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Species-Specific Restriction Site Polymorphism in Root-knot Nematode Mitochondrial DNA[1]

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Abstract: Research was initiated to physically characterize the mitochondrial genomes of several *Meloidogyne* spp. and host-races, to address questions regarding their systematics and dispersal, and to assess the possibility of developing molecular diagnostics for these nematodes. Techniques were developed for purification and rapid detection of mitochondrial DNA from root-knot nematodes. Mitochondrial DNAs among *Meloidogyne* spp. were demonstrated to exhibit extensive divergence. The potential for using the rapidly diverging mitochondrial genomes as a diagnostic assay for *M. incognita, M. hapla, M. arenaria,* and *M. javanica* is discussed.

Key words: Meloidogyne spp., molecular diagnostics, hybridization probe.

One important application of contemporary biotechnology is molecular diagnostics, which involves the detection and quantitation of specific genes by nucleic acid hybridization procedures. With the advent of molecular cloning strategies, highly sensitive and specific DNA hybridization probes can be constructed for detecting target organisms in crude, mixed sample populations. The utility of this approach was demonstrated in the health sciences for the diagnosis of viral, bacterial, and protozoan infections (2). This strategy now has been extended to identifying viral infections in agronomically important crops (5,8). The experiments described here are intended to introduce this rapidly emerging technology to the detection of soil-dwelling, plant-parasitic nematodes and more specifically to the economically important root-knot nematodes.

To develop a reliable and rapid diagnostic assay for root-knot nematode species and host races, we focused our attention on nematode mitochondrial DNAs (mtDNAs). This small, extranuclear genome was selected for several reasons. MtDNA evolves rapidly; specific mitochondrial gene loci evolve 10 to 100 times faster than single copy nuclear genes resident in the same organism (1). Consequently, DNA sequence polymorphisms accumulate in the mtDNA, resulting in useful genetic markers for population studies. In addition, mtDNA is usually present in eukaryotic cells as a highly amplified, small (15–20-kilobase [kb]) circular molecule, thereby facilitating isolation in preparative yields. We believe each of these features will contribute to the development of a sensitive, precise and rapid molecular assay for nematode species and possibly host races as well.

Our objectives were to generate information on the isolation and characterization of mtDNA from root-knot nematodes and to demonstrate dramatic sequence divergence among the mitochondrial genomes of different *Meloidogyne* species.

MATERIALS AND METHODS

Nematode isolates: All nematodes were maintained on tomato (*Lycopersicon esculentum* L. cv. Tropic) or pepper (*Capsicum frutescens* L. cv. Yolo Wonder) plants in the University of California, Riverside, greenhouse facilities. Original isolations were as follows: *M. hapla* from San Bernardino, Calif.; *M. javanica* from Indio, Calif.; *M. arenaria* from Riverside, Calif.; *M. incognita* races 1 and 3 from Riverside, Calif.; and races 3 (NCSU no. 108) and 4 from North Carolina.

Isolation of mtDNA: Mitochondrial DNA was extracted from ca. 0.25 ml of packed nematode eggs, obtained by the method of Hussey (3). Eggs were homogenized in a hand-held Dounce homogenizer for 5–10 minutes in 5 ml MSB-EDTA-proteinase K (0.20 M mannitol, 0.07 M sucrose, 0.05 M tris-HCl, pH 7.5, 0.01 M EDTA, 200 μg/ml proteinase K). The total homogenate was centrifuged at 4 C in 15-ml conical

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tubes at 700 *g* for 5–7 minutes. The supernatant was poured into 50-ml round bottom tubes and centrifuged at 14,000 *g* for 20 minutes at 4 C to collect a crude mitochondrial pellet. The pellet was suspended in 3 ml STE (0.1 M NaCl, 0.05 M tris-HCl, pH 8.0, 0.01 M EDTA, pH 8.0), to which 2–3 drops of 25% sodium dodecyl sulfate (SDS) was added, and the mixture was incubated at room temperature for 5 minutes. CsCl and ethidium bromide were added to the lysate (6) and the mixture centrifuged to equilibrium (16–18 hours; 50,000 rpm; Ti70.1 rotor) in a Beckman L8-70M ultracentrifuge. Supercoiled mtDNA banded just below any contaminating nuclear DNA. The mtDNA band was withdrawn from the gradient, and the ethidium bromide was removed by repeated extractions with isopropyl alcohol followed by 24:1 chloroform-isoamyl alcohol and dialyzed exhaustively in 0.01 mM tris, 1 mM EDTA, pH 7.5.

