Low, but Strongly Structured Mitochondrial DNA Diversity in Root Knot Nematodes (Meloidogyne)

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ABSTRACT

Root-knot nematodes (genus Meloidogyne) have been the subject of recent and numerous studies of genetic variation because of the need to develop molecular diagnostics for the four globally distributed, parthenogenetic species that are significant agricultural pests. Our analysis of Meloidogyne mtDNA improves on previous studies: (i) by examining restriction site polymorphism among a large number of isolates also characterized for standard morphological, host range and allozyme phenotypes; (ii) by using higher resolution electrophoretic techniques; and (iii) by mapping variable restriction sites with reference to the complete nucleotide sequence. This revealed fivefold less sequence divergence (<0.6%) between variants than estimated in previous restriction fragment length polymorphism (RFLP) studies, but perfect correspondence between mtDNA haplotype and allozyme (esterase) phenotypes. The mtDNA variation, although limited, is strongly structured with as much divergence between two lineages of *Meloidogyne arenaria* as between either of these and *Meloidogyne javanica*. The low diversity of mtDNAs suggests that these parthenogenetic lineages arose from distinct but closely related sexual females, a pattern seen in other parthenogenetic complexes. In contrast to the concordance between mtDNA and allozyme markers, there were several discrepancies between the traditional methods of identification. We suggest that further studies of these nematodes should focus on well defined genetic groups, whether or not these coincide with existing taxonomic units.

DESPITE the widespread use of mtDNA in population genetic and systematic studies of free living organisms (reviewed by AVISE *et al.* 1987; MORITZ *et al.* 1987; HARRISON 1989), polymorphism in parasitic species has not been extensively examined (but see BLOUIN *et al.* 1992). There have, however, been several studies of mtDNA variation in the root-knot nematode genus *Meloidogyne* (POWERS and SANDALL 1988; HARRIS *et al.* 1990; OKIMOTO *et al.* 1991; POWERS and HARRIS 1993). This genus contains both sexual and parthenogenetic taxa with considerable polyploidy among the latter (TRIANTAPHYLLOU 1985). The relationships among the species and the pathways by which the meiotic and apomictic parthenogenetic taxa evolved from sexual ones are not clear, although the combined cytological and allozyme studies do suggest certain affinities (ESBENSHADE and TRIANTAPHYLLOU 1987).

Of the more than 50 species of *Meloidogyne* described, just four globally distributed parthenogenetic species, *M. arenaria*, *M. javanica*, *M. hapla* and *M. incognita*, are of concern to agriculture (TAYLOR *et al.* 1982). Defined principally by perineal pattern morphology (EISENBACK *et al.* 1981) they have also been subdivided into races according to differential responses to certain test hosts (TAYLOR and SASSER 1978; TAYLOR *et al.* 1982). Among these four species, variation has been detected for a wide variety of DNA loci (*e.g.*, CURRAN *et al.* 1986; POWERS *et al.* 1986; GARATE *et al.* 1991; XUE *et al.* 1992; CENIS 1993). However, with few exceptions these studies compared only a small number of isolates, typically including reference strains from a central (North Carolina State University) collection. In the exceptions (*e.g.*, HARRIS *et al.* 1990; POWERS and HARRIS 1993) the techniques used lacked resolution for phylogenetic analysis and species designations were presumed from perineal pattern morphology only, so that the relationship of DNA polymorphism to morphological, host range and allozyme variation remains to be determined. This requires a population genetic approach, with analysis of the distribution of polymorphism within and among a large series of field isolates identified by most or all of the methods currently used to distinguish and subdivide species.

Particular attention has been given to mtDNA polymorphism because of the rapid evolution of this molecule relative to nuclear genes (*e.g.*, BROWN *et al.* 1979; DESALLE *et al.* 1987; THOMAS and WILSON 1991) and the technical ease with which variation can be assayed (DOWLING *et al.* 1990). POWERS and SANDALL (1988) found considerable variation in mtDNA restriction fragment patterns within and among 12 isolates representing different species and host races of Meloidogyne and

this provided the basis for a subsequent assay using the polymerase chain reaction and diagnostic *Hin*fI polymorphisms (HARRIS *et al.* 1990). However, the data obtained had some significant limitations. A small number of isolates were examined and some of these were misidentified (see HYMAN and POWERS 1991) resulting in an overestimate of within-species variation. Moreover, the analyses of whole-mtDNA genome restriction fragment variation (POWERS and SANDALL 1988; POWERS *et al.* 1986) were potentially confounded by insertion/deletion events, despite the authors' efforts to exclude these when estimating sequence divergence and constructing phylogenies. A recent study using the polymerase chain reaction (POWERS and HARRIS 1993) revealed length variation in the non-coding region adjacent to the large rRNA gene but did not quantify sequence variation.

The analysis and use of restriction fragment length polymorphisms (RFLPs) is greatly enhanced where the variable sites can be located, either by restriction site mapping (DOWLING *et al.* 1990) or by reference to a sequence (*e.g.*, CANN *et al.* 1984). The complete nucleotide sequence of a *Meloidogyne javanica* mtDNA has been determined (OKIMOTO *et al.* 1991; R. OKIMOTO, H. M. CHAMBERLIN, J. L. MACFARLANE and D. R. WOLSTENHOLME, manuscript in preparation). This genome contains the same number of proteins, rRNA and tRNA genes as other nematode mtDNAs (see also WOLSTENHOLME 1992), but is distinguished by an apparently non-coding region of about 7 kb that includes ≈36 copies of a 102-bp sequence and 11 copies of a 63-bp sequence. OKIMOTO *et al.* (1991) also reported variation in the sizes of the repeat-containing restriction fragments among mtDNAs from different races and species of Meloidogyne. They suggested that these differences, most likely caused by variation in repeat copy number, may provide a useful diagnostic tool.

The present paper reports on an analysis of restriction enzyme site polymorphism among Australian isolates of Meloidogyne. Our study extends previous ones in that we have examined a larger series of field-derived isolates identified by a variety of techniques, we have used higher resolution electrophoretic techniques for detecting fragment variation and, most significantly, we have determined the nature of the variation and the exact location of variable sites by comparison with the complete sequence of a *M. javanica* mtDNA. This has provided for a more precise quantification of mtDNA polymorphism and of the distribution of that variation among geographic areas and taxa.

