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Intra- And Interpopulation Genome Variation In *Meloidogyne Arenaria*[1]

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Abstract: The genetic heterogeneity of two *M. arenaria* race 2 populations (designated Pelion and Govan) was examined using RFLP analysis of 12 clonal lines established from single egg masses (six distinct clonal lines from each population). These populations are essentially identical by traditional biochemical and race identification schemes; however, the Govan population is more aggressive than the Pelion population, producing larger galls and exhibiting greater reproductive capabilities on many soybean cultivars and experimental accessions. Variation at the genomic DNA level was examined using probes representative of expressed DNA sequences present in the eukaryotic genome. Ribosomal DNA, interspersed repeated sequences, and cDNA probes were tested for detection of polymorphism within and between single egg mass lines of each population. Cloned cDNAs and ribosomal intergenic spacer sequences detect polymorphism both within and between populations, demonstrating the usefulness of these sequence classes for molecular genetic analysis of population structure and genome evolution.

Key words: biochemical systematics, DNA polymorphism, *M. arenaria, M. hapla, M. incognita, M. javanica,* nematode, RFLP analysis, root-knot nematode.

Plant-parasitic nematodes are major agricultural pests. Worldwide, the estimated crop loss attributable to these pests is 12.3% (21). In the staple crops, the loss ranges from 3.3% (rye) to as much as 19.7% (banana), while in other economically important crops it can range from 8.2% (tea) to 20.6% (tomato) (21).

Although the most cost-effective nematode control measure is the use of resistant plant cultivars, for many plants there are currently no sources of resistance (8). In addition, in many cases nematodes appear to adapt readily to resistant varieties. Plant genetic resistance to some nematodes appears to be a complex interaction between plant resistance genes and nematode genes for parasitism (25). Plant species may exhibit a number of major and minor resistance genes, each matched to nematode alleles for parasitism. As stated by Triantaphyllou (25), "These nematodes have evolved a balanced type of parasitism, presumably through co-evolution with their hosts. As a result of co-evolution of the genomes of hosts and parasites, there is extensive complementarity between genes for resistance and genes for parasitism."

A prominent model for co-evolution of host and pest systems is the "gene for gene" theory originally proposed by Flor, which states that for every plant resistance gene there is a corresponding virulence gene in the pest (11). This model has been applied extensively to agricultural systems (24) and to fungal pathogen-plant interactions in particular. Gene-for-gene (GFG) relationships have also been postulated to exist for phytophagous insects (7) and phytopathogenic nematodes (8,16,25). The relevance of the GFG model has not been tested rigorously in many host-parasite interactions because traditional genetic studies are not possible where one or both interacting parties have no sexual cycle (24).

Pioneering work in potato cyst nematodes using genetic crosses and resistant cultivars has shown that virulence can be described genetically in nematodes that can be crossed (15,16); however, this approach is not applicable to the mitotically parthenogenetic *Meloidogyne* species, which are major agricultural pathogens. As a result, the genetics of host-parasite interactions in these systems has been de-

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scribed only by studies of resistance genes in the plant host.

Genetic variability at the population level most likely plays a critical role in the maintenance of the species under changing host plant selective forces. Molecular marker based technologies such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) have been successfully used for detection of genetic variability in plant-parasitic nematode populations (2,3,5,6, 17–19,22,29–32). Among *Meloidogyne* populations, RFLP variability is present and easily detected in both repetitive and low copy nuclear sequences (3,5,18); however, variability within a population for these sequences has not been extensively investigated. In addition, only recently has interpopulation variability of known gene coding sequences been reported (32). Intrapopulation variability of coding regions is particularly important as they necessarily include sequences responsible for virulence, so this variability could provide a genetic resource for responding to selection pressure from resistant plant cultivars. Although we cannot define genetically distinct virulence loci in these apomictic pests by traditional genetic approaches, we can assess the level of genetic variability in the populations and investigate the influences of resistant host plant varieties on the genetic composition of the nematode population. In addition, with these techniques, we can assess the fitness of genetically distinct components of the population under selection pressures exerted by plant genetic resistance. However, to initiate these studies, sufficient numbers of polymorphic molecular markers must be identified in the population under study. We have shown previously that a clone (pE1.6A) containing the 3′ end of the 26S (rRNA) gene and sequences in the intergenic spacer between the 26S rRNA gene and the 18S rRNA gene (13) detected polymorphism between *M. arenaria* populations (3). Vahidi et al. (26–28) have described the ribosomal repeat region in *M. arenaria*, and they have demonstrated that the intergenic spacer contains the 5S gene and a 129 basepair (bp) tandemly repeated sequence that varies in copy number among ribosomal repeat units.

