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Nucleotide Substitution Patterning within the *Meloidogyne* rDNA D3 Region and Its Evolutionary Implications

P. Chen,[1] P. A. Roberts,[1] A. E. Metcalf,[3] and B. C. Hyman[2]

Abstract: Evolutionary relationships based on nucleotide variation within the D3 26S rDNA region were examined among a collection of seven *Meloidogyne hapla* isolates and seven isolates of *M. arenaria, M. incognita,* and *M. javanica.* Using D3A and D3B primers, a 350-bp region was PCR amplified from genomic DNA and double-stranded nucleotide sequence obtained. Phylogenetic analyses using three independent clustering methods all provided support for a division between the automictic *M. hapla* and the apomictic *M. arenaria, M. incognita,* and *M. javanica.* A nucleotide sequence character distinguishing *M. hapla* from the three apomictic species was a 3-bp insertion within the interior of the D3 region. The three apomictic species shared a common D3 haplotype, suggesting a recent branching. Single *M. hapla* individuals contained two different haplotypes, differentiated by a *Sau*3AI restriction site polymorphism. Isolates of *M. javanica* appeared to have only one haplotype, while *M. incognita* and *M. arenaria* maintained more than one haplotype in an isolate.

Key words: evolution, *Meloidogyne arenaria, M. hapla, M. incognita, M. javanica,* phylogeny, rDNA, reproductive modes, root-knot nematodes, speciation.

Root-knot nematodes (*Meloidogyne* spp.) are one of the most important pests of agricultural crops worldwide (Esbenshade and Triantaphyllou, 1987; Roberts, 1995; Trudgill and Blok, 2001). Four major species, *M. arenaria, M. hapla, M. incognita,* and *M. javanica,* are widely distributed, and early molecular techniques applied to *Meloidogyne* were used to establish diagnostic tools for differentiating species (Castagnone-Sereno et al., 1994; Fargette et al., 1996; Gárate et al., 1991; Guirao et al., 1995). Where taxonomic goals were addressed, the focus was mainly on host-parasite relationships rather than evolutionary affinities (Hyman and Powers, 1991), as studies were not centered on common characters within, or evolutionary relationships between, species. Studies of phylogenetic relationships within nematodes are not only a basis for stable and predictive taxonomy but also contribute to understanding the biology of nematodes as agricultural pests (Subbotin et al., 2000).

Within *Meloidogyne,* species exhibit three modes of reproduction, termed amphimixis, automixis, and apomixis (Triantaphyllou, 1979). *Meloidogyne arenaria, M. incognita,* and *M. javanica* are apomictic and thus undergo only mitotic parthenogenesis. In these obligate parthenogens, meiosis is absent and it has been suggested that gene flow and recombination must be minimized because from the moment meiosis ceases, the genome essentially freezes; any subsequent changes arise as mutations. Each copy of every gene becomes an independent locus in the sense that it evolves independently of its homologue and former meiotic partner (Judson and Normark, 2000). The considerable ability of these three species to adapt in the environment and their success as parasites are hard to explain given this mode of reproduction (Trudgill, 1997; Trudgill and Blok, 2001). Studies on other invertebrates that reproduce by mitotic parthenogenesis, such as bdelloid rotifers, revealed a great sequence divergence between paired copies of the same gene within an individual, suggesting that parthenogenesis will fix non-lethal mutations and cause a broad divergence within a "species" (Welch and Meselson, 2000). Such a model provides some insight into the possibilities of how obligate, mitotic parthenogenetic species maintain their "heterogeneity." *Meloidogyne hapla* has two cytological races that undergo apomictic and automictic reproduction, respectively, and this species has been described as an evolutionary intermediate among the *Meloidogyne* spp. (Triantaphyllou, 1979). In automixis, oocytes undergo meiotic division. When sperm is available, the egg and sperm nuclei fuse. In the absence of the sperm nucleus, the second polar body nucleus fuses with the egg pronucleus and restores the 2n chromosome number (Triantaphyllou, 1966). This mode of reproduction would maintain homozygosity or heterozygosity in subsequent generations through reuniting of genetic material from a single individual (Judson and Normark, 2000).

