

Genotypic Differentiation of *Meloidogyne* Populations by Detection of Restriction Fragment Length Difference in Total DNA¹

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Abstract: Detection of EcoRI restriction fragment length differences in repetitive DNA sequences permitted the rapid diagnosis, by genotype, of randomly selected populations of *Meloidogyne incognita*, Races 1, 2, 3, and 4; *M. javanica*; *M. arenaria*, Races 1 and 2; and *M. hapla*, Races A and B.

Key words: *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, *M. javanica*, race differentiation, repetitive DNA, restriction fragment, DNA.

Several biochemical techniques (3-7) have been applied to identify and differentiate between the four major species of *Meloidogyne*: *M. incognita* (Kofoid and White, 1919) Chitwood, 1949; *M. javanica* (Treub, 1885) Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; and *M. hapla* Chitwood, 1949. These techniques analyzed phenotypic characters (e.g., total soluble proteins and isozymes) and enabled the differentiation of *Meloidogyne* species (3-7) and in some cases populations within species (3-5), but they have not successfully distinguished the races. A drawback that diminishes the diagnostic value of such phenotypic characters for use in taxonomy is that they are inherently subject to developmentally or environmentally induced variation.

Direct analysis of genotype by detection of differences in DNA restriction fragment lengths avoids these problems and enables precise identification based directly on the genome. This technique has been successfully applied to several nematode groups (2) and is applied here to enable differentiation of populations of races of four *Meloidogyne* spp.

MATERIALS AND METHODS

Populations of *Meloidogyne* species and races were obtained from J. N. Sasser, Department of Plant Pathology, North Car-

olina State University. They included *M. incognita*—Race 1 (NCSU #68), Race 2 (NCSU #E1135), and Race 4 (NCSU #401); *M. javanica* (NCSU #7-2); *M. arenaria*—Race 1 (NCSU #352) and Race 2 (NCSU #480); *M. hapla*—cytological Race A (NCSU #86) and cytological Race B (NCSU #48). *Meloidogyne incognita*, Race 3 (8), was maintained in the greenhouse on chile pepper plants (*Capsicum frutescens* L.) and the other species or races on tomato (*Lycopersicon esculentum* Mill.). Stock cultures were maintained by inoculating these hosts with approximately 10,000 eggs of the appropriate nematode population and propagating in a greenhouse.

Subsequently, eggs were released from the egg masses and collected (9). These cleaned eggs were washed through a 63- μ m-pore sieve and concentrated with repeated washings on a 24- μ m-pore sieve, resuspended in distilled water containing 50 ppm chlorhexidine gluconate, and sealed in hypo-vials for shipment from Tucson to Vancouver.

Upon arrival in Vancouver 2 days later, the eggs were washed in distilled water and the total DNA extracted. Washed eggs of each isolate were pelleted in a microcentrifuge, frozen in liquid nitrogen, and ground by mortar and pestle cooled in liquid nitrogen. The resulting fine powder was digested in approximately six volumes of a 2-mg/ml buffered solution of proteinase K (Sigma) (0.1 M tris buffer pH 8.5, 0.05 M EDTA, 0.2 M NaCl, and 1% sodium dodecyl sulfate) at 65 C for 5-15 minutes. The viscous solution was extracted three times with an equal volume of redistilled phenol (saturated with 0.01 M tris pH 8.0, 0.001 M EDTA buffer) and once with chloroform-isoamyl alcohol (24:1), 5-15 minutes per extraction with occasional

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of 10 mg/ml DNase-free RNase and incubated at room temperature for 15–30 minutes. The solution was then extracted with an equal volume of chloroform–isoamyl alcohol (24:1), and the DNA was precipitated and resuspended as described. This procedure extracts both genomic and mitochondrial DNA (= total DNA). Yields of 50–100 µg of total DNA were obtained from approximately 500 mg wet weight of eggs.

One-microgram aliquots of total DNA were digested with 10 units of EcoRI (ICN Nutritional Biochemicals) in 0.1 M tris-HCl pH 7.2 buffer containing 5 mM MgCl₂, 50 mM NaCl, and 2 mM 2-mercaptoethanol, incubated at 37 C for 1 hour, and heated to 65 C for 10 minutes to stop the reaction.

Restriction endonuclease digested DNA samples were mixed with loading buffer (to a final concentration of 5% glycerol, 0.025% bromophenol blue) and placed in 5-mm × 1-mm slots of a 0.7% agarose gel containing 0.5 µg/ml ethidium bromide. Gels were electrophoresed at 20 V for 16 hours in 0.089 M tris-borate, 0.089 M boric acid, and 0.002 M EDTA running buffer. EcoRI/Hind III cut C1857 lambda DNA was used as size marker. The gel was photographed by 260-nm transmitted irradiation, and line drawings were traced from the negative to overcome the loss of detail incurred in producing the positive print.