Nematode minilysates: Twenty female nematodes were handpicked into a 1.8-ml microcentrifuge tube containing 50 µl proteinase K (200 µg/ml) and 1.0% SDS in MSB. The nematodes were manually disrupted and incubated at 65 C for 30 minutes. The lysate was treated with 5 µl RNAse (10 mg/ml) for 30 minutes at 37 C, phenol extracted, and DNA was concentrated by ethanol precipitation. Cellular DNA obtained in this fashion can be cleaved by all restriction enzymes tested.

Root-knot spot blot: A single root gall was hand homogenized in 100 µl reaction buffer (as described for minilysates) and incubated at 65 C for 1 hour. The crude lysate was spotted directly onto nitrocellulose filters. The filters were overlaid on 0.5 M NaOH, 1.5 M NaCl for 30 minutes, then neutralized with 0.5 M tris (pH 7.5), 3.0 M NaCl and washed twice in 2× SSC (0.3 M NaCl, 0.03 M Na citrate). Hybridization and autoradiography was conducted according to published procedures (4,7,9).

Restriction analysis: Restriction enzymes were obtained from Bethesda Research Laboratories, Bethesda, Maryland, and used according to manufacturer's recommendations. Restriction digests were fractionated on 0.7–1.0% agarose gels. Electrophoresis was conducted in tris-borate-EDTA buffer (6); DNA was stained by ethidium bromide (0.5 µg/ml) and visualized on a Fotodyne ultraviolet transilluminator.

Hybridization: DNA fractionated in agarose gels was transferred to nitrocellulose filters (9). We prepared ^{32}P-labeled *Meloidogyne incognita* (race 1) mtDNA, labeled in vitro by nick translation according to Rigby (7). DNA-DNA hybridization and autoradiography were carried out as described by Hyman et al. (4). Hybridization was carried out at 65 C for 36 hours followed by two washes in 4× SSC for 45 minutes at 65 C and two washes in 2× SSC, 0.1% SDS for 45 minutes at 65 C. Filters were exposed to X-ray film for 24 hours, using a DuPont Cronex intensifying screen.

Results

MtDNA isolation: We successfully isolated intact mtDNA from several *Meloidogyne* spp. in preparative yields, using both whole nematode lysates and purified egg preparations. However, because of the absence of contaminating carbohydrate (that co-purifies with mtDNA in our isopycnic gradients), mtDNA was most efficiently isolated from nematode eggs. The yield from 0.25 ml packed eggs was generally 10–30 µg purified mtDNA.

Restriction enzyme analysis: MtDNA was prepared from *Meloidogyne incognita* (race 1), *M. hapla*, *M. arenaria*, and *M. javanica*. Each preparation was independently digested with several different restriction enzymes. Restriction fragment polymorphisms were easily observable among the four *Meloidogyne* spp. (Figs. 1, 2). MtDNA from four *Meloidogyne* spp. were digested with the restriction enzymes HindIII or HindIII plus HincII (Fig. 1). Several features are notable in these digestions. *M. hapla* (Fig. 1, lanes 2, 6) does not share common restriction fragments with the other three species. The mitochondrial genome of this *M. hapla* isolate must have diverged from the other *Meloidogyne* spp. Digestion with numerous additional restriction enzymes confirms this observation (unpubl.). *M. javanica*, *M. incognita* (race 1), and *M. arenaria* (Fig. 1, lanes 3–5) share two lower molecular weight HindIII digestion products. However, the largest HindIII fragment differs slightly in mobility between these species. This fragment

