Journal of Nematology 24(1):23–28. 1992.

Genomic RFLP Analysis of *Meloidogyne arenaria* Race 2 Populations

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Abstract: Traditional morphological methods of *Meloidogyne* identification have been unsuccessful in distinguishing three South Carolina, USA *Meloidogyne arenaria* race 2 populations—Govan, Pelion, and Florence. These populations differ greatly in reproductive rate and aggressiveness on soybean hosts. Total genomic DNA from eggs of each population was digested with the restriction endonuclease Eco RI and Southern hybridization analyses were performed with single-copy and interspersed multi-copy cloned probes. Probes were isolated from a genomic library of Eco RI, *M. arenaria* DNA fragments cloned into pUC8. One probe, designated pE1.6A, when hybridized to Southern blots of *M. arenaria* genomic DNAs, displayed an interspersed repetitive pattern, and the RFLPs distinguished the Govan population from the Pelion and Florence populations. Another clone, pE6.0A, carrying moderately repeated sequences, distinguished the Pelion and Florence isolates. This communication demonstrates the utility of genomic RFLP analysis for distinguishing populations of the same race within the same species. To test the possible utility of these moderately repeated sequence probes for detecting the presence of nematode DNA in DNA samples from roots inoculated with varying numbers of nematodes, dot blot hybridization analyses were performed. It is possible to detect as few as 30 nematodes per root sample with these cloned probes.

Key words: DNA, *Glycine max*, *M. arenaria*, nematode, restriction fragment length polymorphism.

Root-knot nematodes (*Meloidogyne* spp.) are aggressive plant pathogens of many economically important crops in South Carolina (SC). The extent of damage often depends on the host cultivar and nematode species or race. Four species of economic importance in the state include *M. arenaria* (Neal) Chitwood, *M. hapla* Chitwood, *M. incognita* (Kofoid & White) Chitwood, and *M. javanica* (Treub) Chitwood. Identification of these species and especially races is difficult, time-consuming, and often subjective. Recently, *M. arenaria* has been identified more frequently in SC on important crops such as soybean and tobacco (6). In field and microplot tests, populations of *M. arenaria* race 2 differ significantly in aggressiveness and reproductive rates on selected soybean cultivars. These populations, however, are identical in perineal pattern morphology, reproduction on differential hosts, chromosome counts of immature oocytes, and esterase phenotypes on thin-slab polyacrylamide gels (3).

Because of the shortage of effective and inexpensive nematicides, identification of pathotypes is essential in plant breeding and in making profitable crop management decisions. The emerging technologies of recombinant DNA and restriction fragment length polymorphism (RFLP) studies provide reasonably simple, sensitive, and reproducible methods for distinguishing species, biotypes, and even individuals within a population.

Initial molecular approaches to nematode species and race identification have focused on three sequence classes of the eukaryotic genome. These are mitochondrial DNA (mtDNA), nuclear high copy DNA, and nuclear low copy DNA. Within the last several years, a number of reports have illustrated the use of molecular technologies for distinguishing species and biotypes of various nematodes on the basis of mtDNA restriction fragment polymorphisms (7,11–13). Other researchers have utilized complete genomic DNA for the distinction of races and species (1,4,8). In this investigation, we demonstrate the utility of cloned genomic fragments as probes for distinguishing populations of *M. arenaria* race 2. These populations have been described previously (3) and are indistinguishable by traditional methods.

Received for publication 22 March 1991.

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Materials and Methods

Isolation of nematode DNA: Three *M. arenaria* race 2 SC populations (Govan, Florence, and Pelion) were cultured on tomato (*Lycopersicon esculentum* L. cv. Rutgers) at 25–30 C for 45 days in Wisconsin water tanks in the greenhouse facilities at Clemson University. Eggs were harvested from each population with the NaOCl method (5) and homogenized for 5 minutes in a chilled 2-ml ground glass handheld homogenizer containing equal volumes of eggs and pH 8.0 DNA isolation buffer (0.2 M NaCl, 0.05 M Tris-HCl, 0.01 M EDTA) [ethylenediaminetetraacetic acid], 0.2% SDS [sodium dodecyl sulfate]).