RESULTS

Several distinct bands were visualized for each *Meloidogyne* population in ethidium bromide stained gels viewed under 260-nm irradiation (Fig. 1). Identical results were obtained from 12 gels using DNA samples from the same or different egg batches. These bands, the number of which varied with each population, represent multiple copies of repetitive DNA sequences in a background of single copy or low copy number sequences. Certain of these repetitive DNA bands have restriction fragment lengths unique to each *Meloidogyne* population, and these differences can be compared and used as diagnostic characters; e.g., the possession of a large (> 15 kb) repetitive DNA band distinguishes the two *M. hapla* populations from all the other species studied, and a size difference in this large repetitive DNA band separates *M.*

TABLE 1. Selected diagnostic repetitive DNA bands of *Meloidogyne* spp. visualized by UV irradiation on ethidium bromide stained agarose gel (see Fig. 1A).

<i>Meloidogyne</i> isolate		Approximate size of diagnostic repetitive DNA bands (kilobases)
<i>M. arenaria</i>	Race 1	5.8
	Race 2	6.8, 5.7
<i>M. hapla</i>	Race A	15, 6.6, 5.8
	Race B	15.5, 6.1, 5.9
<i>M. incognita</i>	Race 1	5.6, 1.9
	Race 2	5.8, 4.8, 2.1
	Race 3	5.7, 2.1
	Race 4	6.2, 5.6, 2.1
<i>M. javanica</i>		6.7, 6

hapla Race A from *M. hapla* Race B (Table 1). The approximate size (in kilobases) of diagnostic repetitive DNA bands for each species and race studied are listed in Table 1, marked by arrows in Figure 1A, and drawn in Figure 1B. It should be noted that the diagnosis of *M. incognita* Race 1 and Race 3, which have similar repetitive DNA restriction fragment length patterns (Fig. 1, lanes 6 and 8), was confirmed by running EcoRI cut total DNA in a 1.5% gel at 25 V for 20 hours. These conditions gave greater resolution of shorter DNA fragments (at the expense of larger DNA fragments) and gave clear resolution of the approximately 1.9- and 2.1-kb repetitive DNA bands (gel not shown).

DISCUSSION

We demonstrated that direct analysis of genotype by detection of restriction fragment length differences in total DNA enables rapid (< 24 hours) differentiation of populations of four *Meloidogyne* species and their races. However, before DNA restriction fragment differences can be considered taxonomically useful, the nature and extent of variation in repetitive DNA restriction fragment lengths within a particular species and race must be determined. Hence, analysis of total DNA from many populations will be required before a particular restriction fragment length pattern can be considered diagnostic.

The ability of DNA restriction fragment length differences to separate races reflects its high sensitivity, compared with other biochemical methods. This high sensitivity is due to two properties of restriction en-

donuclease analysis, namely 1) the whole genome is examined—whereas protein, isozyme, and antigen analysis assay only coding DNA sequences, which are a small proportion of the total genome, and 2) a large number of DNA fragments are produced. For example, EcoRI has a six-base pair recognition sequence (GAATTC) which by chance would occur every 4^6 base pairs, and in a nematode with a genome size of 8×10^7 base pairs (e.g., *Caenorhabditis elegans*), restriction endonuclease digestion would generate 2×10^4 fragments. All these fragments are potentially useful as taxonomic characters for discriminating *Meloidogyne* populations.

Fortuitously, the *Meloidogyne* populations examined were readily separable by restriction fragment length differences in repetitive DNA sequences. Such differences in repetitive DNA are unusual at the race or strain level between populations of freely interbreeding amphimictic nematodes (2). This may reflect the peculiarities of reproduction in *Meloidogyne* spp. Most *Meloidogyne* species are believed to create genotypically distinct, reproductively isolated populations rapidly by mutation and clonal selection via parthenogenesis (11). Alternatively, if repetitive DNA restriction fragment length differences cannot distinguish all populations of interest, several more sensitive DNA analytical techniques exist which could prove useful. One such technique is DNA hybridization using species-specific cloned DNA fragments, which provide a positive or negative assay for species (1) and may prove useful for reproductively isolated *Meloidogyne* populations. In addition, the use of single copy DNA restriction fragment length differences, by agarose gel electrophoresis and hybridization of labelled cloned DNA fragments, has proved useful for the diagnosis of interbreeding populations of both free-living and animal-parasitic nematodes (1,10) and could be readily applied to *Meloidogyne* populations (our preliminary data on *Meloidogyne* support this). Single copy DNA restriction fragment length differ-

ences provide a more sensitive assay of genotypic divergence between populations since there are many more different single copy sequences than different repetitive DNA sequences. Use of single copy DNA restriction fragment length differences could be extended to detect gene flow between populations within amphimictic or meiotic parthenogenetic *Meloidogyne* populations.

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