Different molecular approaches have been employed to address questions of evolutionary relationships among *Meloidogyne* spp. Typically, these strategies have been based on consideration of morphological characters and host-parasitic relationships. Enzyme profiles supported the traditional taxonomy in that the apomictic and the automictic species were resolved into two different clades (Esbenshade and Triantaphyllou, 1987). The results from mtDNA analyses were mostly congruent with the esterase patterns, but some sequence divergence within species was also observed. Moreover, mtDNA RFLP profiles can be shared by different species (Hugall et al., 1994). In contrast to mtDNA, the ITS region within the nuclear 26S rDNA has little correlation with the taxonomic "species." Three apomictic species, *M. arenaria, M. incognita,* and *M. javanica,* were resolved into two groups using ITS region sequences, while *M. hapla,* containing both apomictic and automictic races, revealed two groups within the species on this basis, but no resolution between the

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[1] Department of Nematology, University of California, Riverside, CA 92521.
[2] Department of Biology, University of California, Riverside, CA 92521.
[3] Department of Biology, California State University, San Bernardino, CA 92407.
E-mail: bradley.hyman@ucr.edu
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two reproduction modes was found (Hugall et al., 1999).

It would be desirable to study additional DNA loci for a more detailed understanding of evolutionary relationships. The D3 expansion region, constituting approximately 350 base pairs located in the nuclear rDNA, has been shown to be useful in examining nematode phylogeny in some taxonomic groups because it contains regions that evolve at different rates (Al-Banna et al., 1997). The D3 region has been used to infer affinities of several nematode genera and families (Al-Banna et al., 1997; Courtright et al., 2000; Litvaitis et al., 2000; Subbotin et al., 2000). For example, divergence of D3 was used to distinguish *Pratylenchus* species, while also defining the genus in relation to other Pratylenchidae (Al-Banna et al., 1997). The D3 region was used to assess relationships among populations of the Antarctic nematode *Scottnema lindsayae* from geographically distinct locations (Courtright et al., 2000). Two D3 haplotypes were found in this species. Interestingly, some *S. lindsayae* individuals were found to have both haplotypes. The focus of the present study of the D3 region was to test for differences in a collection of seven *M. hapla* isolates from northern Europe and the United States. Sequence data from *M. hapla* were compared with the three common apomictic species of *Meloidogyne,* providing a new basis for interpreting phylogenetic relationships among the four economically most important *Meloidogyne* species.

Materials and Methods

Nematode isolates: Fourteen *Meloidogyne* isolates were included in this study and they are listed with their origin and, if known, race designation (Table 1). Nematode cultures were maintained in the greenhouse on susceptible tomato (*Lycopersicon esculentum* cv. UC 82), except *M. hapla* isolates, which were maintained on tomato cultivar VFN-8 that carries the *Mi* gene. Isolates VW4 and JNC were obtained as DNA from material previously identified to species; all other isolates were identified to species according to standard protocols using a combination of perineal pattern, differential host range test, and isozyme profiles (Esbenshade and Triantaphyllou, 1987). All the *M. hapla* isolates were identified as race A based on isozyme profiles using Glutamate-oxaloacetate transaminase (GOT). Race A populations had an additional band of GOT activity that is not detected in race B populations (Esbenshade and Triantaphyllou, 1987).

Table 1. The origin, race designation, and D3 sequence haplotypes of *Meloidogyne* isolates studied.

Species	Origin	Race	Code	Haplotype
M. hapla	San Bernardino, CA	A	SB	1,2
M. hapla	Tulare, CA	A	PX	1,2
M. hapla	Madera, CA	A	K6	1,2
M. hapla	France	A	LM	1,2
M. hapla	England	A	AN	1,2
M. hapla	Tennessee	A	TN	1,2
M. hapla	Wisconsin	A	WI	1,2
M. incognita	Coachella Valley, CA	1	COA	3,4,5
M. incognita	Patterson, CA	1	BEL	6
M. incognita	Denair, CA	1	MUL	5,6
M. javanica	North Carolina State Univ.	—	JNC	5
M. javanica	UC Davis, CA	—	VW4	5
M. javanica	Chino, CA	—	J811	5
M. arenaria	UC Riverside, CA	—	MA	5,7