MATERIALS AND METHODS

Collection and culturing: All populations were collected from the field and maintained on tomato (*Lycopersicon esculentum* cv. Tiny Tim). To ensure that populations were of a single strain and not a mixture, each field isolate was taken through several cycles of culturing from a single egg mass before being used for analysis. Nematode eggs were removed from roots in 0.5% sodium hypochlorite (SOUTHEY 1986) and collected on a 25-μm sieve. Eggs were further purified by discontinuous sucrose gradient centrifugation (SOUTHEY 1986).

Host range testing and identification by perineal pattern: The differential host test was based on that of TAYLOR and SASSER (1978). Four replicate seedlings of capsicum (*Capsicum frutesens*) cv. California Wonder, cotton (*Gossypium hirsutum*) cv. Deltapine 16, peanut (*Arachis hypogaea*) cv. Florunner, tobacco (*Nicotiana tabacum*) cv. NC95 and watermelon (*Citrullus vulgaris*) cv. Charleston Grey, and tomato cv. Tiny Tim as a susceptible control, were inoculated. Plants were then rated as susceptible if they had more than two egg masses and resistant if no more than two. The combination of resistance and susceptibility to the differential hosts determines the assignment of a species or host race. Identification by perineal pattern (EISENBACK *et al.* 1981) was based on at least ten females per isolate.

Typing of esterase phenotype: Esterase isozyme types (EST EC 3.1.1.1) were determined by the method of ESBENSHADE and TRIANTAPHYLLOU (1985) and are described according to their terminology. From water washed tomato roots several mature females were teased, placed in 30 μl of 2% Triton X-100 in 1.5-ml Eppendorf tubes and macerated with disposable plastic pestles. The tubes were then stored at −70° until aliquots were run on 10–15% gradient acrylamide gels on a Pharmacia Phast system using native buffer strips. Gels were stained with α-naphthyl acetate and Fast Garnet at 37° for 30 min. To confirm their identity, the samples having H1 esterase pattern were also stained for malate dehydrogenase as described in ESBENSHADE and TRIANTAPHYLLOU (1985)

Isolation and restriction analysis of mitochondrial DNA: Mitochondrial DNA was prepared from egg masses pooled from single egg mass cultures by differential high speed and ultracentrifugation as described in DOWLING *et al.* (1990), except that pooled egg masses were homogenized by hand with mortar and pestle for 5 min prior to centrifugation.

Restriction enzymes were obtained from New England Biolabs and used according to the manufacturer's recommendations. About 1–5 ng mtDNA were digested to completion in 10 μl of recommended buffer containing about 1 unit of enzyme. DNA fragments were then radioactively end-labeled with [α-^{32}P]deoxynucleotides using the Klenow fragment of DNA polymerase I, and then fractionated on 1.5% agarose or 3.5% polyacrylamide gels in 1 × TBE as described by DOWLING *et al.* (1990). The gels were dried and autoradiographs produced at −70°.

Polymerase chain reaction (PCR) amplification and sequencing: Mitochondrial DNA sequences were amplified from purified mtDNA and from Chelex (Bio-Rad) extracts of esterase preparations in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% Tween 20, 0.01% Nonidet P-40 with 0.2 μM of each primer and 1.5 mM $MgCl_2$, using Taq DNA polymerase I and annealing temperature of 50°. The primers used are those of HARRIS *et al.* (1990) with an additional primer, within the putative large subunit ribosomal RNA gene, for amplifying haplotype A (GAAAAATAAAAAAATTTTGTT, positions 10192–10212, T. O. POWERS personal communication). To investigate some aspects of length variation we designed primers to a 3′ proximal region of the open reading frame (ORF in Figure 3) (ATCGGGGTTTAATAATGGG positions 9298–9316) and the 5′ end of the $tRNA^{His}$ gene (AAATTCAATTGAAATTAATAGCT positions 10018–10040). For restriction enzyme analysis, 5 μl of PCR product was cut with *Hin*fI. For sequencing, the PCR product was first purified from unincorporated primers and dNTPs with MC30 size exclusion spin columns (Millipore) and then 20 ng of the purified product was sequenced with the BRL cycle sequence kit using primers end labeled with [γ-^{33}P]ATP by T4 polynucleotide kinase.

TABLE 1

Description of the Meloidogyne isolates used in this study

Name	Primary host	Region	Perineal pattern	mtDNA	Esterase	Host range
39	Grapevine	FN	arenaria	A	A2	i2
75	Grapevine	VIC	javanica	A	A3	j/a2
NQ1	Tobacco	FN	arenaria	A	A2	j/a2
15	Pinapple	MO	arenaria	A	—	i1
Y	Taro	FN	javanica	A	A2	j/a2
65	Grapevine	NT	arenaria	A	A2	j/a2
G	Thunbergia	FN	—	B	I1	i1
H	Home garden	NO	incognita	B	I1	i2
Z	Banana	FN	incognita	B	I1	i2
79	Pawpaw	MA	arenaria	B	I1	i2
35	Banana	FN	arenaria	B	I1	i1
I	Vegetable	NO	javanica	B	I1	i1
J	Dolichos	NO	incognita	B	I1	—
68	Sweet potato	BU	arenaria	C	A1	—
NQ5	Tobacco	FN	—	C	A1	j/a2
NQ7	Tobacco	FN	arenaria	C	A1	j/a2
33	Pasture	FN	—	C	—	i2
77	Ginger	MO	arenaria	H	A1	j/a2
88	Pomegranate	SA	arenaria	D	—	novel(2)
42	Sugarcane	FN	javanica	D	J3	i2
5	Kiwi fruit	MO	javanica	D	J3	j/a2
11	Grapevine	DD	javanica	D	J3	j/a2
16	Kiwi fruit	MO	javanica	D	J3	j/a2
37	Sugarcane	FN	javanica	D	J3	j/a2
40	Grapevine	FN	javanica	D	J3	—
51	Tomato	BU	arenaria	D	J3	j/a2
72	Lucerne	MO	arenaria	D	J3	j/a2
78	Cucurbits	NO	arenaria	D	J3	novel(2)
83	Lucerne	SA	javanica	D	J3	j/a2
90	Pistachio	SA	—	D	J3	—
93	Ginger	MO	javanica	D	J3	—
C	Banana	FN	javanica	D	J3	—
NQ2	Tobacco	FN	javanica	D	J3	j/a2
V	Duboisia	BU	javanica	D	J3	—
B	Tomato	MO	javanica	D	J3	j/a2
86	Pecan	SA	javanica	D	J3	i2
53	Peanut	MO	hapla	D	J3	—
94	Ginger	MO	javanica	D	J3	j/a2
44	Peanut/maize	BU	arenaria	D	J3	i2
21	Sugarcane	MO	javanica	D	—	j/a2
91	Grapevine	SA	javanica	D	—	j/a2
D	Olive	SW	javanica	D	—	i2
3	Pigeon pea	MO	javanica	D	—	j/a2
A	Tomato	BU	javanica	D	—	novel(3)
82	Lucerne	SA	javanica	D	J3	j/a2
50	Tobacco	FN	—	D	J3	—
98	Grapevine	SA	javanica	D	J3	novel(2)
12	Grapevine	DD	arenaria	G	S2-M1	novel(1)
113	Peanut	MO	hapla	F	H1	novel(5)
114	Peanut	FN	javanica	I	H1	a1
48	Kiwi fruit	MO	hapla	E	H1	hapla
102	Carrot	WA	hapla	J	H1	novel(4)