In this study, we further examine the genome variability of two populations of *M. arenaria* using RFLP analysis of clonal lines established from single egg mass isolates and probes derived from gene coding regions. We present evidence that gene coding sequences (cDNAs and rDNA sequences) detect significant polymorphism between and within the Pelion and Govan populations, enabling the genetic distinction of individual clonal lines within populations.

Materials and Methods

Nematode maintenance: Meloidogyne arenaria race 2 populations from Govan and Pelion, South Carolina, were cultured on tomato (*Lycopersicon esculentum* L. cv. Rutgers) at 25–30 C in a greenhouse. The Pelion isolate was a field population that was cultured for approximately 4 years, and the Govan population was a field population that was cultured for approximately 1 year. Clonal lines of both populations were established by collecting single egg masses from infected tomato roots and potting each egg mass individually with a tomato seedling in sterile soil. After 45–60 days, the plant was replaced with a young seedling and fresh soil was added. The individual clonal lines were allowed to reproduce until enough eggs were produced to extract a usable quantity of DNA (usually achieved in three generations). *Meloidogyne hapla, M. incognita, and M. javanica* eggs came from laboratory cultures generously provided by R. S. Hussey, University of Georgia, Athens. Eggs were collected using the NaOCl method (14) and layered on a 50% sucrose cushion and centrifuged at 900 g for 10 minutes. Eggs were collected from the interface and washed and pelleted in water at 900 g. The eggs were then frozen overnight at −80 C.

DNA isolation and analysis: Total genomic DNA was extracted by homogenizing ap-

proximately 500,000 thawed eggs in 1 ml DNA isolation buffer (100 mM Tris-HCl pH 8.5, 50 mM EDTA, 200 mM NaCl containing 1% sodium dodecyl sulfate [SDS] and 0.8 mg/ml proteinase K) (22) in a chilled, 2-ml ground-glass, hand-held homogenizer for 1 minute on ice. The extracts were incubated at 50 C for 1 hour. DNA was isolated by adjusting the samples to 1.5 g with extraction buffer and adding 1.5 g cesium chloride (CsCl) and 150 μl of a 10-mg/ml ethidium bromide (EtBr) solution. The final volume was adjusted to 3 ml using a 1:1 (w:w) CsCl-H_2O solution. The samples were centrifuged overnight at 60,000 rpm in a TLA 100.3 rotor in a TL-100 (Beckman) ultracentrifuge. The resultant DNA bands were visualized with a UV transilluminator and the DNA removed from the tube with a P-200 pipetter (Gilson). The EtBr was removed by repeated extraction with butanol saturated with TE buffer (0.01M Tris-HCl pH 8.0, 0.001M EDTA), and CsCl was removed by dialysis (three changes of 1,000 ml TE for 2 hours per change). DNA was digested to completion with restriction endonucleases (either EcoR I or Hind III; Promega Biotech, Madison, WI) (20). Digested DNA was electrophoresed in 0.8% agarose in Tris-acetate-EDTA (TAE) buffer (20), stained in 0.5 μg/ml ethidium bromide, and visualized on a UV transilluminator. Gels for hybridizations were blotted (23) using nylon filters (Hybond-N, Amersham, Arlington Heights, IL).

An EcoR I genomic library for *M. arenaria* was constructed in the plasmid pUC8 and recombinants grown on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal 50 μg/ml), isopropyl-thio-β-D-galactoside (IPTG 160 μM), and ampicillin (0.1 mg/ml). Recombinants were randomly selected and classified as repeated (multi-copy) or low copy based on hybridizations with total genomic *M. arenaria* DNA (3).