DNA extraction: Tomato plants were harvested about 10 weeks after inoculation. Nematode eggs were dislodged from the roots by cutting and macerating the roots in 0.5% NaOCl (Hussey and Barker, 1973). The mixture was washed through a series of three screens (pore diameters of 833 μm, 90 μm, and 25 μm, respectively). The eggs were collected from the 25-μm-pore screen and hatched at approximately 24 °C. Bulked second-stage juveniles for initial sequence analysis were collected after 4 days and cleaned by the sugar flotation method before processing through phenol-chloroform extraction (Pableo et al., 1988). DNA from single females for use in restriction analysis of individual nematodes was extracted using the QIAprep spin miniprep kit (Qiagen Inc., Valencia, CA). A single female was dissected from a tomato root and placed in a 1.5-ml centrifuge tube containing 50 μl of Qiagen P1 resuspension buffer solution. The pipet tip was used to crush the nematode, and DNA extracted using protocols supplied by the manufacturer.

PCR amplification: Primers used for amplification of the D3 rDNA region were D3A 5′-GACCCGTCTTGAAACACGGA-3′ and D3B 5′-TCGGAAGGAACCAGCTACTA-3′ (Al-Banna et al., 1997; Courtright et al., 2000; Ellis et al., 1986). Amplification was conducted in a 25-μl reaction mixture containing 1 μM of each primer, 0.5 mM of each dNTP, 1.5 mM $MgCl_2$, 2.5 U of *Taq* polymerase, and the buffer provided with *Taq* polymerase (Promega, Madison, WI). The DNA template was present at approximately 40 ng. The PCR conditions were denaturing at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 45 seconds, 55 °C for 1 minute, and then 72 °C for 45 seconds.

Cloning and sequencing: PCR products were purified using the Concert rapid PCR purification system (GibcoBRL, Grand Island, NY). Purified PCR products were ligated to the plasmid vector pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, CA) and DNA introduced into the Invitrogen *Escherichia coli* host supplied with the kit using protocols provided by the manufacturer. Candidate recombinant plasmid DNA were purified through QIAprep spin miniprep kit as previously described. The dye-primer sequencing kit from Visible Genetics (Visible Genetics Inc., Toronto, Canada) was

used to sequence both strands of the cloned D3 inserts using flanking primers specifically designed for the plasmid vector pCR2.1 and labeled with the fluorescent dyes Cy 5.0 or Cy 5.5. Cycle-sequencing conditions were as follows: PCR at 94 °C for 2 minutes and 15 seconds, followed by 35 cycles at 94 °C for 45 seconds, 50 °C for 45 seconds, 72 °C for 2 minutes and 15 seconds, and 72 °C for 2 minutes and 15 seconds. The PCR products were sequenced on a Visible Genetics (Toronto, Canada) Long-Read tower sequencer. Initially, three or four individual plasmid clones from each isolate were sequenced; in later experiments, only two clones were sequenced per isolate if both duplicates matched an existing haplotype. Two to four clones from each isolate were sequenced to assess the intra- and interspecific variation and to eliminate artificial polymorphism generated in the PCR reaction. DNA sequences were deposited in Genbank with accession numbers AY335815, AY335816, and AY355411 through AY355415 for haplotypes 1 to 7, respectively.

Sequence alignments and phylogenetic inference: Nucleotide sequences were aligned using ClustalX (Thompson et al., 1997). Three independent phylogenetic analyses were conducted using PAUP*4.0 (Swofford, 2002), and the D3 sequence of *Pratylenchus vulnus* was obtained from GenBank (accession number U47547) and served as an outgroup. Genetic distances among the taxa were evaluated using three methods: a maximum likelihood tree using a branch and bound search, a maximum likelihood distance tree using minimum evolution with exhaustive search, and maximum parsimony analysis using an exhaustive search. All characters were unordered with equal weight.

Restriction enzyme analysis: The sequenced D3 region was propagated in the pCR2.1 vector plasmid. Cloned insert DNA fragments could be released from the vector plasmid using *Eco*R I. Typical reactions included: 2 µl of 10× buffer, 1 µl *Eco*R I enzyme (8 U), 2 µl of 10 mg/ml bovine serum albumin, 5 µl of sterilized water, and 10 µl of DNA. The reaction was incubated at 37 °C overnight. Reaction products were fractionated on 1% (w/v) agarose gels at 100 volts for 30 minutes. The desired D3 rDNA product was a band of approximately 350 bp in size. The target D3 rDNA band was excised from the agarose gel using a surface-sterilized razor blade and extracted from the gel plug using Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (BIO-RAD Laboratories, Hercules, CA).