The mtDNA haplotypes, except for the last four, are defined by restriction enzyme site changes shown in Figure 5, esterase patterns as described in ESBENSHADE and TRIANTAPHYLLOU (1985) and host range types by the standard host range test (TAYLOR and SASSER 1978). In the host range codes the letters a, i and j refer to *M. arenaria*, *M. incognita* and *M. javanica* with the number denoting the host race; the test cannot distinguish between j and a2. In the esterase codes, A, I, J and H indicate patterns commonly seen in *M. arenaria*, *M. incognita*, *M. javanica* and *M. hapla*, respectively. Region codes, which refer to collection site are described in the legend to Figure 1. Primary host is the plant the single egg-mass culture was originally taken from in the field. A dash means the relevant information is not available. Novel standard host range responses 1, 2, 3, 4 and 5 will be described elsewhere (J. STANTON manuscript in preparation).

Inferring site changes and mapping variable sites: Sizes of constant and variable fragments were measured relative to known size markers run on each gel. The location and nature of cleavage site changes were inferred from the restriction site map obtained from the complete *M. javanica* mtDNA sequence. The nucleotide position numbers used in this paper start at the beginning of the $tRNA^{f\text{-}met}$ gene, the first tRNA gene after the 63-bp tandem repeats (R. OKIMOTO, H. M. CHAMBERLIN, J. L. MACFARLANE and D. R. WOLSTENHOLME, manuscript in preparation).

Statistical analysis of sequence polymorphism: From the restriction site data, sequence divergence, haplotype and nucleotide diversity were calculated using the REAP computer package (MCELROY *et al.* 1992). The proportion of nucleotide

diversity distributed among populations, G_{ST}, was estimated and its significance evaluated via randomization tests as described by PALUMBI and WILSON (1990).

RESULTS

Characterization of field isolates: From a total of 110 isolates collected from various hosts in the field and maintained as single egg masses on tomato in the greenhouse, 64 have been typed for mtDNA restriction enzyme site variants, 56 by perineal pattern, 44 for esterase phenotype and 68 by standard host range. Fifty-two of these, covering isolates from 26 different primary hosts and comprising the most complete set of data, are described in Table 1. The collection represents a wide geographical area within Australia (Figure 1). Twenty-one of the 52 isolates examined in detail are from tropical north Queensland (*i.e.*, north of the Tropic of Capricorn), 21 are from southern Queensland (subtropical), one is from the Northern Territory, one is from Western Australia, and the rest are from temperate southern Australia.

The host range test employed was unable to distinguish between *M. javanica* and race 2 of *M. arenaria* and revealed five combinations not previously reported (novel-1, 2, 3, 4, 5; Table 1): these novel host ranges will be described elsewhere (J. STANTON, manuscript in preparation). All of the isolates with the H1 esterase pattern also had the H1 phenotype for malate dehydrogenase, which is characteristic of *M. hapla* (ESBENSHADE and TRIANTAPHYLLOU 1990). In general, the agreement among different methods of identification is poor (Table 1). There are several conflicts (9/35 tests) between host-range and esterase identification, and even more between perineal pattern and either host range (18/41) or esterase phenotype (13/41).

Variation in mtDNA: In preliminary surveys, a selection of mtDNAs (20) was screened with up to 16 restriction enzymes to identify those revealing polymorphism. Some 6-bp recognizing enzymes (*Bam*HI, *Bgl*II, *Ava*II, *Bcl*I, *Spe*I) and 4-bp recognizing enzymes (*Hin*PI, *Msp*I) had few if any sites, presumably because of the extremely high A + T content (83.6%) of the genome (R. OKIMOTO, H. M. CHAMBERLIN, J. L. MACFARLANE and D. R. WOLSTENHOLME, manuscript in preparation). Others such as *Alu*I, *Dde*I and *Dra*I revealed some variation but did not give consistently clear results after end-labeling. All 52 samples considered here (Table 1) were screened with three 6-bp (*Hin*dIII, *Xba*I, *Eco*RI) and three 4-bp recognizing enzymes (*Mbo*I, *Taq*I and *Hin*fI) which assayed a total of 88 sites.