Total genomic DNA was isolated from eggs by phenol-chloroform extraction and ethanol precipitation (9). The DNA was then purified by RNAse and proteinase K digestions or by ethidium bromide–cesium chloride gradient centrifugation; the DNA was then digested completely with the restriction endonuclease Eco RI (Promega Biotech, Madison, WI) (9). Restriction digests were electrophoresed on 0.8% agarose gels. Electrophoresis was conducted in tris-acetate-EDTA buffer (TAE) (9), and DNA was stained by ethidium bromide (0.5 μg/ml) and visualized on an ultraviolet transilluminator. Lambda DNA digested with Hind III was included to determine fragment size.

Construction of pUC8 partial genomic library: A shotgun pUC8 library of *M. arenaria* Govan DNA digested with Eco RI was constructed by calcium chloride-rubidium chloride transformation of *E. coli* strain JM83 (9). Cells containing plasmids with inserts were selected after plating on plates containing 100 μg/ml ampicillin and 50 μl of 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), and 20 μl of 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Selected clones were stored at −20 C in titre plates containing 75% LB media and 25% glycerol. Plasmid DNA from these clones was prepared with standard mini-lysate procedures (9).

Identification of clones carrying multi-copy sequences: For this purpose, clones were selected at random from the library and their plasmid DNAs were isolated. Individual plasmid samples were digested with Eco RI to release the insert, and digestion products were electrophoresed on 0.8% agarose gels, Southern blotted and hybridized with ^{32}P nick-translated *M. arenaria* genomic DNA. In each screening experiment, the insert of a clone known to be extensively repeated in the genome as determined in a genomic Southern analysis was included as a standard for comparative purposes. All loadings were standardized internally by hybridization intensity to the vector in each case. In our similar experiments in other systems, it is not uncommon for genomic DNA as a probe to show some limited homology to pUC 8 under our hybridization conditions.

Inserts of selected plasmids with multicopy sequences were electroeluted from agarose gels and radiolabeled with α-[^{32}P]dCTP by nick translation or random-priming oligo-labelling methods (9). Radiolabeled fragments were hybridized to Southern blots (Hybond-N transfer membranes, Amersham, Arlington Heights, IL) of Eco RI-digested genomic DNA of the three *M. arenaria* populations. Hybridizations were performed at 65 C for 24 hours followed by four washes of 2× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) for 2 hours at 65 C and one wash of 0.3× SSC for 30 minutes at 65 C. Filters were exposed to X-ray film using a Dupont Cronex intensifying screen. Filters were rehybridized up to three times after stripping in a 50% formamide, 20× SSPE, 20% SDS solution at 65 C for 45–90 minutes with a 1-minute rinse at room temperature in 0.1× SSC, 0.1% SDS solution.

Quantitative dot-blot analysis: Soybeans (cultivar DP506) were germinated on germination paper. After 48 hours, seedlings were transferred to another piece of germination paper, and *M. arenaria* second-stage juveniles (J2) were pipetted directly onto the seedlings (10). Inoculation rates

were 0 (uninfected control), 50, 100, 200, and 400 J2 per seedling. After 48 hours, seedlings were collected and DNA was extracted following the same method used with the *M. arenaria* eggs, except the roots were ground in a chilled mortar. Total DNA from inoculated roots, control roots, and *M. arenaria* eggs was spotted equally onto two identical Hybond-N filters. Southern hybridizations were then performed on the dot blots with a highly repeated *M. arenaria* probe containing a 1.5-kb insert (designated pE1.5A) and pSR1.1 (a gene clone for the soybean 25S ribosomal subunit, a gift from R. Meagher) as a DNA loading control. After inoculation, a subset of roots was stained histochemically and J2 were counted under a microscope. The average number of counted nematodes was then compared to the inoculation rate for calculation of the percentage of infection. For comparative purposes, each nematode hybridization signal was expressed as a ratio of nematode signal to that of the pSR1.1 to correct for any differences due to sample loading.