Meloidogyne incognita cDNA libraries were constructed in the vector lambdaZap (Stratagene) with poly A^+ mRNA of eggs or mature females, using a one-tube cDNA synthesis kit (random primers for females and oligo dT for eggs, Pharmacia). RNA was extracted from approximately 2 ml of eggs or mature females using a phenol-SDS-LiCl procedure (1); however, the LiCl precipitation step was omitted, and the extract was precipitated directly with ethanol. The poly A^+ fraction was obtained from the precipitated nucleic acids by oligo dT cellulose column chromotography. The cDNA libraries of mature females contained 20,000 primary recombinants; the egg cDNA library contained 200,000 primary recombinants.

Antibody screening experiments or differential hybridization was used to select cDNA clones from the libraries. Clones pCRH3 and pCRH5 were selected from an initial screening of the mature female library with monoclonal antibodies to nematode stylet exudates in collaboration with R.S. Hussey, University of Georgia, Athens. Clone MiJ2E6 was selected from the egg library by a differential hybridization with cDNA probes constructed to juvenile and egg RNA extracts. LambdaZap clones were converted into pBluescript plasmids following the manufacturer's protocol.

Cloned insert DNA was labelled using the random priming method (9), except that 15 μCi [^{32}P] dCTP (3,000 Ci/mmole) was used per reaction instead of 50 μCi dCTP. Hybridizations were conducted at 65 C overnight in 6x SSPE (1.08 M NaCl, 0.06 M sodium phosphate, and 6 mM EDTA pH 7.7), 10× Denhardt's, 1% SDS, and 20 μg/ml boiled salmon sperm DNA (20). Filters were rinsed four times in 2 × SSPE or 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) at 65 C for 20 minutes each and one rinse in 0.3 × SSPE or 0.3 × SSC at 65 C for 20 minutes. Filters were wrapped in plastic film and exposed at −80 C to Kodak X-Omat XAR-5 X-ray film with an intensifying screen until bands were detectable.

Results

To examine variation in or around gene coding sequences in *M. arenaria* popula-

tions and among the major *Meloidogyne* species, we used 10 different *M. incognita* cDNA sequences as probes for genomic RFLP analysis. These cloned expressed sequences had been selected from antibody or differential screening experiments designed to identify genes involved in parasite development and the establishment or maintenance of giant cells. Characterization of these clones included investigation of their variability within and between species. Within 12 *M. arenaria* single egg mass lines—six each from the Govan and Pelion populations—the cDNA probes hybridized to only a few fragments in the Southern analysis. Four of the 10 (including one not shown) detected variation between (Fig. 1A) or within (Pelion: Fig. 2A; Pelion and Govan: Fig. 3A) populations. In Figure 2A, the polymorphism is evident as the appearance of a new fragment in lanes one and three, with concomitant decrease of signal at the smallest fragment size. We interpret this result to suggest that there are at least two identical alleles for this region in the other lanes (2, 4–6) in this population; however, the egg mass lines in lanes 1 and 3 now display an RFLP in one of these alleles with one remaining unchanged. We do not think the new allele is resultant from a partial digestion because the same filter has been hybridized with other cloned sequences, and no evidence of partial digestion was seen in these lanes. Similarly, in Fig. 3A, two additional high-molecular weight bands are detected in lanes 1–3, 8, 9, and 11 that are not seen in the remaining lanes, where the corresponding sequences either are absent or are concealed in the common, strongly hybridizing band. The level of detectable polymorphism in or around gene sequences in the single egg mass lines is worth noting (four in 10 cloned cDNA inserts examined), particularly as these sequences are generally expected to demand