The survey of all samples with these six enzymes revealed two broad groups of mtDNAs: one large group (N = 48) with very similar fragment patterns and another very divergent and diverse group (N = 4; *e.g.*, population 48 in Figure 2). The latter appear to be much larger genomes (*ca.* 27 kb) and shared so few fragments with

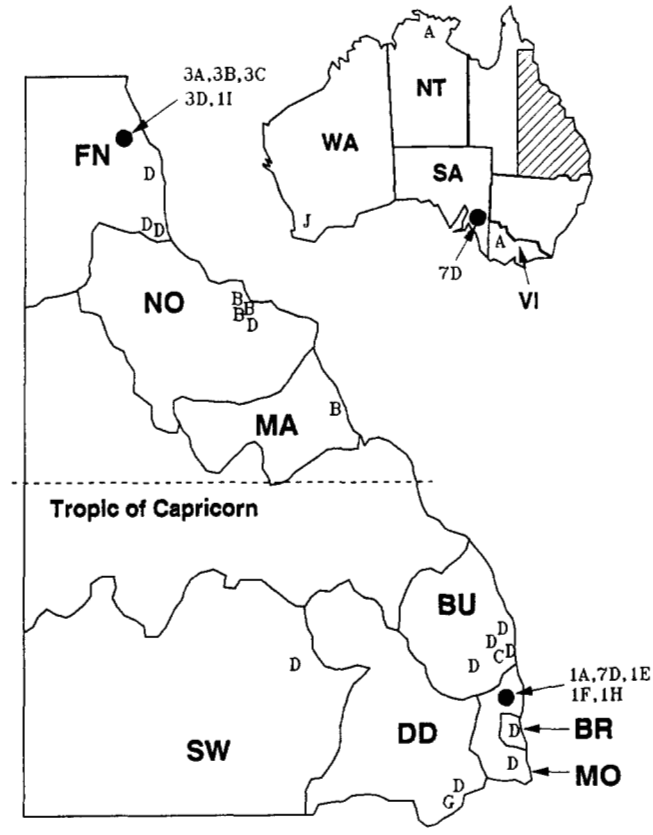

FIGURE 1.—Locations of collection sites of Meloidogyne field isolates. Smaller letters (A–J) indicate mtDNA haplotypes. Larger letters indicate the states of Australia and region codes within Queensland. These are: VIC, Victoria; WA, Western Australia; SA, South Australia; NT, Northern Territory; and in Queensland: FN, far North Queensland; NO, North Queensland; BU and MA, central Queensland; MO, SW, DD, all Southern Queensland.

the former group that, combined with dispersed insertion/deletion variation, sequence divergence between the two groups of genomes cannot be reliably estimated (see UPHOLT 1977; DOWLING *et al.* 1990). The isolates in this second group were all identified as *M. hapla* on the basis of esterase phenotypes: the structure and diversity of mtDNAs from *M. hapla* will be reported elsewhere (A. F. HUGALL, J. STANTON and C. MORITZ, manuscript in preparation). The remainder of the analyses concern just the common mtDNA group found in 48 isolates.

Among these 48 isolates there was considerable variation in fragment patterns, most apparently due to high frequency changes in repeat copy number. For example, digests with *Mbo*I (*e.g.*, Figure 2 and 3.5% acrylamide gels not shown) produced 17 fragments, of which two showed extensive variation in length, and often multiple non stoichiometric bands, apparently due to variation in the number of 102- and 63-bp repeats, respectively. Considering only the fragment changes that did not appear to be due to variation in the number of 63- and 102-bp repeats or other deletions (see below), there were six different

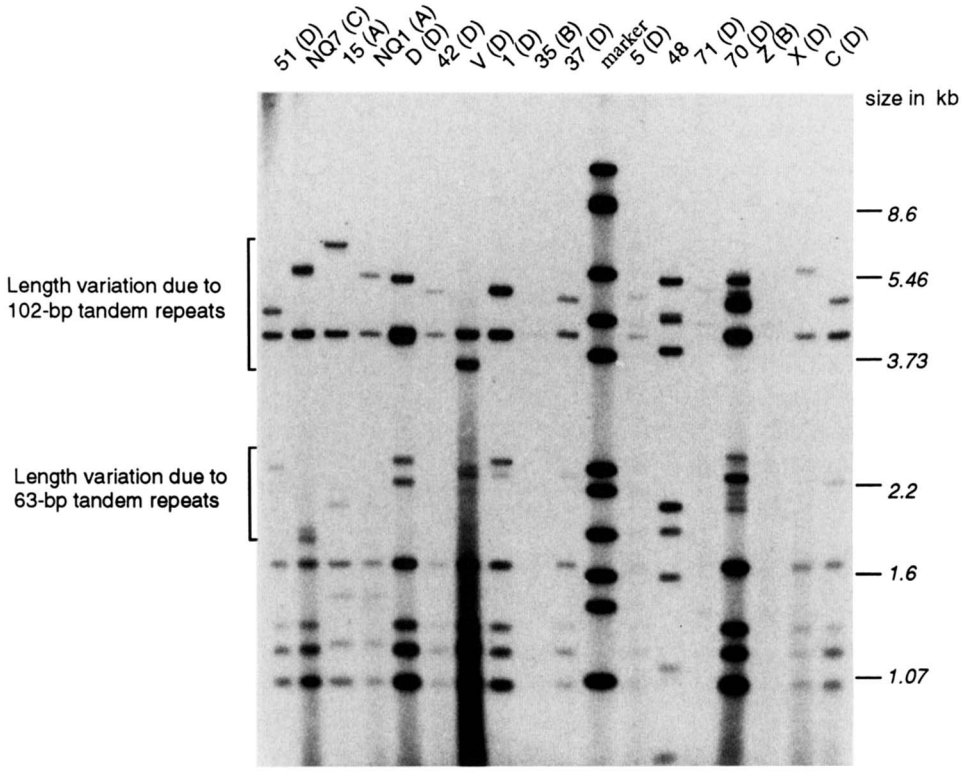

FIGURE 2.—Restriction fragment length polymorphisms among Meloidogyne mtDNAs as revealed by digestion with *Mbo*I. Lanes are identified by population names and derived haplotype code listed in Table 1. Note the distinctive fragment profile for sample 48, an isolate of *M. hapla*. The size marker is *Ava*II/*Bgl*II cut λ DNA. All agarose gels were partnered by 3.5% acrylamide gels to resolve bands shorter than 1 kb. In the case of *Mbo*I these revealed three more polymorphic bands not seen here.