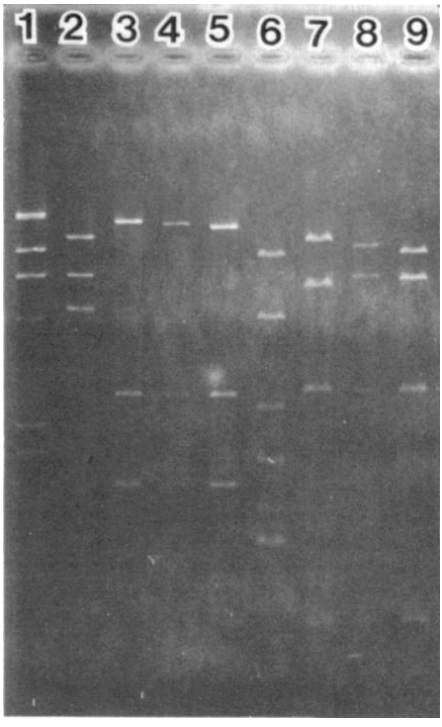

FIG. 1. Restriction endonuclease digestion of *Meloidogyne* spp. mtDNA. Lane 1: Lambda DNA standard digested with HindIII. Lanes 2–5: *M. hapla, M. javanica, M. incognita* (race 1), *M. arenaria* mtDNA digested with HindIII. Lanes 6–9: Same mtDNA samples digested with a combination of HindIII and HincII. The DNA preparations were electrophoretically fractionated on a 0.7% gel and visualized by ethidium bromide staining.

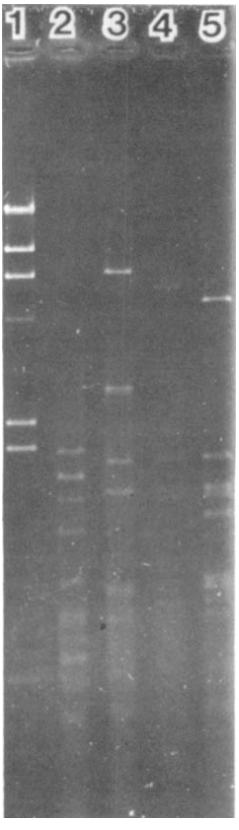

FIG. 2. Restriction endonuclease digestion of *Meloidogyne* spp. mtDNA. Lane 1: Lambda DNA standard digested with HindIII. Lanes 2–5: *M. hapla, M. javanica, M. incognita* (race 1), *M. arenaria* mtDNA digested with HinfI. The DNA samples were electrophoretically fractionated on a 0.7% agarose gel and visualized by ethidium bromide staining.

is ca. 16 kb pairs in size, largest in *M. javanica* (Fig. 1, lane 3) and smallest in *M. arenaria* (lane 5). The difference in mobility of this fragment may indicate slight differences in total genome size. Lanes 6–9 are mtDNA from the same species cut with a combination of HindIII and HincII. Again the divergence of the mitochondrial genome of *M. hapla* from the mtDNA of the other species is evident (Fig. 1, lane 6). By cleaving the DNAs with HindIII plus HincII, the subtle differences between *M. javanica, M. incognita,* and *M. arenaria* become accentuated. The highest molecular weight fragment in HindIII digestion is now cleaved by HincII, resulting in two fragments of ca. 10.0 and 6.0 kb (Fig. 1, lanes 7–9). The mobility of the largest of these two fragments is diagnostic for these three *Meloidogyne* spp. In *M. javanica,* it appears that a second HincII site further reduces the size of the second high molecular weight fragment (Fig. 1, lane 7).

Figure 2 displays mtDNA from the same *Meloidogyne* spp. cut with the restriction enzyme HinfI. HinfI recognizes considerably more restriction sites in these genomes and therefore produces fragments of a smaller average size. More than 12 fragments are generated in these digestions. At this level of resolution, an even greater number of fragment differences are revealed between *M. javanica, M. incognita,* and *M. arenaria* (Fig. 2, lanes 3–5).