Results and Discussion

Two strategies can be used to search for DNA polymorphism in *Meloidogyne* populations. In one case, a number of clones carrying low copy number sequences can be used as probes on Southern blots of restriction-digested nematode DNA samples, utilizing a reasonable number of restriction enzymes with each probe. Another approach is to identify clones that contain moderately repeated sequences and then use these "fingerprint" type clones as probes on Southern blots of restriction enzyme-digested DNAs. In the first case, a fine-structure analysis of a particular region reveals polymorphism at particular restriction sites, whereas in the other case a greater portion of the genome is examined but less detail is obtained on any particular region. For our initial efforts, we utilized the second approach. For this purpose, clones carrying moderately repeated sequences were sought using a prescreening assay. In this assay, *M. arenaria* genomic DNA was used as a probe on a Southern blot of restriction-digested plasmid DNA clones (Fig. 1). Cloned inserts that displayed hybridization signal in this analysis were chosen for further study. Hybridization to the vector sequences was used to standardize the gel loadings internally.

One cloned probe containing a 1.6-kb insert (designated pE1.6A), when hybridized, displayed an interspersed repetitive pattern, and the RFLPs present distinguished populations within a race (Fig. 2A). Specifically, the Govan population was distinguished from the other populations. In field, microplot, and greenhouse tests, the Govan population has a higher reproductive rate and produces larger galls on soybean hosts than the Florence and Pelion populations (3). Another clone containing a 6.0-kb insert (designated

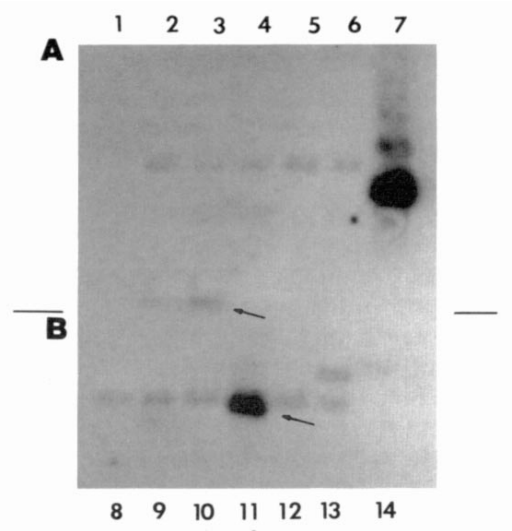

Fig. 1. Autoradiogram of Southern hybridization of Eco RI-digested plasmid DNAs from a shotgun library of *M. arenaria* DNA, hybridized with ^{32}P-labelled, total genomic *M. arenaria* race 2 Govan DNA. Digested plasmid DNA preparations were loaded in two places on the gel to allow a greater number of samples to be screened. The line marks the second set of wells. Lanes 1–6A and 8–13B were uncharacterized plasmids, Lane 7A and 14B were undigested multi-copy and single-copy standards, respectively. Arrows denote inserts carrying multi-copy sequences. The hybridizing common fragment in lanes 1–6 and 8–13 is the vector that shows a limited homology to the probe.