TABLE 2

Description of RFLPs due to point mutations used for haplotype analyses

Enzymes	Haplotypes						Fragment changes
	A	B	C	D	G	H	
*Eco*RI							
(i)	1	1	1	1	0	1	1.71 + 6.34(V) → ~8.5 kb
(ii)	0	0	0	1	0	0	1.05 kb → 0.525 + 0.533 kb
*Hin*fI							
(i)	1	1	1	0	1	1	1.5 + ~1.0(V) → 2.66 kb
(ii)	1	0	0	0	0	0	1.8 → 0.9 + 0.88 kb
(iii)	0	1	0	0	0	0	1.9 → 1.5 + 0.3 kb
(iv)	1	1	1	0	1	1	1.9 + 0.8 → 2.73 kb
*Taq*I							
(i)	0	0	0	0	0	1	1.10 → 0.9 + 0.23 kb
(ii)	1	1	0	1	1	0	0.43 + 1.52 → 1.95 kb
*Mbo*I							
(i)	0	1	1	1	1	1	1.19 + 0.27 → 1.46 kb
(ii)	1	1	0	1	1	0	0.35 → 0.10 + 0.25 kb

The presence of any particular site is indicated by a "1" and absence by "0". The (V) indicates variable length band due to the tandem repeats, the specific sizes are for the *M. javanica* sequence of OKIMOTO *et al.* (1991). The position of each site is shown on Figure 3. *Mbo*I (ii) and *Taq*I (ii) are considered the same, and the gain of *Eco*RI (ii) is synonomous with the loss of *Hin*fI (iv). The deletion in haplotype A contains an *Eco*RI, *Mbo*I and *Hin*dIII site; variation previously reported for these sites (*e.g.*, *Hin*dIII RFLPs; POWERS and SANDALL 1988) is therefore correlated and not due to individual point mutations.

haplotypes, with all enzymes except for *Xba*I and *Hin*dIII showing the gain/loss of restriction sites (Table 2).

Four of the six haplotypes (A–D) are, on the basis of published fragment patterns, the same as those reported from North American isolates (POWERS and SANDALL 1988). Most (41) of the isolates were also typed using the PCR diagnostic test devised by HARRIS *et al.* (1990). As previously reported, no PCR amplification was achieved from mtDNAs with the A haplotype, while B, C and D haplotypes could be distinguished by digestion of the 1.7-kb amplification product with *Hin*fI (data not shown). Use of a nested primer in the L-rDNA (T. O. POWERS, personal communication.) allowed amplification of the mtDNA from the A haplotype, but digestion of this truncated product with *Hin*fI could only distinguish the B haplotype from the rest. Of the variants identified in the later PCR assay of POWERS and HARRIS (1993), their *M. arenaria*, *M. incognita* and *M. javanica* groups correspond to our haplotypes A, B and D; whereas our C, G and H haplotypes were not diagnosable.

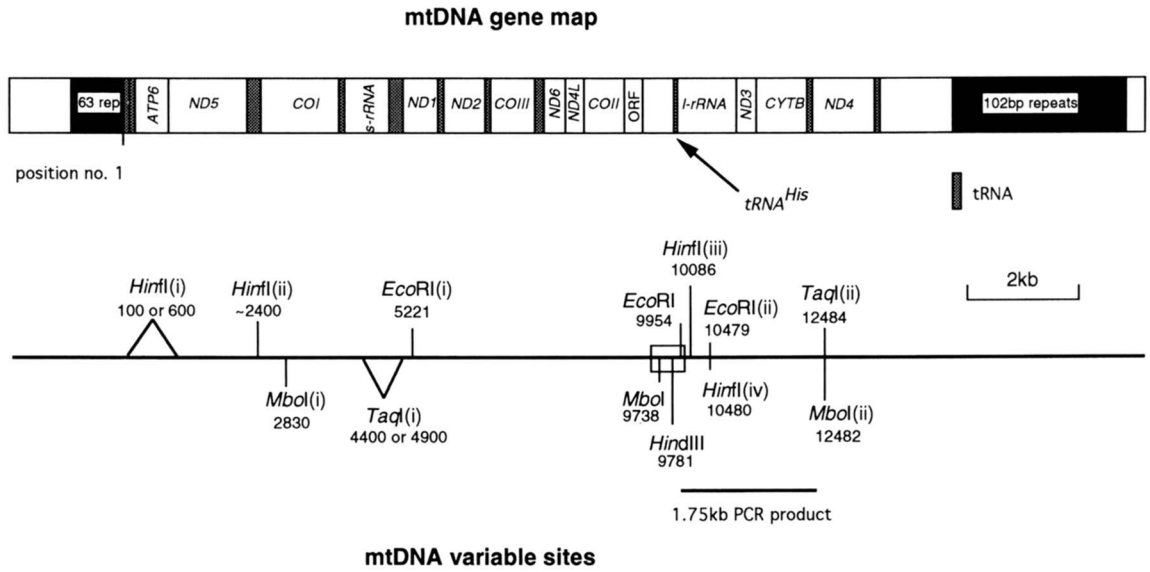

FIGURE 3.—Positions of the 10 polymorphic restriction enzyme sites. The gene map and nucleotide position numbers are from R. OKIMOTO, H. M. CHAMBERLIN, J. L. MACFARLANE and D. R. WOLSTENHOLME (manuscript in preparation). This positions $tRNA^{His}$ 5′ to the L-rRNA gene. The deletion in haplotype A, shown as a box, encompasses an *Eco*RI, a *Hin*dIII and an *Mbo*I site. *Hin*fI (iii) and (iv) lie within the 1.75-kb PCR product and are responsible for the RFLPs shown in Figure 5 of HARRIS *et al.* (1990). The exact positions of *Hin*fI (i), *Hin*fI (ii) and *Taq*I (i) have yet to be determined.

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                                        MnlI
D     AAATTTAATAATAAATGTTTTTTAAATTCTTTGAGGTTTTGATTTTTGATTTTTTGTTTATGCTCATTGTTAAAGAAAAGCA
H,C   ....................................A.....................................................
B     .....................................................................................
A     .....................................................................................
G     .....................................................................................