Spot-blots: To demonstrate the feasibility of using mtDNA-based diagnostic probes for the rapid detection of *Meloidogyne* spp., galls from infected tomato plant roots were isolated and gall macerates prepared by digestion with proteinase K. Samples (containing a mixture of plant host and nema-

tode DNAs) were spotted directly onto nitrocellulose filters and hybridized with ^{32}P-labeled *M. incognita* (race 1) mtDNA. Strong hybridization signals were obtained with as little as 1 μl macerate from *M. arenaria* or *M. javanica* infected roots (Fig. 3). No hybridization was observed with the *M. hapla* preparation. This observation is consistent with results derived from comparative restriction digestions which indicates a high degree of divergence between these mitochondrial genomes. However, cross-hybridization was observed between the *M. incognita* mtDNA probe and DNA within *M. arenaria* and *M. javanica* gall macerates (Fig. 3, lanes 1 and 2) or purified mtDNA control samples (lanes 3 and 4) obtained from these same *Meloidogyne* spp. This cross-hybridization is most likely the result of conserved mtDNA sequences within the mitochondrial genome of these three species.

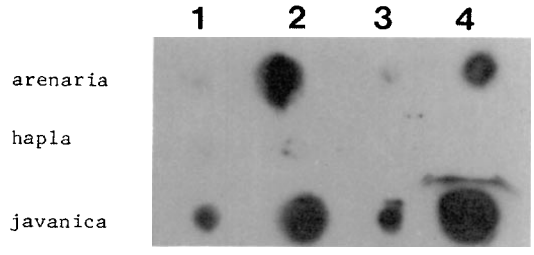

FIG. 3. Diagnostic root-knot spot blot. Crude root macerate, prepared from *Meloidogyne* spp. galled root as described in text, was spotted onto a nitrocellulose filter. Lanes 1 and 2 contain 1 and 5 μl, respectively, of gall macerate. Lanes 3 and 4 contain 8 and 40 ng of purified mtDNA from each *Meloidogyne* spp., as noted. Samples were denatured in situ with NaOH and hybridized with ^{32}P-labeled *M. incognita* (race 1) mtDNA. *M. incognita* (race 1)-infected root macerates generated strong hybridization signals when tested in parallel experiments (data not presented).

Analysis of M. incognita host-races using nematode minilysates: We recently developed a rapid procedure to isolate total cellular DNA from one or very few nematode specimens. The DNA obtained by this procedure is suitable for digestion by restriction enzymes. Using our nematode minilysate technique, we screened mtDNAs from *M. incognita* host races for restriction site polymorphisms. Total cellular DNA was isolated from nematodes of three host races and subjected to cleavage by HindIII or EcoRI. After fractionation by agarose gel electrophoresis, the DNA was transferred to nitrocellulose filters and hybridized with ^{32}P-labeled *M. incognita* mtDNA to visualize the mtDNA component of total nematode cellular DNA. The autoradiogram presented in Figure 4 reveals that the mtDNAs from host races 1, 3, and 4 generate a similar but not identical restriction pattern with these two enzymes. Treatment with additional enzymes has further demonstrated that some differences between host races exist. The extent of these differences is currently under investigation.

DISCUSSION

In order to assess the feasibility of constructing diagnostic root-knot nematode molecular probes, we have begun to study the physical and genetic organization of mitochondrial genomes from several species and host races of the genus *Meloidogyne*. Of these species, *M. incognita* is considered to be the most common and most destructive. We present here information on the isolation, analysis, and detection of *Meloidogyne* mtDNA.

Our initial studies revealed that mtDNAs from *M. incognita, M. arenaria, M. hapla,* and *M. javanica* have diverged to a sufficient extent that several polymorphisms were revealed by the first two enzymes employed. Size variations in generated fragments were diagnostic for species and indicated that refined probes for individual *Meloidogyne* spp. could be generated. Preliminary sizing of the mtDNA restriction products revealed a mitochondrial genome size of approximately 20 kb, within the size range common among most animal mtDNAs.

It is not surprising that root-knot nematode species can be identified on the basis of their mtDNA restriction patterns. Given the length of time that these species probably have been reproductively isolated (10) and the rapid evolution of mtDNA (1), there should be considerable accumulation of restriction site polymorphism. For the purposes of developing reliable diagnostic probes, however, it will be necessary to identify divergent regions of species-specific mtDNA. These regions can then be incorporated into suitable cloning vectors and used as diagnostic molecular

