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# Isolates of *Meloidogyne hapla* with Distinct Mitchondrial Genomes

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*Abstract:* Because two conflicting reports of the structure of the *Meloidogyne hapla* mitochondrial genome exist, we compared the mitochondrial DNA (mtDNA) purified from two isolates of *M. hapla:* one from San Bernardino County in southern California (BRDO) and the other from England. The authenticity of the BRDO isolate in particular was confirmed by examination of morphological characters, isoenzyme analysis, and differential host range tests. Restriction analysis revealed that mtDNA from the BRDO and English isolates corresponded to only the structure first reported, although significant differences between the two isolates were apparent. Southern blots probed with cloned, cytochrome oxidase I (*cox-1*) DNA from *Romanomermis culicivorax* mtDNA confirmed that the analyzed DNA was of mitochondrial origin. Thus, *M. hapla* has at least two distinct but presumably related mitchondrial genomes, plus at least one very different structure. These data are discussed with reference to recent molecular diagnostic and phylogenetic analyses of *Meloidogyne.*

*Key words:* DNA, *Meloidogyne hapla,* mitochondrial DNA, molecular diagnostics, nematode, phylogeny, restriction fragment length polymorphism.

Species of *Meloidogyne* are usually discriminated by the analysis and comparison of variable morphological characters (4), differential host range tests (4), or isoenzyme analysis (2,3). These methods are time-consuming and can produce inconclusive results (5).

Using restriction enzyme analysis, Powers et al. (11) demonstrated readily scorable differences between the mitochondrial genomes of the major *Meloidogyne* species. *Meloidogyne hapla* appeared the most divergent, a finding consistent with previous isoenzyme data and thus providing weight to the proposal that mitochondrial DNA (mtDNA) polymorphisms might form the basis of a diagnostic assay (11). This work was subsequently expanded to include more isolates and provide a quantitative assessment of divergence (12). In this study, *M. arenaria* was the most divergent of the four major species, with *M. hapla* very similar to *M. incognita* and *M. javanica.* Significantly, the pattern presented for the San Bernardino, California, isolate of *M. hapla* (*M. hapla* BRDO) differed from that previously reported.

To resolve this disparity, we re-examined the restriction patterns of mtDNA purified from an isolate of *M. hapla* BRDO maintained at this university. For comparison, we also examined mtDNA from another isolate, *M. hapla* Angleterre, and discovered a striking degree of intraspecific variation.

## Materials and Methods

*Nematode maintenance and identification: Meloidogyne hapla* BRDO was originally isolated from an alfalfa field in San Bernardino County, California, by H. Ferris (formerly of the University of California at Riverside); *M. hapla* Angleterre was originally isolated in England and was obtained from P. Roberts (University of California at Riverside). Host range, perineal patterns, and isoenzyme profiles of the latter had recently been confirmed by P. Roberts and W. Matthews. Both strains were maintained in the greenhouse on tomato (*Lycopersicon esculentum* Mill. 'VFN-8').

The host range of *M. hapla* BRDO was determined essentially as previously described (4). An inoculum of $4 \times 10^4$ eggs was applied to three pots each of the following plants: tomato ('Rutgers'), pepper (*Capsicum frutescens* L. 'California Wonder'), watermelon (*Citrullus vulgaris* Schrader 'Charleston Grey'), cotton (*Gos-*

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*sypium hirsutum* L. 'Deltapine 61'), tobacco (*Nicotiana tabacum* L. 'NC95'), and peanut (*Arachis hypogaea* L. 'Florunner'). The number of egg masses on each was determined 52 days later. Parallel tests were concurrently performed on other *Meloidogyne* species (*M. incognita, M. javanica*).

Isoforms of malate dehydrogenase (Mdh) and nonspecific esterase (Est) in individual *M. hapla* BRDO females were determined by electrophoretic assay (2,3), with *M. javanica* females as controls. Perineal patterns were cut from 25 adult females and mounted in glycerin (4) before microscopic examination with Nomarski optics.