Template DNA from single *Meloidogyne* females of each isolate was purified and the D3 region was amplified as described earlier. The D3 PCR products were purified using the QIAprep spin miniprep kit, digested with *Sau*3A I, and fractionated on 1.8% (w/v) agarose gel at 80 volts for 30 minutes. Electrophoretically fractionated DNA was visualized by ethidium bromide staining (0.5 µg/ml) on a UV transilluminator.

Results

Cloned D3 amplification products were sequenced and a consensus sequence generated from each isolate to assess intra- and interspecific variation. A total of seven D3 haplotypes were observed among the four *Meloidogyne* species (Table 1). A distinguishing feature of the *M. hapla* haplotypes was a 3-bp insertion at position 204 in *M. hapla,* not found in the other species analyzed (Fig. 1).

There were a total of nine parsimony informative sites, not including the 3-bp gap, found in the 300-bp sequences aligned in Figure 1. By the maximum likelihood analysis using a branch and bound search, a single tree was generated (Fig. 2). A maximum likelihood distance analysis using minimum evolution with exhaustive search also produced the same tree. Maximum parsimony exhaustive search also produced one tree that was congruent with the previous two analyses. Thus, three independent methods recovered the same tree in each case. The D3 sequence data of the species *P. vulnus,* from a different family (Pratylenchidae) in Tylenchida, was used as an effective outgroup to root the *Meloidogyne* tree (Fig. 2). The D3 sequence data from *S. lindsayae,* another tylenchid nematode, did not form an appropriate outgroup due to a lack of congruency between the different methods of phylogenetic analysis (data not shown). The trees from the three searches supported the separation of automictic (*M. hapla*) and apomictic species (*M. arenaria, M. javanica,* and *M. incognita*) (Fig. 2). This result was consistent with the presence/absence of the 3-bp insert that also separated *M. hapla* from the three apomictic species when considered as a single character.

Sequences from 19 randomly sampled clones representing D3 sequences from seven *M. hapla* isolates were found to be of two types, termed haplotype 1 (four clones) and haplotype 2 (15 clones). The two haplotypes were differentiated by a transversion at position 192, and two transitions at positions 202 and 214 (Fig. 1). In the haplotype 2 sequence, the C-to-T transition at position 214 eliminated a *Sau*3A I restriction enzyme-cleavage site. Therefore, *Sau*3A I digestion could be used as a diagnostic test for the presence of one or both haplotypes. For example, *Sau*3A I restriction profiles generated three fragments of approximately 175, 150, and 25-bp sizes for haplotype 1 (Fig. 3, lane 6) and two fragments of approximately 325 and 25-bp sizes for haplotype 2 (Fig. 3, lane 5). Isolates TN, SB, WI, and PX carried only haplotype 2 when examined by sequence analysis. However, when D3 amplification products derived from each of these four isolates were analyzed further by *Sau*3A I restriction enzyme digestion, haplotype 1 was also found to be present in all four samples (data not shown). To determine whether the two D3 haplotypes occurred in different individuals of an isolate or carried within the same individual, template

