

Characterization of Species and Races of the Genus *Meloidogyne* by DNA Restriction Enzyme Analysis¹

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Abstract: Total DNA of three species of *Meloidogyne* spp., including four subspecific races of *M. incognita*, were digested separately with EcoR I, Cla III, and Hind III and probed with ³²P-labelled total genomic DNA from *M. incognita* race I in Southern hybridizations. Short exposures of Southern blots after Hind III digestion revealed patterns that were useful for separating the species. Race differences were seen after longer exposures. The DNA fragment patterns obtained were scanned with a laser densitometer and the data were subjected to principal coordinate and cluster analyses. The likelihood of cloning species and race-specific DNA probes is discussed.

Key words: cluster analysis, DNA, DNA probe, *Meloidogyne arenaria*, *M. incognita*, *M. javanica*, nematode, principal coordinate analysis, restriction fragment length polymorphism, RFLP, root-knot nematode, Southern blot.

Extensive recent efforts in plant nematology to use biotechnological methods for diagnosis of species and host-specific races have included monoclonal antibodies (9) to identify *Meloidogyne* spp. and cloned DNA probes to identify *Globodera* spp. (4). In addition, several studies of genomic and mitochondrial restriction site polymorphisms among populations (6, 10, 15, 16) have been conducted. In terms of speed and ease of use, the immunological methods have a distinct advantage over molecular biological methods, but the former have not been successful in detecting subspecific differences among nematodes. Accurate identification is economically important because it determines the cultivars specified in crop rotation or control programs.

Molecular biological methods should provide clear results and be rapid and inexpensive; it is likely that cloned DNA

probes will fulfill these criteria. The specificity obtainable with DNA probes permits discrimination among closely related populations, e.g., *Leishmania* complexes (1), and their sensitivity allows the detection of small numbers of parasites, e.g., one *Onchocerca gibsoni* microfilaria (8). Related methods, however, such as those described in this paper, should also be of value in that they could reveal information on interspecific or intraspecific genetic diversity and on the likelihood of specific probe isolation from DNA libraries. All of the molecular techniques described have been successfully applied to the identification of both human and animal parasites, protozoa as well as helminths (for review, see Barker [2]).

Restriction fragment length polymorphism (RFLP) of repetitive genomic DNA has been effective in discriminating a number of species and strains of plant-parasitic nematodes (6, 10). Use of this technique in conjunction with Southern (17) hybridization with a radiolabelled probe has been described only for pathotypes of the pine-wood nematode, *Bursaphelenchus xylophilus* (3). Furthermore, only one restriction enzyme has been used in studying genomic RFLP of *Meloidogyne* species (6). This study presents the results of using three restriction enzymes to investigate genetic diversity among single populations of three species and four host races of root-knot nematodes. A radiolabelled total genomic DNA probe is used to reveal any polymorphism and repetitive DNA frequency.

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MATERIALS AND METHODS

Nematodes: Populations of *Meloidogyne* species were originally obtained from the Department of Plant Pathology, North Carolina State University. They included *M. incognita* race 1 (NCSU #401), race 2 (NCSU #E1135), race 3 (NCSU #285), and race 4 (NCSU #401); *M. javanica* (NCSU #7-2) and *M. arenaria* race 1 (NCSU #351). Nematodes from these pure cultures were reared on *Lycopersicon esculentum* cv. Mon-eymaker in a greenhouse at 25 C. After 55 days, eggs and juveniles were collected by maceration and centrifugal flotation (12), cleaned on a sucrose gradient, and repeatedly washed in sterile 100 mM NaCl before freezing in a minimal volume of the same solution at 45 C.