                                  MnlI                    EcoRI(ii)
D     CTTTTAGCGTGATTGGTCAAAGGTAGCGAGGTAATTTGTTTTTTTATTGAATTCTAGTATGAATGGATTTTTTGTTATTAAA
H,C   ..............................................G.....G..............................
B     ...........................A..................G.....G..............................
A     ..............................................G.....G..............................
G     ..............................................G.....G..............................
                                                    HinfI(iv)
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FIGURE 4.—Sequence from putative L-rDNA positions 10349–10512, showing several nucleotide substitutions. These include an A ↔ G transition accounting for two RFLPs; *Eco*RI (ii) at 10479 and *Hin*fI (iv) at 10480. This section has been sequenced for representatives of all six haplotypes using the primers of HARRIS *et al.* (1990). It also includes two *Mnl*I sites distinguishing haplotypes B from C from the rest.

The locations of the 13 variable restriction sites were determined by comparison with the complete *M. javanica* mtDNA sequence (Figure 3). Eight of the 13 variable restriction sites were present in this sequence (= haplotype D, see Table 2) and were mapped precisely. Another two, *Hin*fI (iii) and (iv), were located by sequencing of parts of the 1.75-kb PCR product (Figure 4). The other three sites are less precisely located; the novel *Hin*fI (i) and *Taq*I (i) sites could each be at either of two locations. Of particular interest is the concentration of restriction site changes within or near to the large subunit ribosomal RNA gene, the region spanned by the PCR amplification test devised by HARRIS *et al.* (1990).

However, the mapping of variable restriction sites revealed several features which combine to reduce the estimate of sequence divergence below that apparent from comparison of fragment patterns. First, there are two cases of multiple restriction site changes derived from a single base substitution. In the first case, variable *Mbo*I (ii) (GATC) and *Taq*I (ii) (TCGA) sites overlap at positions 12482–4 in the sequence so that the loss of both sites in haplotype C is likely to be due to a single base substitution. In the second case, sequencing of part of the 1.75-kb PCR product spanning these sites from representatives of haplotypes B, H and G (Figure 4) demonstrated that the gain of the *Eco*RI-(ii) site and the loss of the *Hin*fI-(iv) site in haplotype D is due to a single G ↔ A transition at position 10480 in the sequence.

Most significantly, the loss in haplotype A of the *Eco*RI, *Hin*dIII and MboI sites near the junction of the L-rDNA and non-coding region (Figure 3) is due to a single event, a deletion of 529 bp encompassing these three

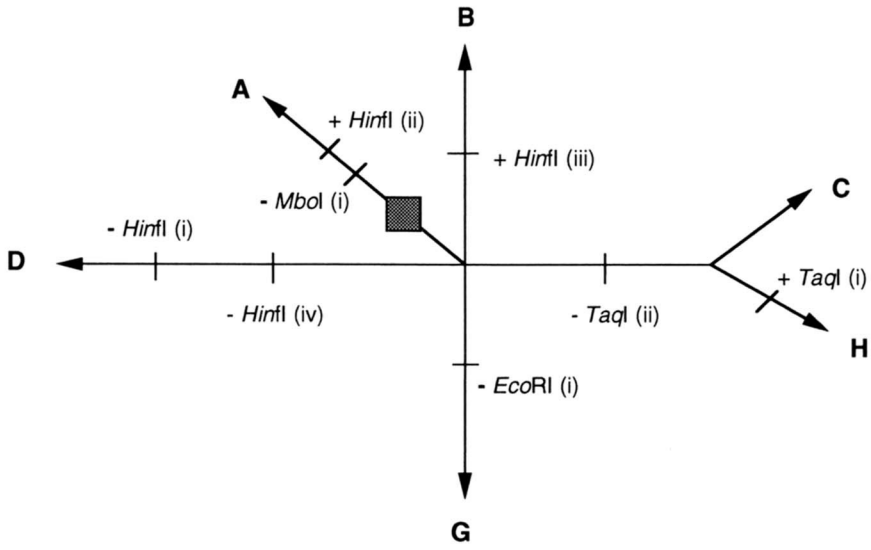

FIGURE 5.—Minimum length unrooted network of mtDNA haplotypes A, B, C, D, G, and H based on the distribution of variable sites. Restriction enzymes sites (see Figure 3) are identified by numbers in parentheses; – or + indicates loss or gain of that site relative to the direction indicated by the arrows. As *Mbo*I (ii) is considered synonymous with *Taq*I (ii) it has not been included. Similarly the *Hin*fI (iv) site change accounts for the variable *Eco*RI (ii) site. The box represents the 529-bp deletion.

restriction sites as well as one of the primer sites selected by HARRIS *et al.* (1990). Aside from modifying the fragment pattern for these enzymes (*e.g.*, for *Mbo*I, 1.32 + 0.44 → 1.26 kb; samples 15 and NQ1 in Figure 2), this deletion also altered fragment patterns for all other enzymes (*e.g.*, *Hin*fI, 1.9 → 1.4 kb; *Taq*I, 2.51 → 2.0 kb). The exact position and size of the deletion was determined for two representatives of haplotype A, 15 and NQ1, by sequencing from either side (data not shown). It encompasses positions 9478 to 10006, extending from 145 bp 3′ of the open reading frame to 10 bp 5′ to the $tRNA^{His}$ gene (see Figure 3) As expected,the deletion eliminates the primer site at 9808–9827.

Removing the redundant site changes and excluding the 529-bp deletion reduces the number of restriction site changes due to independent base substitutions from 13 to just eight (Table 2), with between one and four site changes separating haplotypes (Figure 5). The estimates of sequence divergence between haplotypes are all less than 0.6%, ranging from 0.58% between H and A to 0.14% between C and H (Table 3). The nucleotide diversity across the 48 isolates with this group of mtDNAs is low (0.0028). Comparisons of the sequences from the L-rRNA and cytochrome *b* genes also revealed low divergences among haplotypes, ranging from zero to 0.46% (Table 3).

Stimulated by the indication that it might be possible to use the number of copies of tandem repeats to identify different species and host races of Meloidogyne (OKIMOTO *et al.* 1991), we examined the distribution of the sizes of the repeat-containing fragments among the different haplotypes (Figure 6). The number of copies inferred represent the fragment length less the known non-repeat sequences. The dominant *Mbo*I fragment containing the 102 bp tandem repeats ranged in size from 3.50 kb (≈19 copies) to 6.55 kb (≈49 copies), whereas the *Mbo*I fragment containing the 63 bp tandem repeats varied from 1.85 to 2.50 kb (≈3–14 copies).