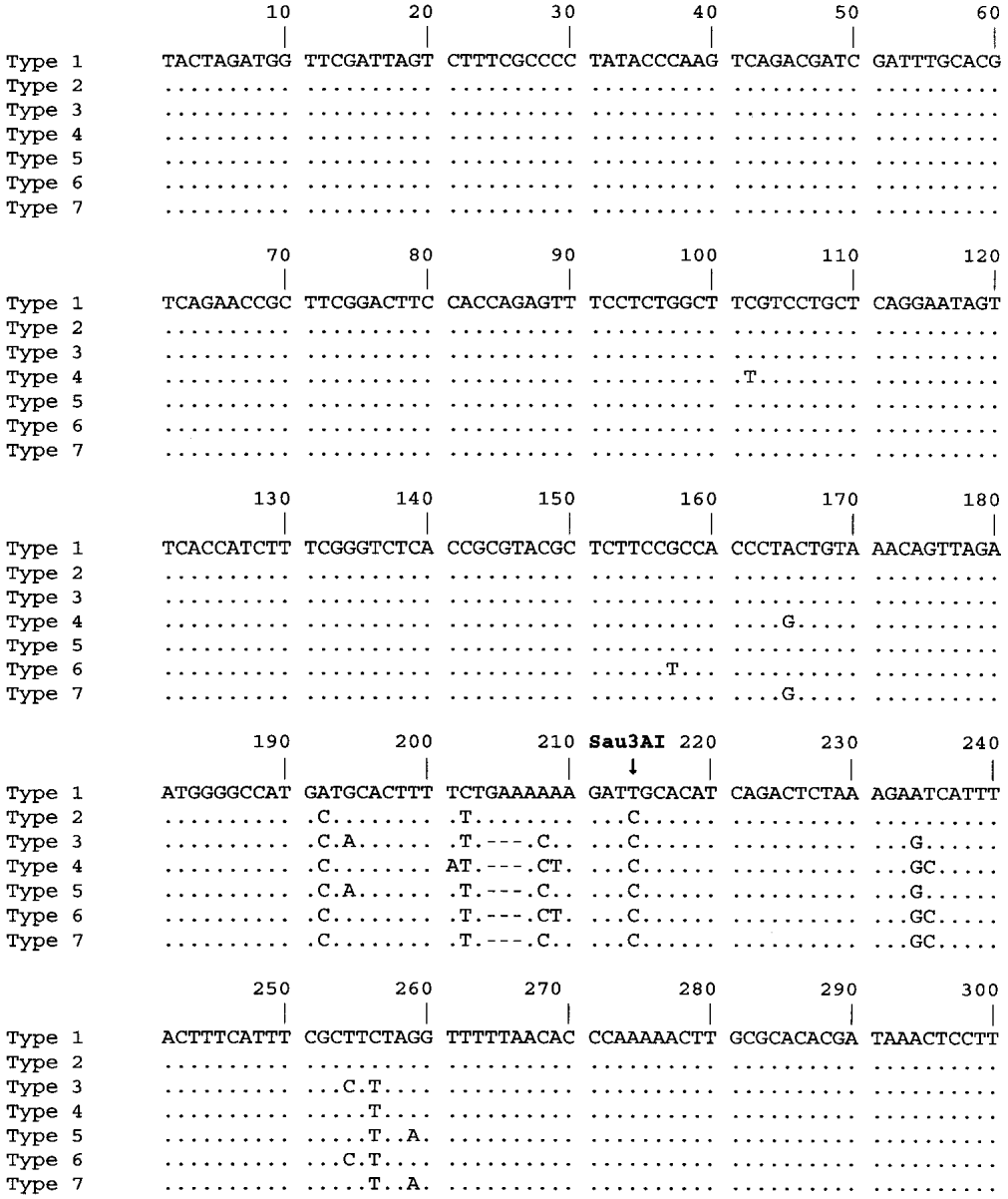

FIG. 1. Seven sequence haplotypes of rDNA D3 region (Types 1 and 2 belong to *Meloidogyne hapla,* and Types 3–7 belong to the apomictic species group). Numerals above each line of sequence represent nucleotide position number. The *Sau*3A I restriction site polymorphism is indicated by an arrow at nucleotide 214. GenBank accession numbers for the seven haplotypes are listed in Materials and Methods.

DNA from single females of isolates AN, TN, K6, and WI were used for D3 amplification. After digesting the D3 PCR products from individual females with *Sau*3A I, both D3 haplotypes were found to occur in the same individual. For example, in three randomly chosen females representing isolate K6, both haplotype 1 (digestion products of 175, 150, and 25 base pairs) and haplotype 2 (325 and 25 base pairs; Fig. 3, lanes 2–4) are present. Two out of three *M. incognita* isolates and the *M. arenaria* isolate also had more than one haplotype in the same isolate, but there was no suitable restriction enzyme available to assess whether these different haplotypes existed in a single individual. All three isolates of *M. javanica* had only one haplotype, the D3 haplotype 5, which was also found in *M. incognita* and *M. arenaria* (Table 1).