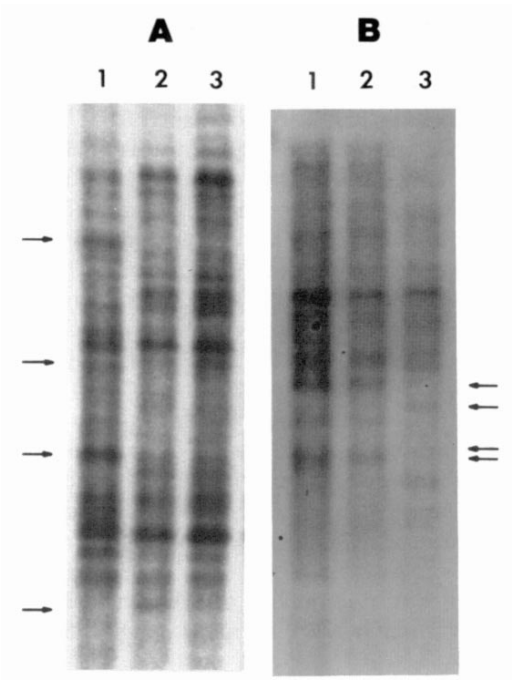

FIG. 2. Autoradiogram of a Southern hybridization using repeat sequence probes on Eco RI-digested DNAs of South Carolina *M. arenaria* populations. Lane 1: Govan. Lane 2: Pelion. Lane 3: Florence. Arrows mark band positions where obvious differences occur among the profiles. A) Probed with pE1.6A. B) Probed with pE6.0A.

pE6.0A) distinguished the Pelion and Florence populations (Fig. 2B). These patterns are reproducible and clearly demonstrate the utility of this type of cloned probe for distinguishing populations of a particular race of *M. arenaria*. It is interesting to note that these differences occur in populations closely related geographically.

These *M. arenaria* populations originated from composite field samples in order to reflect naturally occurring genetic diversity. Therefore, the question of individual polymorphism cannot be answered by examining these populations. Single egg-mass isolates of each of the above *M. arenaria* populations are being cultured in order to address this question and to examine the influence of host selection pressure on the genetic structure of these populations.

One of the potential applications of RFLP technologies is the use of molecular probes for determining the number and type of nematodes in field populations. We have tested the use of genomic repeat DNA probes in a dot blot analysis to determine if *M. arenaria* larvae could be quantified on roots of inoculated plants. No hybridization occurred when DNA of uninfected soybean roots was probed with a nematode probe (Fig. 3, lane C). *Meloidogyne arenaria* DNA (no soybean DNA) showed a strong signal with the nematode probe and some homology to the soybean ribosomal probe (pSR1.1). Ribosomal genes are highly conserved; therefore, strong homology between diverse organisms is not uncommon. The dot blot signals compare favorably with the actual histochemical counts of the nematodes in the soybean roots (Table 1). Infection of all plants would not be expected, and infection for this experiment was approximately 50% (number of nematodes in the roots compared to number used for infection). The number of nematodes within roots increased with increased inoculation rate in approximately the same proportion to the mean intensity ratio of the dot blot signal.

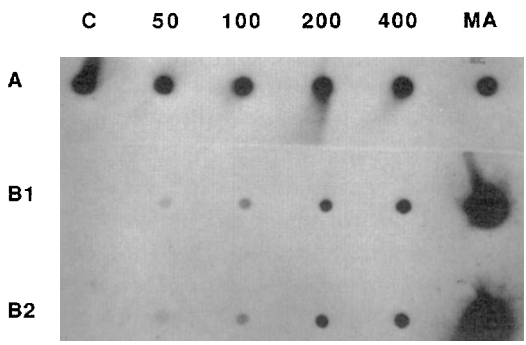

FIG. 3. Autoradiogram of a quantitative dot-blot estimation of soybean root infection by *M. arenaria* juveniles with multi-copy *M. arenaria* probe (pE1.5A). Columns: (C) control uninfected root DNA, (50) DNA from roots inoculated with 50 nematodes/root, (100) DNA from roots inoculated with 100 nematodes/root, (200) DNA from roots inoculated with 200 nematodes/root, (400) DNA from roots inoculated with 400 nematodes/root; (MA) *M. arenaria* DNA control. Rows: (A) DNA Samples probed with pSR1.1 soybean 25S ribosomal clone, (B1 and B2) replicates probed with pE1.5A, a highly repeated sequence clone.