DNA preparation and restriction endonuclease digestions: Total DNA from each population was extracted from approximately 33 μ l packed nematodes. The frozen pellet of eggs was ground to a fine powder with a mortar and pestle, cooled on dry ice. The powder was transferred to a 50-ml plastic tube (Falcon), 2 ml DNA extraction buffer (50 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0) was added, and the tube was shaken for 3 minutes at 37 C. Another 1.8 ml extraction buffer was added with 0.2 ml 10% sodium dodecyl sulphate (SDS), and the tube was shaken for 3 minutes at 37 C. Then 2 mg proteinase K (Boehringer Mannheim, Mannheim, Germany) was added, and the digestion proceeded for 3 hours at 37 C. The resultant solution was extracted three times with an equal volume of equilibrated phenol (11), and the aqueous phase was extracted once with chloroform : isoamyl alcohol (24:1). The RNA was removed by adding 700 ng Ribonuclease A (Sigma) and incubating for 1 hour at 37 C in an orbital shaker. Phenol and chloroform extraction was repeated, and DNA was precipitated from the final aqueous phase overnight at -20 C by addition of 0.1 volumes 3 M sodium acetate and 2.5 volumes cold absolute ethanol. The DNA was pelleted in a Corex centrifuge tube at 22,600 g at 4 C for 30

minutes, washed with cold 70% ethanol, and resuspended in 100 μ l TE buffer (100 mM Tris, 1 mM EDTA, pH 8.0). Nucleic acid concentration was determined spectrophotometrically at 260 nm.

Twenty micrograms of DNA from each nematode was digested overnight at 37 C with EcoR I (in the presence of spermidine), Cla I, and Hind III (Boehringer Mannheim) in the buffers suggested by the manufacturer and heated for 10 minutes at 65 C to stop the reaction.

Radiolabelling DNA and Southern blotting: Five micrograms of each digestion mixture was mixed with gel loading buffer (11), and the samples were loaded into wells of a horizontal 0.8% agarose gel (15 \times 25 cm) containing 0.8 μ g/ml ethidium bromide in 1 \times TAE buffer (11). Electrophoresis was performed at 2 V/cm in a 1 \times TAE running buffer for 16 hours. Hind III-digested lambda DNA (Boehringer Mannheim) was used for molecular weight markers.

DNA fragments were transferred from the agarose gel to nitrocellulose paper (Schleicher and Schuell) by capillary blotting (17) and hybridized with radiolabelled total genomic DNA from *M. incognita* race 1. The probe was labelled by nick translation (Boehringer Mannheim) with [α -³²P]dCTP (Amersham) to an activity of approximately 5 \times 10⁶ cpm/ μ g. Efficiency of uptake was determined by removal of unbound radiolabel with a Centri-Sep column (Advanced Laboratory Techniques, Southborough, UK) and precipitation with trichloroacetic acid. The specific activity of the probe was measured by scintillation counting. Southern blots were prehybridized by shaking with 20 μ g/ml boiled, sheared salmon sperm DNA in 4 \times SSC (0.6 M NaCl, 0.06 M sodium citrate, 4 \times Denhardt's solution [7], 0.1% SDS) for 3 hours at 65 C in a water bath. Just before hybridization, the radioactive probe was denatured by boiling for 10 minutes and maintained at 65 C during transfer to the hybridization chamber. After hybridization at 65 C for 16 hours, the filter was rinsed once with 0.1 \times SSC containing 0.05% SDS and then washed three times