DISCUSSION

D3 region as an effective marker for phylogenetic analysis: The choice of DNA loci is crucial to meaningful phylogenetic analysis. The region should exhibit sufficient variability to allow estimation of the historical relationships among taxa, but not so much that more distant ancestor-descendant relationships are obscured. Inferred phylogenetic relationships within and between the four *Meloidogyne* spp. based on sequence comparisons of the rDNA D3 expansion region are congruent with several previous studies. Three different phylogenetic methods of analysis used here all provided strong support that *M. hapla,* the only species that undergoes automixis (facultative parthenogenesis) among the four species studied, formed a separate clade, to the

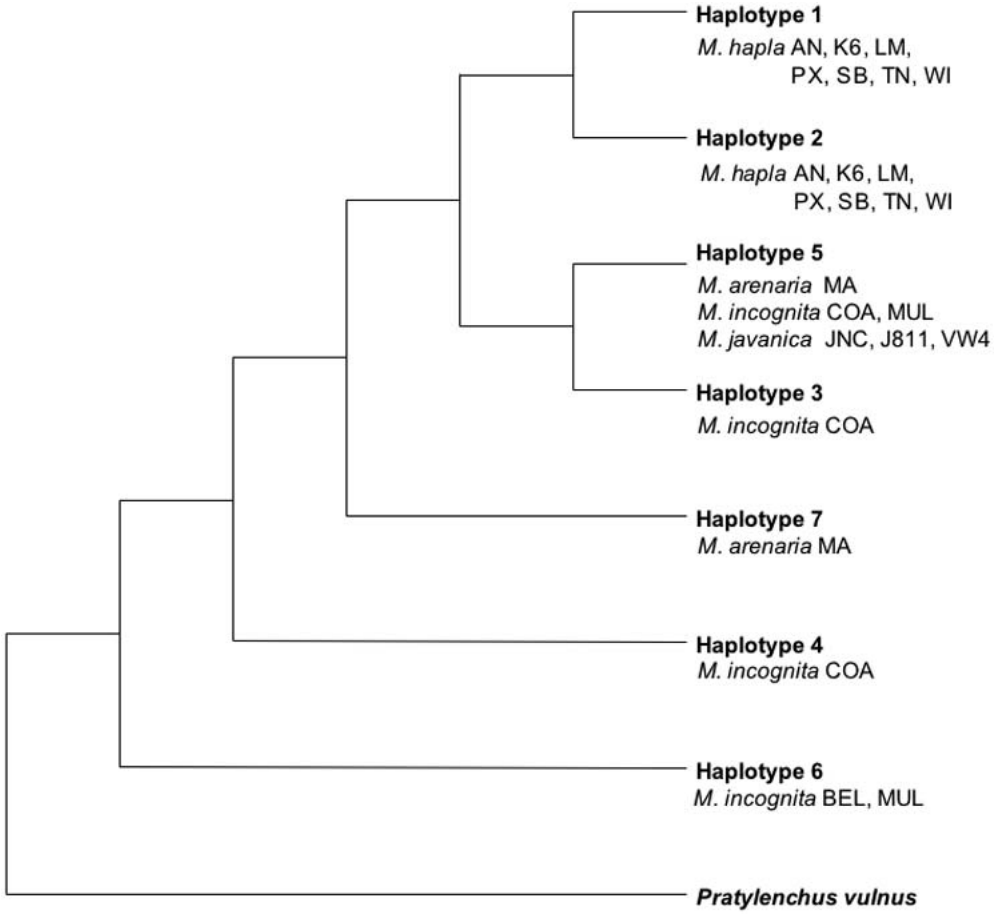

FIG. 2. Maximum likelihood tree generated from analysis of rDNA D3 region sequence for seven haplotypes in 14 isolates of *Meloidogyne* species using a branch and bound search with *Pratylenchus vulnus* as the outgroup (-Ln likelihood = 685.788). Maximum likelihood distance analysis using minimum evolution with exhaustive search (Minimum evolution score = 0.20269) and maximum parsimony with exhaustive search (Consistency Index CI = 0.9123; Homoplasy Index HI = 0.0877) produced the same tree.