*Mitochondrial DNA analysis:* Mitochondrial DNA was isolated and CsCl-purified from sucrose-banded eggs as previously described (6,11). Aliquots of DNA preparations (typically 200 ng) were cleaved with either Ava II, Cla I, Eco RI, Hind III, or Xba I before electrophoretic separation on 1% agarose. Enzymes were obtained from Life Technologies, Inc., Gaithersburg, Maryland. Gels were stained with ethidium bromide, and the digestion products were visualized under 300 nm illumination. Sizes of DNA fragments were determined by comparison with the electrophoretic mobility on the same gel of DNA standards of known size (Bst EII digest of bacteriophage λ DNA). When the yield of mtDNA recovered was low, 20-ng aliquots were restricted and the resultant overhang was partially filled using the Klenow fragment of *E. coli* DNA Polymerase I and an appropriately labeled dNTP; $\alpha[^{32}P]$dGTP was used following Ava II digestion, $\alpha[^{32}P]$dCTP following Cla I or Xba I digestion, and $\alpha[^{32}P]$dATP after digestion with Eco RI or Hind III. After electrophoresis on 1% agarose, gels were air-dried in a fume hood and bands were detected by autoradiography.

After examination of ethidium bromide-stained bands, DNA was blotted to a nitrocellulose membrane filter (14). The filter was prehybridized and hybridized with a DB#6 insert that had been labeled to $10^8$ dpm/μg by nick translation (13), essentially as described by Wahl et al. (15), except formamide concentrations were reduced to 10%. DB#6 is a plasmid recombinant containing a 700 base pair Xba I–Eco RI insert encoding part of cytochrome oxidase I (*cox-1*), a mitochondrial gene cloned from *Romanomermis culicivorax* (7) and kindly made available by B. Hyman. This probe was chosen to establish the mitochondrial origin of the bands, as *cox-1* sequences in metazoans occur only in mtDNA (1). Filters were washed to final stringency of 330 mM $Na^+$/42 C and autoradiographed.

## Results

*Nematode identification:* Analyses of *M. hapla* BRDO were consistent with a diagnosis of *M. hapla*. The perineal patterns of *M. hapla* BRDO contained "wings" and punctations at the tail terminus, features diagnostic for *M. hapla*. In the differential host test, the mean numbers of *M. hapla* BRDO egg masses on each host were as follows: tomato, 144 (range: 117–163); pepper, 78 (55–104); watermelon, 0 (0); cotton, 0 (0); peanut, 118 (83–145); and tobacco, 36 (28–50). This test was performed two times with similar results, reflecting a typical host range for *M. hapla*. Polyacrylamide gels (Fig. 1) stained to reveal isoforms of Mdh and esterase in individual females of *M. hapla* BRDO and *M. javanica* contained patterns typical for each species. The esterase patterns for the BRDO and Angleterre (not shown) isolates identified them as "a" and "b" types of *M. hapla*, respectively.

*Analysis of mtDNA:* Restriction patterns of CsCl-gradient-purified mtDNA from *M. hapla* BRDO and *M. hapla* Angleterre are shown on different gels in Figures 2 and 3. In some lanes, most noticeably the Ava II digestion of *M. hapla* Angleterre mtDNA, faint, nonstoichiometric bands are evident. It is conceivable that these molecules are of nuclear origin, possibly some class of repeat. To unambiguously confirm the mitochondrial origin of the major, stoichiometric DNA species, a Southern filter of the digests was chal-

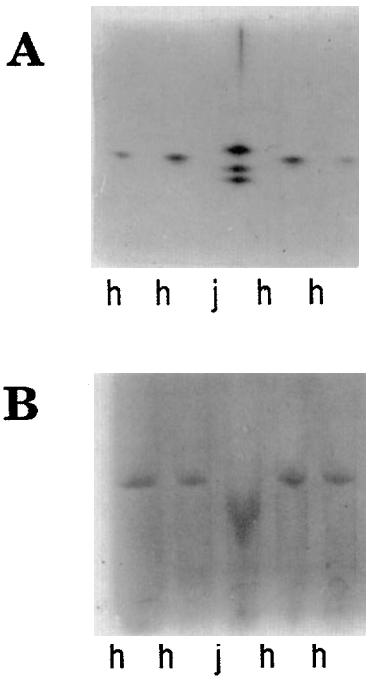


FIG. 1. Isoenzyme analysis of single *Meloidogyne hapla* BRDO (h) and *M. javanica* (j) females stained to reveal iso-forms of malate dehydrogenase (A) or non-specific esterases (B).