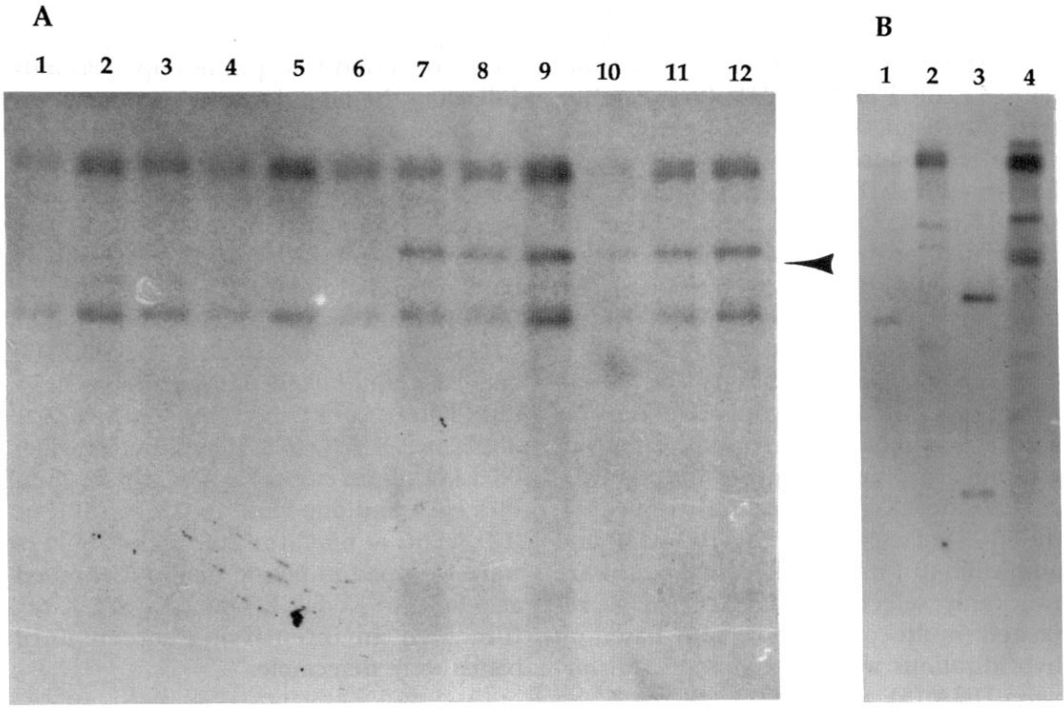

FIG. 1. Autoradiograms of Southern hybridizations using the *M. incognita* cDNA probe pCRH3 A) Hind III digested genomic DNA from clonal lines of Pelion (P) and Govan (G) populations of *M. arenaria*. Lanes: (1) P1; (2) P2; (3) P7; (4) P8; (5) P9; (6) P11; (7) G2; (8) G4; (9) G5; (10) G8; (11) G9; (12) G11. B) Hind III digested genomic DNA from: (1) *M. javanica;* (2) *M. hapla;* (3) *M. incognita;* (4) *M. arenaria.* Arrow indicates band present in Govan, but absent from Pelion lines.