exclusion of the apomictic species. Within the rDNA D3 region, all *M. hapla* isolates carried three extra base pairs (GAA) at position 204, which are absent in the other species. This distinguishing feature of the apomictic group further supports the separation of *M. hapla* into a clade distinct from the other grouping composed of the three apomictic species (Fig. 2). The apomictic species shared a common D3 haplotype 5 in this study (with the exception of one *M. incognita* isolate, BEL) and were resolved into one clade. A similar grouping of the apomictic species was found in other studies that used RFLP analysis, as well as by phylogenetic analysis of mtDNA RFLP profiles and rDNA ITS regions (Hugall et al., 1994, 1999).

The D3 region has been used to study phylogenetic relationships in many different nematodes, and it has provided varying levels of resolution in different nematode taxa (Al-Banna et al., 1997; Litvaitis et al., 2000; Subbotin et al., 2000). D3 sequence analysis was used to differentiate species of *Pratylenchus* and was used to generate a phylogenetic tree among additional Pratylenchidae (Al-Banna et al., 1997; Carta et al., 2001). However, the D3 sequence failed to differentiate *Globodera* at the species level, with no variation among the eight populations studied, whereas the rapidly divergent ITS region of approximately 1194 bp was shown to be divergent within species (Subbotin et al., 2000). When the D3 region was used for constructing a phylogenetic tree of Adenophorea, the tree generated from the D3 sequences was ambiguous and only 59% of the characters were parsimony informative (Litvaitis et al., 2000). In the present study, the D3 region differentiated the four common *Meloidogyne* spp. to a level based on their reproduction modes. Furthermore, the D3 region provided some resolution within the apomictic group, indicating *M. incognita* as more variable than, and slightly distinct from, *M. arenaria* and *M. javanica* with respect to numbers of unique haplotypes. Developing a robust phylogenetic hypothesis of *Meloidogyne* spp. clearly will be served by sequences from additional genomic regions, a broader range of species of *Meloidogyne* and outgroups, as well as an increasingly sophisticated understanding of sequence evolution.

Heterogeneity of rDNA D3 and its evolutionary implications: The occurrence of two distinct D3 haplotypes in *M. hapla* individuals is relevant to evaluating current