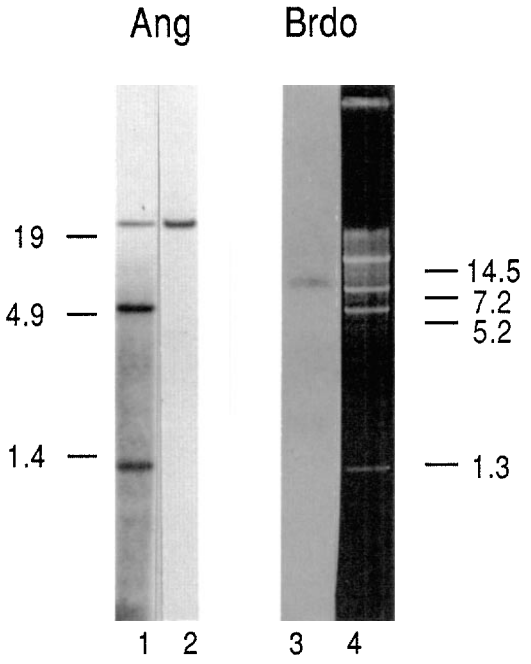


FIG. 2. Restriction analysis of mitochondrial DNA isolated from *Meloidogyne hapla* Angleterre (Ang) or *M. hapla* BRDO (Brdo). Lanes 1 and 4 show Hind III profiles. Lanes 2 and 3 are autoradiographs of Southern blots of the same digests, probed with DB#6, a mitochondrial-specific probe. Sizes are indicated in kb.

lenged with the cloned *R. culicivorax cox-1* probe. This probe detected the 19.0-kb Hind III fragment in the *M. hapla* Angleterre mitochondrial genome and the 7.2-kb Hind III fragment in *M. hapla* BRDO mtDNA (Fig. 2).

## DISCUSSION

The objective of this research was to investigate a discrepancy in the literature regarding the structure of the mitochondrial genome of *M. hapla* BRDO by performing a restriction analysis on mtDNA isolated from a population of *M. hapla* BRDO maintained at this university. Based on three independent criteria, viz., perineal patterns, isoenzyme profiles, and host range preference, we unambiguously confirmed our isolate as being *M. hapla*.

The profile we obtained following Hind III digestion of mtDNA from our *M. hapla* BRDO isolate closely resembles that originally presented for this strain (11). Although the original study did not include restriction-fragment sizes, from the published figure we have calculated the Hind III fragments to be approximately 12, 7.0, 4.8, and 1.3 kb. Although these values are not identical with those we obtained in this study (14.5, 7.2, 5.2, and 1.36 kb), we consider them to lie within the normal range of gel-to-gel variation. Thus, we have confirmed the original observation of Powers et al. (11).

A subsequent study (12) yielded another Hind III profile for mtDNA from *M. hapla* BRDO. We have estimated these digestion products to be approximately 15, 2.6, and 1.65 kb. This profile substantially differs from the *M. hapla* BRDO pattern obtained by both Powers et al. (11) and us (Fig. 2), both in fragment number and size. These size differences are inconsistent with either simple loss or gain of a restriction enzyme cleavage site, or incomplete digestion at a particular site(s). Powers and Sandall (12) also presented an Eco RI pattern for *M.*