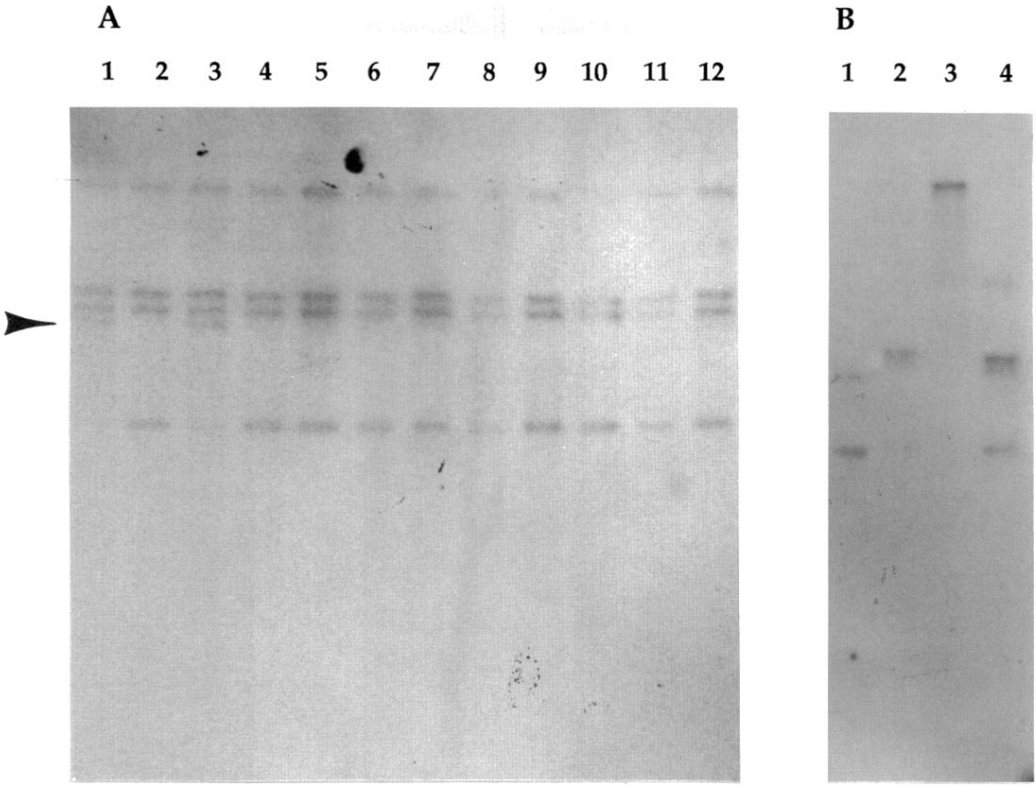

FIG. 2. Autoradiograms of Southern hybridizations using the *M. incognita* cDNA probe pCRH5. A) EcoR I digested genomic DNA from clonal lines of Pelion (P) and Govan (G) populations of *M. arenaria.* Lanes: (1) P1; (2) P2; (3) P7; (4) P8; (5) P9; (6) P11; (7) G2; (8) G4; (9) G5; (10) G8; (11) G9; (12) G11. B) EcoR I digested genomic DNA from: (1) *M. javanica;* (2) *M. hapla;* (3) *M. incognita;* (4) *M. arenaria.* Arrow indicates band present in Pelion lines P1 and P7, but absent from the remaining Pelion and all Govan lines.

higher sequence conservation. Hybridization patterns of the cDNA clones were also variable among the four major root-knot nematode species (Figs. 1–3B).

We subcloned the 3′ end of the 26S rRNA gene and a copy of a 129-bp repeat sequence present in the intergenic spacer from our clone pE1.6A, which had been shown previously to detect polymorphism between *M. arenaria* populations (3). Figure 4A shows a Southern hybridization of DNA from the single egg mass lines, digested with Hind III and probed with sequences specific for the 3′ end of the 26S rRNA ribosomal gene of *M. arenaria,* subcloned from pE1.6A. The 26S rRNA gene-specific probe detected interpopulation but not intrapopulation variation in these egg mass lines (Fig. 4A); however, when a probe specific for the 129 bp repeat sequence was hybridized on the same Southern blot, both interpopulation and intrapopulation variation were evident (Fig. 4B). We do not know if all of these variant repeat regions are present on ribosomal repeats or are at other non-ribosomal gene sites in the genome. Both probes detected many Hind III fragments of different sizes that were invariant among the egg mass lines and between the populations.

Probes carrying interspersed repeat sequences can be useful for these analyses since they target sequences dispersed at many sites, increasing the possibility of detecting polymorphisms. We have examined plasmid cloned inserts preselected as containing repeat sequences and have demonstrated polymorphism with these probes (3). One such clone, designated IG3, produced an interspersed repeated