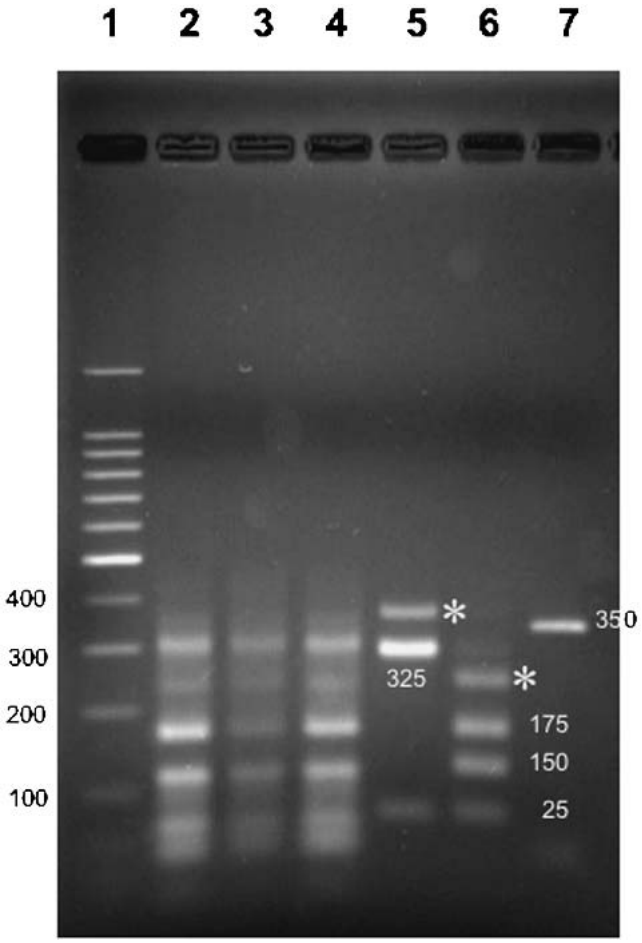

FIG. 3. *Sau*3A I restriction enzyme profiles of single females. Lane 1: 100-bp ladder (MW marker); Lanes 2–4: single females of isolate K6; Lane 5: cloned haplotype 2, excised from the cloning vector and digested with *Sau*3A I; Lane 6: haplotype 1, excised from the cloning vector and digested with *Sau*3A I; Lane 7 is an uncut control. Digestions in lanes 5 and 6 are incomplete; partial products are marked with an asterisk (*). The slight difference in migration of the purified uncleared PCR product (lane 7) and the undigested partial product of the reaction in lane 5 is due to variation in salt conditions between purified PCR products and restriction enzyme reaction conditions. Numbers adjacent to or beneath DNA bands correspond to size of the DNA fragment in nucleotide pairs.

hypotheses of speciation within *Meloidogyne*. In most animals, a single sequence is found in the vast majority of rDNA gene copies. When multiple haplotypes are shared among reproductively isolated groups, sufficient time after lineage separation would be required to accumulate and fix nucleotide substitutions in all rDNA copies, as suggested by Courtright et al. (2000) in their study of *S. lindsayae*. Moreover, two isolates of *M. incognita* and an isolate of *M. arenaria* also carry several D3 haplotypes (Table 1), although we do not yet know whether the multiple haplotypes are present within an individual genome. These multiple haplotype arrangements could have arisen through hybridization in ancestral forms. In support of this hypothesis, Triantaphyllou (1983) suggested that the genus *Meloidogyne* might have originated from hybridization of ancestors for which the chromosome number $n = 9$ was the common form. Furthermore, ITS analysis of *M. hapla* rDNA revealed two distinct groups (Hugall et al., 1999), and our finding that two distinct D3 forms occur in the same individual supports the possibility that *M. hapla* may have evolved from interspecific hybridization.

Previous studies using RAPD-PCR, restriction enzyme analysis, and isozyme comparisons on three apomictic species suggested that *M. javanica* and *M. arenaria* are more closely related to each other than either is to *M. incognita* (Castagnone-Sereno et al., 1994; Esbenshade and Triantaphyllou, 1987; Gárate et al., 1991). This hypothesis is not contradicted by the haplotype relationships developed from the D3 sequences in the present study. Triantaphyllou (1979) stated that "*M. incognita* has a special cytological feature which suggested it to be monophyletic, and also, its diploid form represents an intermediate step of evolution." The D3 data showed *M. incognita,* with one common (haplotype 5) and three unique haplotypes (haplotypes 3, 4, and 6), to be somewhat distinct from *M. arenaria* and *M. javanica*. It can be hypothesized that the branching of *M. incognita* presumably is earlier and, although it may have monophyletic origin, separation may have been distant enough to accumulate mutations. However, the divergence of *M. incognita* could also represent an origin from multiple parental lines. Previous studies on *M. arenaria* suggested that it is polyphyletic (Castagnone-Sereno et al., 1994), so the presence of multiple haplotypes in the *M. arenaria* isolate is consistent with that view. With the exception of one *M. incognita* representative (BEL), the apomictic species isolates included in the present study also shared a common profile (haplotype 5), which is the closest branch to the *M. hapla* clade. This evidence supports the cytological interpretation of species relationships that the apomictic species were evolved from the automictic group (Table 1). The three *M. javanica* isolates had only one D3 sequence haplotype, suggesting that D3 is conserved or homogeneous in this species; other studies based on isozyme and mtDNA also showed that *M. javanica* had the least variation among these common *Meloidogyne* species (Esbenshade and Triantaphyllou, 1987; Hugall et al., 1994). In previous studies, the apomictic forms also were found to comprise only one clade without resolution between the species, and this clade was significantly distinct from the clade of the automictic group (Castagnone-Sereno et al., 1994; Gárate et al., 1991; Hugall et al., 1994, 1999).

The results of the present study demonstrate the utility of rDNA D3 sequence analysis for addressing affinities within this complex genus that contains widely different reproduction modes. Confirmation of species relationships offered here and their association with reproductive mode will require sequence analysis of multiple nuclear loci and additional representatives across *Meloidogyne*. Such studies will facilitate an in-

creased understanding of the variety of reproduction strategies in nature.

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