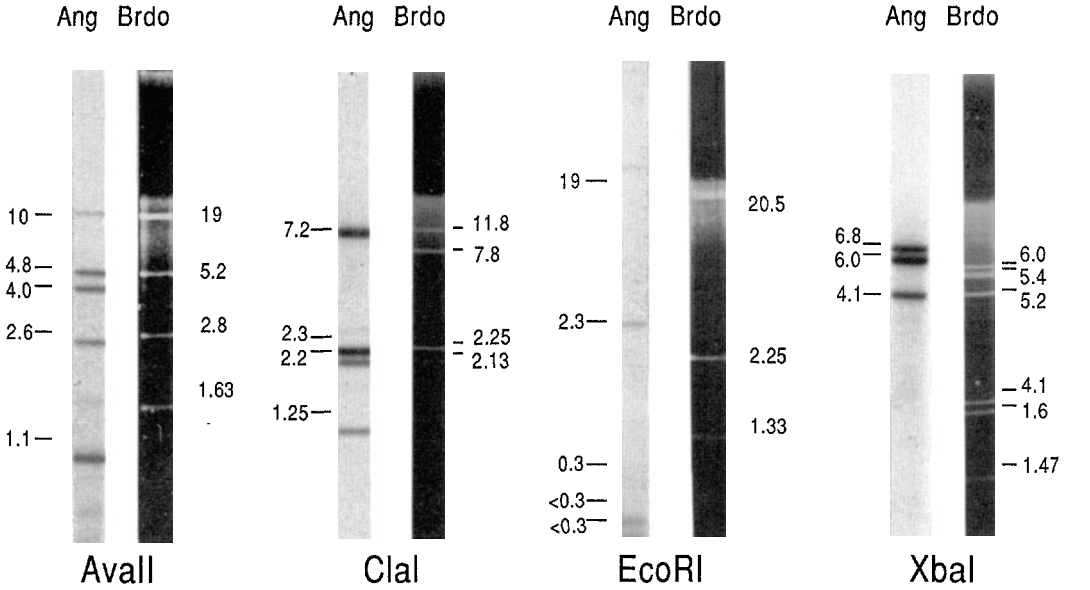


FIG. 3. Restriction analysis of mitochondrial DNA isolated from *Meloidogyne hapla* Angleterre (Ang) or *M. hapla* BRDO (Brdo). Sizes are indicated in kb.

*hapla* BRDO mtDNA, with fragments estimated by us as 6.6 (possibly a doublet), 2.2, 1.8, 1.05, 0.96, 0.74, 0.66 and <0.2 kb. These values greatly differ from the fragment sizes of Eco RI digested *M. hapla* BRDO mtDNA obtained in the present study (20.5, 2.25, 1.33, 0.53, and 0.25 kb; note that the smaller two fragments are not readily apparent in Figure 3) and again indicate a significantly different organization of the mitochondrial genomes from two isolates supposedly representing the same nematode line.

We also examined restriction profiles of mtDNA from *M. hapla* Angleterre, which were different from patterns from our *M. hapla* BRDO isolate (and from the isolate used by Powers and Sandall [12]). In some instances, these differences are consistent with simple loss or addition of restriction sites. For example, addition of a Hind III site in the 19-kb fragment of *M. hapla* Angleterre would result in a profile much like that for *M. hapla* BRDO. Although performed to confirm the mitochondrial origin of our DNA samples, the Southern blots with *cox-1* sequences are consistent with this interpretation. In contrast, a more complex explanation is required to interpret the results of Ava II digestion, which indicated that the *M. hapla* Angleterre mitochondrial genome is smaller than that of the BRDO isolate and also contains numerous, nonstoichiometric fragments. These fragments may represent dispersed, acquired clusters of Ava II sites that either give different fragments with similar sizes or are subject to partial digestion. Using partial Taq I digestion, Okimoto et al. (9) identified two short repeats in a number of *Meloidogyne* species, including a "b" type of *M. hapla*. However, these repeats lack Ava II sites (9), and are tandemly arranged, not dispersed.

The pairwise comparisons of mtDNA restriction profiles of the two "lines" of *M. hapla* BRDO and of *M. hapla* BRDO with *M. hapla* Angleterre suggest a significant degree of intraspecific polymorphism within the *M. hapla* mitochondrial genome. A study comparing restriction profiles of mtDNA from many isolates of *M. hapla*, including all "lines" of *M. hapla* BRDO, might reveal the extent of these polymorphisms, although, like Hyman and Powers (8), we urge caution in drawing sweeping conclusions from limited amounts of restriction data. We encourage a detailed, se-

quencing-based investigation of mtDNA, which would provide a discrete set of unambigious characters.

We have previously described interspecific matings because *M. hapla* males and *M. incognita* and *M. javanica* females. These matings produce genomic hybrids with maternal mitochondrial genomes (10). We are repeating these experiments to estimate the mating frequency and to assess the stability of the resultant phenotypes under the ensuing parthenogenetic mode of reproduction. One outcome that we predict to arise from these matings will be an *M. hapla* mtDNA genome not unlike that reported by Powers and Sandall (12).

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