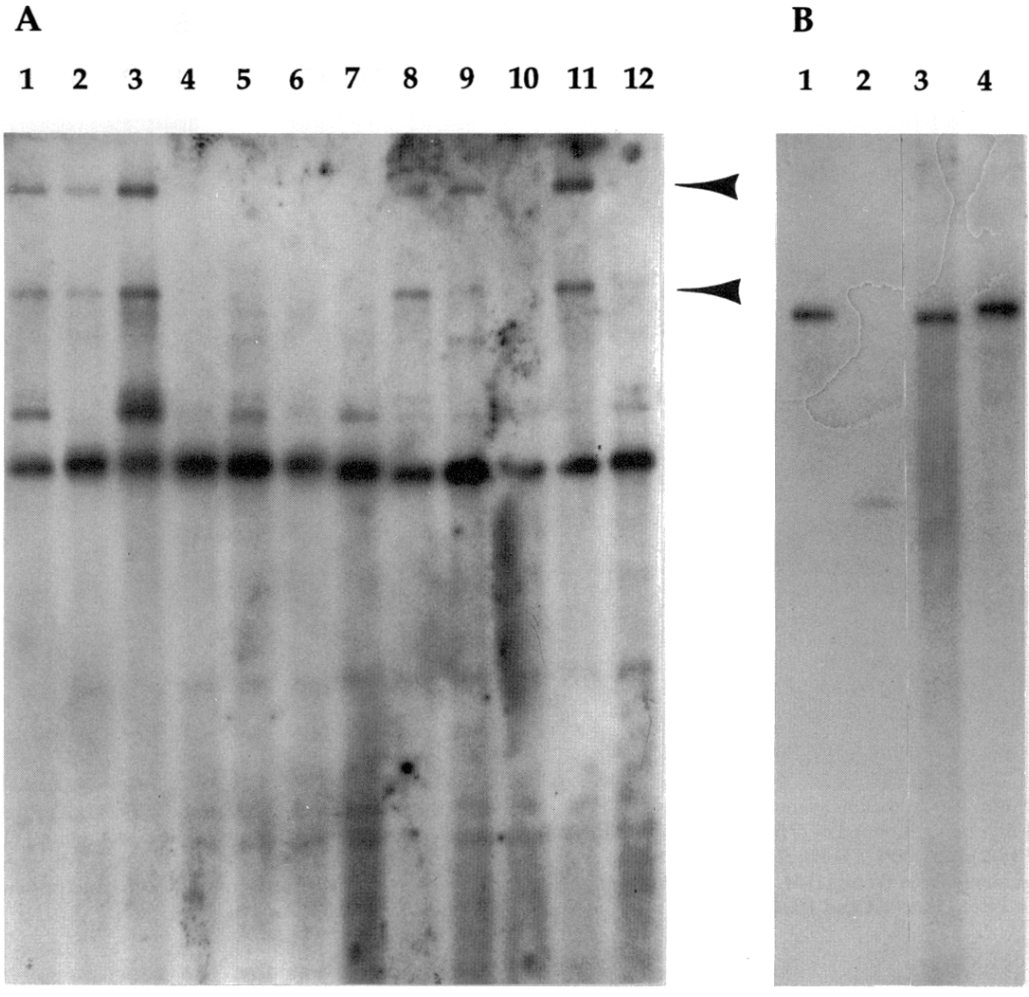

FIG. 3. Autoradiograms of Southern hybridizations using the *M. incognita* cDNA probe MiJ2E6. A) EcoR I digested genomic DNA from clonal lines of Pelion (P) and Govan (G) populations of *M. arenaria*. Lanes: (1) P1; (2) P2; (3) P7; (4) P8; (5) P9; (6) P11; (7) G2; (8) G4; (9) G5; (10) G8; (11) G9; (12) G11. B) Hind III digested genomic DNA from: (1) *M. javanica;* (2) *M. hapla;* (3) *M. incognita;* (4) *M. arenaria*. Arrows indicate bands present in Pelion lines P1, P2, and P7 and Govan lines G4, G5, and G9, but absent from the remaining Pelion and Govan lines.

pattern on the restriction digested samples of the different clonal lines. With this probe, polymorphisms were evident between but not within the populations (Fig. 5). Although these differences are reproducible, the patterns can be complex, with many faint background signals, and therefore difficult to score reliably on many different samples. For this reason, cDNA sequence variation could be more useful for distinguishing individuals within and between these populations.

DISCUSSION

The results presented in this communication demonstrate the utility of RFLP analysis for examining variation in different classes of genomic sequences of *M. arenaria* populations and for observing genome variation within a population as well as among species of root-knot nematodes. The two *M. arenaria* populations used in this study are both race 2 as determined by host differential tests (4); however, differ-

