sometimes with induction of adventitious roots, and some yield reduction or field damage, were reported in Brazil (Lordello, 1982). Coffee was originally imported to Hawaii in 1825 from the São Paulo area of Brazil (Pendergrast, 2000), but *M. hapla* is not listed among current infestations on coffee in that area (Lordello et al., 2001; Oliveira et al., 2001). It is possible that the current use of *C. robusta* in Brazil in place of older *C. arabica* due to other disease problems (Medina-Filho et al., 1999) may have made a potential *M. hapla* problem irrelevant. Coffee was originally brought to Brazil from Ethiopia (Pendergrast, 2000), and the morphological similarities of the Maui and the African population might indicate a genetic relationship due to historical transport with the plant. In the coffee fields of Kilimanjaro, Tanzania, *M. hapla* was commonly found on the same tropical-American invasive *Bidens pilosa* weed as in Maui (Medeiros et al., 1993) and appeared not to affect nearby coffee plants (Whitehead, 1969). Neither *C. arabica* nor tomato are hosts of *M. exigua,* common on coffee in Central and South America (Lehman and Lordello, 1982). *Meloidogyne exigua* has not been detected in Hawaii (Rodríguez et al., 1998; Schenck and Schmitt, 1992).

The cultivar-population interaction should be important in predicting pathogenicity. A California population of *M. javanica* virulent on tomato failed to reproduce on *C. arabica* 'Catuai,' even though other populations of *M. javanica* parasitized *C. arabica* (Araya and Caswell-Chen, 1995). It is particularly interesting that Healani tomato containing the *Mi* gene was moderately resistant to this population (1) of *M. hapla.* While other tomato cultivars with the *Mi* gene have not shown appreciable resistance to various populations of *M. hapla* (Brown et al., 1997), resistance to at least one other population of *M. hapla* (pop. 25) was demonstrated by *Mi*-containing tomato 'VFN8' and 'Rossol' in excised root culture; the same plants were heavily attacked in pots (Molinari and Miacola, 1997).

In summary, morphology and at least four DNA sequences place this population within *M. hapla.* Until more comparable information is gathered on other populations worldwide, it is impossible to know whether this population is native to Hawaii and whether the interesting host interactions of this population arose recently (Trudgill and Blok, 2001). However, some distinguishing features of this population may aid that search. It will be interesting to observe other locations for any morphological and molecular character associations, and to investigate the role cytogenetics may play in the interactions.

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