TABLE 3

Percent sequence divergence between Meloidogyne haplotypes

	A	B	C	D	G	H
A	—	0.43	0.43	0.57	0.43	0.58
B	0.13	—	0.29	0.43	0.29	0.43
C	0.13	0.37	—	0.43	0.29	0.14
D	0.26	0.46	0.37	—	0.44	0.57
G	0.13	0.37	0.19	0.28	—	0.43
H	0.13	0.28	0.00	0.37	0.28	—

Above the diagonal, sequence divergences are from RFLP data, while below they are from combined known nucleotide sequences of L-rRNA and cytochrome *b* genes (1080 bp). As one primer cannot be used for haplotype A, only 780 bp has been sequenced. Values from RFLPs were calculated using the REAP program for six enzymes as described in Table 2 but excluding *Mbo*I (ii) and *Hin*fI (iv), which have been accounted for by *Taq*I (ii) and *Eco*RI (ii), giving a total of 78 constant and eight variable sites. *Mbo*I sites due to the 102-bp tandem repeats have not been counted in the number of constant sites. The 529-bp deletion in A has been disregarded.

Assuming that the fragment size variation results from differences in the numbers of repeated sequences, then the copy number of these repeats appears to be non-randomly distributed among the common haplotypes (Figure 6) with haplotypes A and C having the most copies of the 102-bp sequence and haplotype B the least. However, there appears to be a broad spread of copy number for the 102-bp sequence in haplotypes B and D, less so for A and C, and little partitioning of numbers of the 63-bp repeat.

Geographic and taxonomic distribution of mtDNA variation: In contrast to the low nucleotide diversity, the haplotype diversity among the 48 isolates (0.603) is moderately high. Haplotype D is the most common, occurring in more than half (29/48) of the isolates, haplotypes A, B and C are of similar but lower abundance (4–7/48), and haplotypes G and H were restricted to single isolates (Table 1).

Considering just the samples from Queensland (N = 38; sample sizes elsewhere are too small), the mtDNA polymorphism is geographically structured with the B

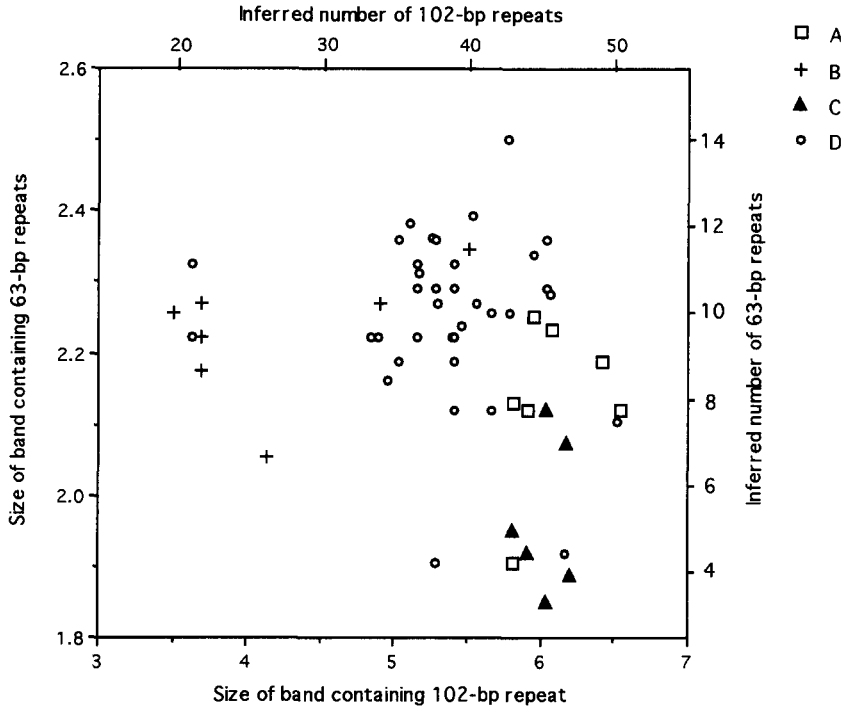

FIGURE 6.—Correlation of the lengths of the *Mbo*I fragments (in kb) containing the 63- and 102-bp repeats for each of the common mtDNA haplotypes (A–D). The other scales indicate the number repeats required to give such a size after subtracting non-repeat sequences (OKIMOTO *et al.* 1991). For samples with multiple bands containing repeats, each point is determined from the dominant band.

type being common in north Queensland and rare elsewhere (Figure 1). This impression is substantiated by quantitative comparison of samples from north (N = 20) and south (N = 18) of the Tropic of Capricorn (Figure 1). The nucleotide diversity between these two areas (0.0032) is as large or larger than values within either area (north, 0.0032; south 0.0022). The proportion of diversity distributed among areas, G_{ST}, is 0.130, a significant value being larger than all but 1 of 100 randomization tests (*i.e.*, P = 0.01).

The correlation between the mtDNA haplotype and the taxonomic identity of the isolate depends on the method of identification (Table 1). There is very close correspondence between mtDNA haplotype and esterase phenotype across the 39 isolates for which both kinds of genetic data are available. The *M. incognita* esterase phenotype I1 coincides with mtDNA type B (N = 7), *M. javanica* esterase J3 with mtDNA type D (N = 23), *M. arenaria* esterase A1 with mtDNA types C and H (N = 4), and the *M. arenaria* A2 and A3 esterases with mtDNA type A (N = 5).

The inability of the standard host range test to discriminate between *M. javanica* and race 2 of *M. arenaria*, and the presence of several unique host ranges limits the extent to which the fit between mtDNA haplotype and host range can be tested. The host ranges are congruent with the mtDNA types in some places; *e.g.*, all isolates with the B haplotype had I1 or I2 host ranges. However, the I2 host range was also observed for isolates having the A, C and D haplotypes, and the I1 host range for A and B haplotypes. No host range type has a single haplotype. The correspondence between perineal pattern and mtDNA haplotype is poor, with each mtDNA haplotype except for C being associated with multiple perineal pattern types.