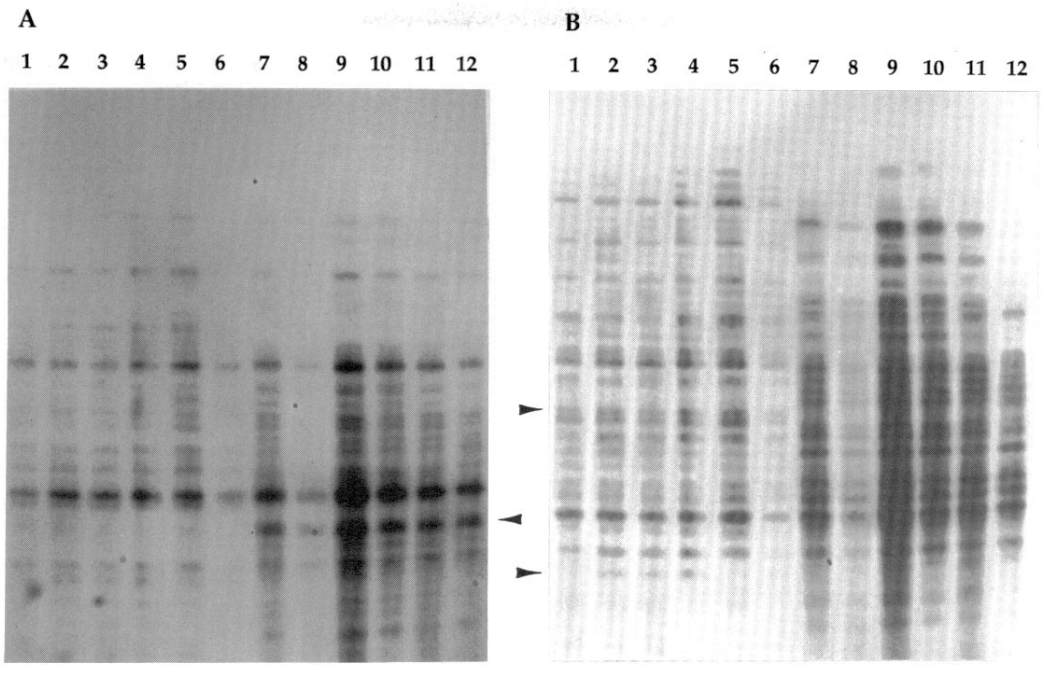

FIG. 4. Autoradiogram of a Southern hybridization of Hind III digested genomic DNA from clonal lines of Pelion (P) and Govan (G) populations of *M. arenaria* probed with (A) a fragment from the 3′ end of the *M. arenaria* 26S rRNA gene or (B) a 129-bp repeated sequence from the intergenic spacer region of the ribosomal repeat. Lanes: (1) P1; (2) P2; (3) P7; (4) P8; (5) P9; (6) P11; (7) G2; (8) G4; (9) G5; (10) G8; (11) G9; (12) G11. Arrows: (A) Band present in Govan, but absent from Pelion lines. (B) Band present in Pelion, absent from Govan lines (upper arrow); band present in Pelion lines P2, P7, and P8, but absent from remaining Pelion and all Govan lines (lower arrow).

ences between these two populations have been documented (4). The Govan population is more aggressive on a wider range of hosts than the Pelion population. The two populations are also distinguished by RFLP analysis (3); however, intrapopulation variation had not been previously documented. We established single egg mass lines from these populations to search for intrapopulation variation by RFLP analysis and to examine their clonal composition.

Tandemly repeated probes and probes of randomly selected low copy genomic sequences (unpubl. results) show few differences between or within populations. This result is perhaps not unexpected because these populations are very similar. Interspersed repeated probes demonstrate polymorphisms between populations and sometimes even within populations; however, analysis of such patterns can be difficult and subject to misinterpretation.

In the case of ribosomal genes, we have detected many variant forms within the individual lines. Most of these variant forms appear consistently throughout the clonal lines within a population. Because *M. arenaria* reproduces by mitotic parthenogenesis, it would appear that these variant forms are present in a single individual. On the population level, the other three species show a similar degree of variability in ribosomal RNA genes (results not shown), and a limited investigation of *M. incognita* and *M. javanica* also found that most variant forms of rRNA genes were shared between lines established from single egg masses (12). Ribosomal RNA genes in eukaryotes are usually both highly repeated and highly homogenized within a genome; on Southern blots, rDNA probes typically hybridize intensely to a limited number of bands, and the detection of variant forms requires overexposing the