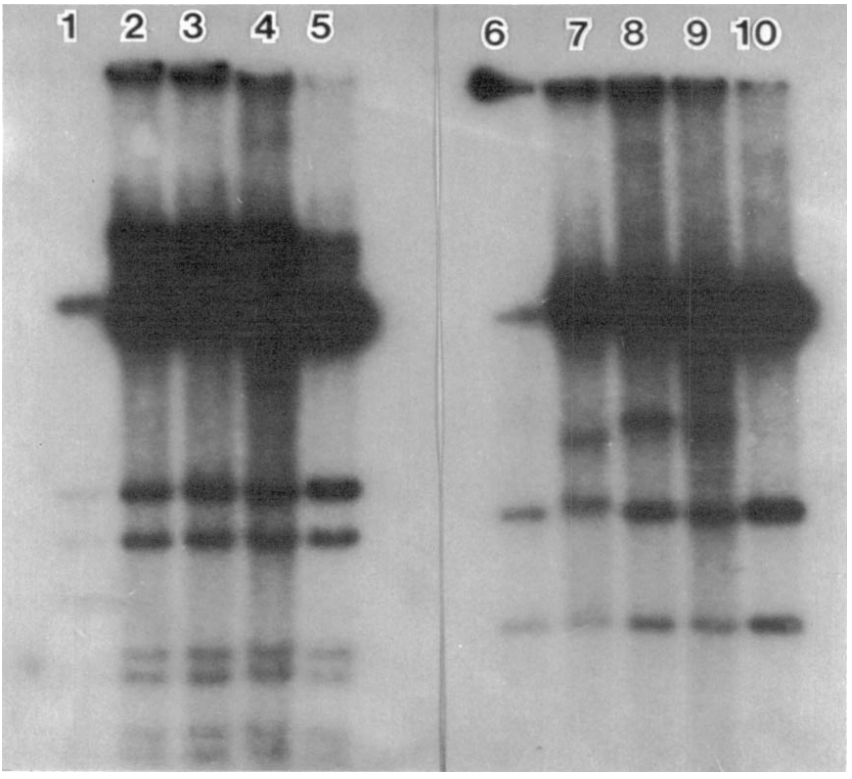

FIG. 4. Restriction enzyme analysis of *Meloidogyne incognita* host races using minilysates. Twenty females were subjected to the rapid lysis procedure as described. The total cellular DNA samples so obtained were digested with EcoRI (lanes 1–5) and HindIII (lanes 6–10). Digests were electrophoretically fractionated on a 0.7% agarose gel, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled *M. incognita* race 1 mtDNA. Lanes 1, 6: Race 3 DNA from California. Lanes 2, 7: Race 3 DNA from North Carolina. Lanes 3, 8: Race 4 DNA from North Carolina. Lanes 4, 9: Race 4 DNA from North Carolina, obtained from a crude gall macerate. Lanes, 5, 10: Race 1 from California.

probes. With the advent of nonradioactive labels, development of field kits for rapid identification of species is envisioned. Since multiple probes may be used and hundreds of samples screened simultaneously, the time and expense of diagnosis will be considerably lessened.

MtDNAs from individual *M. incognita* host races were also subjected to restriction analysis. In contrast to the polymorphisms detected among *Meloidogyne* spp., less divergent banding patterns were generated among the three host races tested, suggesting that host-race development is a relatively recent evolutionary event. More detailed studies using numerous enzymes will be required in order to further address divergence among host races. It may be necessary to incorporate S1 nuclease treatments, mtDNA heteroduplexing, and DNA sequencing to address fine-structure variation among host races.

The types of analyses presented here demonstrate the utility of using mtDNA (as a "reporter") for nematode diagnostics. The highly amplified nature of mtDNA in nematode cells enabled us to obtain a prominent hybridization signal in two types of mixed sample preparations. Specific hybridization to mtDNA was easily observed in DNA transfer experiments using *Meloidogyne* minilysates, where nematode chromosomal DNA was present in vast excess over mtDNA. The sensitivity of our hybridization procedure is also exemplified by the spot-blot experiments where nematode mtDNA was identified in crude gall macerates that contained plant host cellular DNA in addition to nematode chromosomal and mtDNA.

The experiments presented here represent a small number of possible field samples. Numerous additional isolates need to be compared to assess intraspecific varia-

tion. In addition, it will be important to assess any correlation of intraspecific variation with variations in nematode pathogenicity.

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