DISCUSSION

Level and distribution of sequence variation: The combination of using high resolution electrophoretic techniques to separate restriction fragments and characterizing the observed variants by comparison with the complete sequence of *M. javanica* mtDNA has greatly increased the precision with which variation in mtDNAs from Meloidogyne can be assessed. This revealed two highly divergent groups of mtDNAs, one associated with *M. hapla* and the other, more common group associated with *M. arenaria*, *M. incognita* and *M. javanica*. The overall level of sequence divergence within the latter group was low, with estimates ranging from 0.14–0.58%.

These values are considerably lower than previous estimates among haplotypes A-D based on fragment comparisons (up to 3.1%; POWERS and SANDALL 1988). This is because (i) we identified two cases where a single base substitution caused multiple restriction site changes, and (ii) several fragment changes distinguishing haplotype A from the others were found to be due to a single deletion of 529 bp. Recognition of the deletion has a major effect on the analysis, reducing the estimates of sequence divergence between the A type and the other types from 2.1–3.1% to just 0.25–0.40%. This is significant because it changes the relationships among mtDNA haplotypes, affecting the interpretation of sequence variation within and among species (see below). It also removes the apparent conflict between relationships inferred from mtDNA and those from nuclear variants (*e.g.*, CASTAGNONE-SERENO *et al.* 1993)

The deletion eliminates most (529 of 684 bp) of the noncoding region between a 115 codon open reading

frame and the $tRNA^{His}$ gene. In addition to the three restriction sites discussed above, the deletion also includes one of the primer sites (positions 9808–9827) used in the PCR amplification test designed by HARRIS *et al.* (1990), which accounts for the inability in this and the previous study to amplify the informative segment of mtDNA from haplotype A. By relocating the primer to the cytochrome oxidase II gene, POWERS and HARRIS (1993) were able to amplify haplotype A and, consistent with our result, produced a fragment ≈600 bp shorter than in types B and D.

Despite the low overall nucleotide diversity and the apparent identity of mtDNAs from Australian and United States isolates, the variation within Queensland has strong geographic structure. This unusual genetic structure could be due to a combination of rapid spread via commensalism and local differentiation through selection among parthenogenetic lines. It could also be that haplotype B has only just been introduced into North Queensland and is still to spread within Australia.

Evolution of parthenogenetic Meloidogyne: The congruence between mtDNA variants and previously recognized taxonomic entities within Meloidogyne is difficult to evaluate because of the ambiguity or poor resolution of the non- molecular methods of identification that have been employed. However, the perfect concordance between the isozyme phenotypes previously used to diagnose different types of root-knot nematode (ESBENSHADE and TRIANTAPHYLLOU 1985, 1990) and the mtDNA types suggests that the major mtDNA variants track genetically distinct lineages. Considering all of the evidence, the esterase-based identifications in particular, we suggest that mtDNA types A, C and H correspond to *M. arenaria*, mtDNA type B to *M. incognita* and mtDNA haplotype D to *M. javanica*. The variation within *M. arenaria* is also structured among lineages, with haplotype A nematodes having the A2 or A3 esterase patterns and those with haplotypes C and H the A1 esterase pattern.

The contention that the major mtDNA variants correspond to genetically distinct parthenogenetic lineages appears to conflict with the high levels of within-taxon mtDNA variation reported by POWERS and SANDALL (1988). In particular, they reported high divergence within *M. hapla* and within *M. incognita*. Subsequently, HYMAN and POWERS (1991) indicated that the isolates of *M. hapla* in the earlier study had been misidentified, two of them actually being *M. arenaria*. With this correction, they had two principle types of mtDNA within *M. arenaria*, corresponding to our A and C haplotypes. While the sequence divergence within *M. arenaria* remains substantial compared to that occurring between other named taxa, this variation is correlated with isozyme differences and is much less in absolute terms than was estimated by POWERS and SANDALL (1988).

The concern about the fit between patterns of mtDNA variation and existing taxonomic units (species and races) may be misplaced given the difficulty of applying species (let alone race) concepts to asexual lineages (see also TRIANTAPHYLLOU 1985; HYMAN and POWERS 1991). According to recent interpretations of the evolutionary species concept (*e.g.*, FROST and WRIGHT 1989), parthenogenetic lineages with independent origins constitute separate evolutionary entities and should be recognized as separate species. The limited understanding of the evolutionary history of parthenogenetic Meloidogyne makes it difficult to apply this concept.

From the mtDNA evidence, it appears that *M. hapla* has a distinct maternal ancestry, consistent with the presence of both sexual and parthenogenetic forms within this taxon (TRIANTAPHYLLOU 1985), distinctive allozyme profiles (ESBENSHADE and TRIANTAPHYLLOU 1987) and their unique ecology of populating cool climates and higher elevations in the tropics. These data support the recognition of *M. hapla* as a distinct evolutionary species. In contrast, the other lineages have very similar mtDNAs, and thus closely related maternal ancestors, a pattern also seen in other parthenogenetic organisms (*e.g.*, DENSMORE *et al.* 1989; MORITZ *et al.* 1992). The dichotomy of mtDNA within *M. arenaria* is particularly notable, as is the correspondence between the mtDNA types and different esterase phenotypes. Previous studies of isozyme variation (ESBENSHADE and TRIANTAPHYLLOU 1987) revealed very low diversity within each of *M. javanica*, *M. incognita* and *M. hapla*, but high diversity within *M. arenaria*. Together, these data suggest that the *M. arenaria* as presently defined is composite, including lineages at least as distinct from each other as each is from *M. javanica*.

The close agreement between patterns of variation in mtDNA and esterases, representing independent genetic systems, indicates the presence of discrete genetic entities within the species *M. incognita*, *M. javanica* and *M. arenaria*, but not ones that fully correspond to existing taxonomic boundaries defined largely by host ranges and morphology. Accordingly, we suggest that further attempts to define diagnostics for, or biological attributes of, different types of Meloidogyne should focus on corroborated genetic groupings rather on the existing taxonomic units.

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