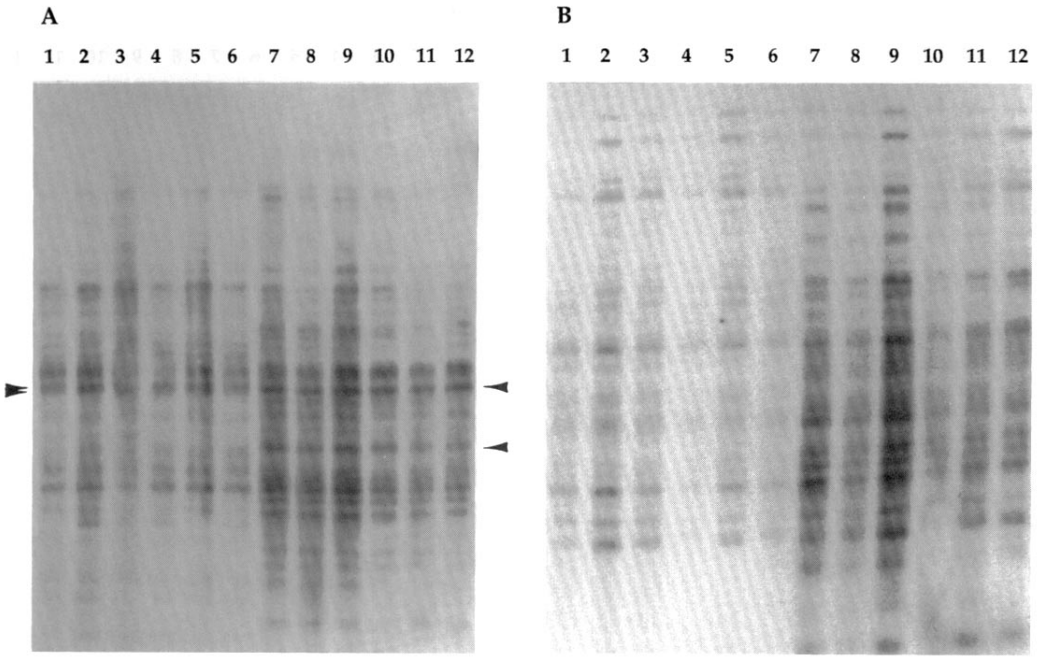

FIG. 5. Autoradiogram of a Southern hybridization of EcoR I (A) and Hind III (B) digested genomic DNA from clonal lines of Pelion (P) and Govan (G) populations of *M. arenaria* eggs using the *M. arenaria* interspersed repeated probe IG3. Lanes: (1) P1; (2) P2; (3) P7; (4) P8; (5) P9; (6) P11; (7) G2; (8) G4; (9) G5; (10) G8; (11) G9; (12) G11. Arrows: (A) Doublet band in Pelion, singlet in Govan lines (upper arrows); band present in Govan, but absent from Pelion lines (lower arrow).

autoradiogram. This is the case for *L. esculentum, Prunus persica* Batsch, *Glycine max* (L.) Merrill cvs. Deltapine 506 and Braxton, *Festuca arundinacea* Schreb. cv. Kentucky 31 and its endophyte *Acremonium coenophialum* Morgan-Jones and Gams, *Drosophila melanogaster,* and *Heliothis virescens* (13), as well as *Caenorhabditis elegans* (10) and *Panagrellus redivivus* (Georgi, unpubl.), among others. The degree of variation we have found in the major *Meloidogyne* species is highly unusual.

Interestingly, sequences in or around gene coding regions exhibit variation within (Figs. 2,3) and between (Fig. 1) these populations, as evidenced from the use of cDNA probes in our analysis. Of all sequences examined, these appear to hold the greatest promise for the reliable easy detection of genetic variability within these populations. Furthermore, cDNA sequences are of great interest as they necessarily include sequences required for parasitism. The degree of variability found with the cDNA probes used in the present study is particularly intriguing, because the probes were isolated in screens for genes involved in preparasitic development and feeding site induction. One of these probes (MiJ2E6, Fig. 3A) detected intrapopulation variation among Govan clonal lines, where we have not previously detected variation with interspersed repeated sequence probes. As anticipated, all the cDNAs we have tested hybridized to all four major Meloidogyne species: expressed sequences can be expected to be more highly conserved than "junk" DNA. Nonetheless, considering the inter- and intrapopulation variation, it is not surprising that these same cDNA sequences detect considerable polymorphism among the major species. We are currently converting these cDNA sequences to sequence tagged sites to enable detection of polymorphism by PCR in population genetic studies. We are also using these cDNAs to extract clones from a genomic library for an on-

going investigation into the nature of the variability of these sequences in the genome.

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