TABLE 1. Integration optical densities from autoradiograms of DNA from uninfected soybean roots, soybean roots infected with 50, 100, 200, or 400 *Meloidogyne arenaria* second-stage juveniles, and *Meloidogyne arenaria* DNA, probed with a ribosomal gene clone from soybean (pSR1.1) and a repeated clone (pE1.5A) taken from a pUC8 partial genomic library of *Meloidogyne arenaria* DNA.

	Soybean control	50	100	200	400	*M. arenaria* control
			pSR1.1			
Replicate 1	61,726.9	7,599.9	7,639.6	13,201.3	12,000.0	10,343.6
Replicate 2	29,483.6	9,112.0	8,702.2	10,568.2	11,256.0	8,209.0
Mean	45,605.3	8,355.9	8,170.9	11,884.7	11,628.0	9,276.3
			pE1.5A			
Replicate 1	0.0	265.8	691.4	2,259.1	3,339.3	492,976.3
Replicate 2	36.8	342.1	634.2	2,021.6	2,573.9	672,569.1
Mean	18.4	303.9	662.8	2,140.3	2,956.6	582,772.7
			Nematodes per seedling			
		31†	54	106	196	

† Seven to nine seedlings were histochemically stained and counted under a microscope to determine the average number of second-stage juveniles penetrating the roots.

RFLP analysis is a powerful tool for the measure of genetic relatedness. This approach has been used to distinguish species of *Meloidogyne* and, more recently, populations (2). Examining mitochondrial DNA organizations, Powers et al. (12) demonstrated that *M. incognita, M. arenaria, M. hapla,* and *M. javanica* could be distinguished clearly through use of only two restriction enzymes. More recently, Powers and Sandall (11) have reported the use of restriction enzyme analysis coupled with end labeling to demonstrate interspecies as well as intraspecies polymorphisms among *Meloidogyne* populations. Although the four races of *M. incognita* were distinguishable, the *M. arenaria* races were indistinguishable from each other.

Similar analyses demonstrated the utility of RFLP approaches for the distinction of *Heterodera glycines* and *H. schachtii* Schmidt (13), which are difficult to distinguish with morphological characteristics. In these studies, molecular hybridization of cloned probes was an extremely important tool in assigning restriction fragment relationships (homologies) in the different species patterns of mitochondrial DNA. The results clearly demonstrated the sensitivity of molecular hybridization technologies in estimating species divergence.

Restriction enzyme digestion of genomic DNA produces an unresolvable smear of fragments, but sequences with moderate to high repetition can be resolved as bright, distinguishable bands superimposed on the unique sequence fragments. Curran et al. (4) demonstrated the use of restriction enzyme digestion patterns of total genomic DNA as diagnostic for distinction of races and species of *Meloidogyne* populations.

Bolla et al. (1) utilizing genomic RFLP analysis demonstrated that host specific pathotypes of *Bursaphelenchus xylophilus* differed in genomic organization. In this study, restriction digestion analysis was coupled to Southern hybridization with genomic DNA as a probe. Because of the high copy number of repeat sequences, distinct fragments resulting from this approach distinguished species and races. Similarly, *H. glycines* races were distinguished by inspection of restriction-digested total genomic DNA isolates (8).

Multi-copy probes may have application as field diagnostic tools. We have tested the sensitivity of these probes for determining the level of infection of roots by *M. arenaria* under controlled conditions. With our assay we can easily determine the presence of 30 nematodes per root. For this approach to have any field application, one must be able to detect a single nematode and identify it to the species level. Currently, we have no probes specific for *M. arenaria* DNA over the other *Meloidogyne*

species but are searching our cloned material for such sequences. We are also examining the use of PCR technologies using oligonucleotides constructed to the repeat element in pE1.6A. Because this sequence appears to be quite variable, it may give species-specific PCR products.

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