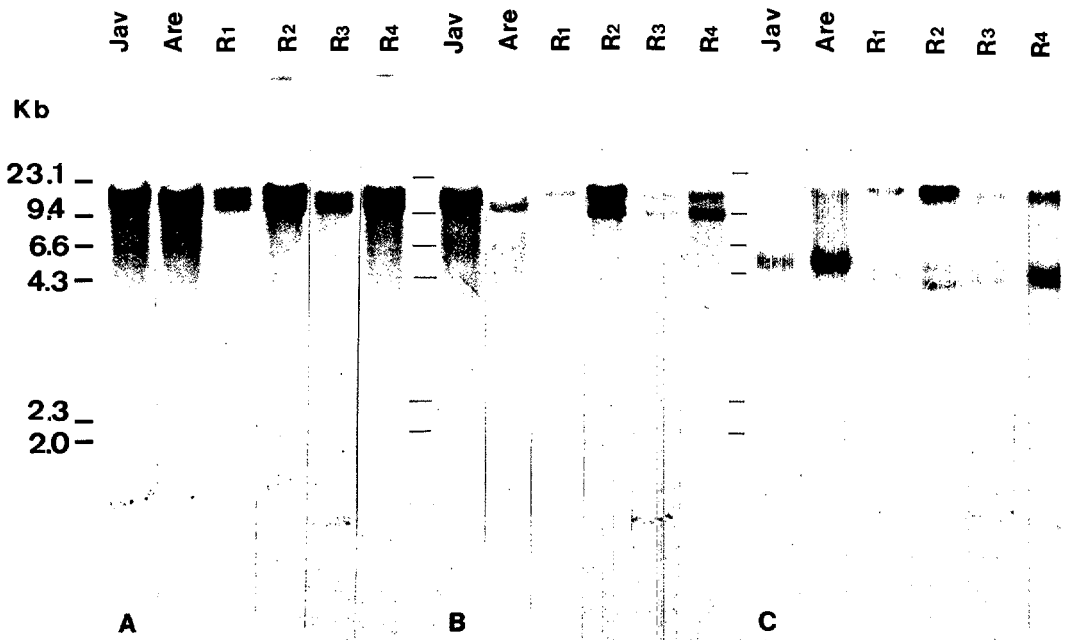


FIG. 1. Southern blot hybridization patterns of *Meloidogyne* spp. DNA with ^{32}P -labelled *M. incognita* race 1 DNA as a probe and 6-hour exposures following digestion with three restriction endonucleases. A) Cla I. B) Hind III. C) EcoR I. Jav = *M. javanica*, Are = *M. arenaria* race 1. R1–R4 = *M. incognita* races 1–4. Fragment sizes are shown in kilobases.

for 30 minutes each in the same buffer at 52 C. After air drying, the filter was exposed to Fuji X-ray film with an intensifying screen for 6, 24, and 72 hours in order to detect restriction fragment length polymorphism in high, moderate, and low frequency repetitive DNA fragments as determined by hybridization intensity. Autoradiographs after 6, 24, and 72 hours were scanned with an LKB Ultrascan XL densitometer.

Statistical analysis: Principal coordinate analysis (PCO) and cluster analysis as determined by average linkage was carried out with the Genstat 5 statistical program (13). The PCO was performed for each of the restriction enzyme digest patterns and for the combined total data. Cluster analysis was performed on total data and data from the 6-hour exposure only.

RESULTS

There was no cross-reactivity between the ^{32}P -labelled total DNA probe from *M. incognita* and total DNA extracted from tomato plants, the nematode hosts (data not

shown). Figures 1 and 2 show the repetitive fragment band patterns after short and long exposures, 6 and 24 hours, respectively. The combined hybridization patterns, after scanning with a laser densitometer of 6, 24, and 72 hours, are shown in Figure 3. The radiolabelled total DNA probe produced 16 discernable fragments after restriction enzyme digestion (Figs. 1–3). Characteristic band patterns allowed all but race 1 and race 3 of *M. incognita* to be discriminated from the other host races and species. As depicted in PCO analysis (Fig. 4), Hind III digestion was most successful for species and race identification in these populations. *Meloidogyne arenaria* and *M. javanica* were clearly separated from each other as well as from the races of *M. incognita*. Race 2 of *M. incognita* was consistently distinguishable from the other races of this species by two bands at 5.5 kb and 4.0 kb in the EcoR I digest. Races 1 and 3 were separable from each other by only one band in this digest and from race 4 by two bands at approximately 4.3 kb. Numerical investigation of these DNA hybridization pat-

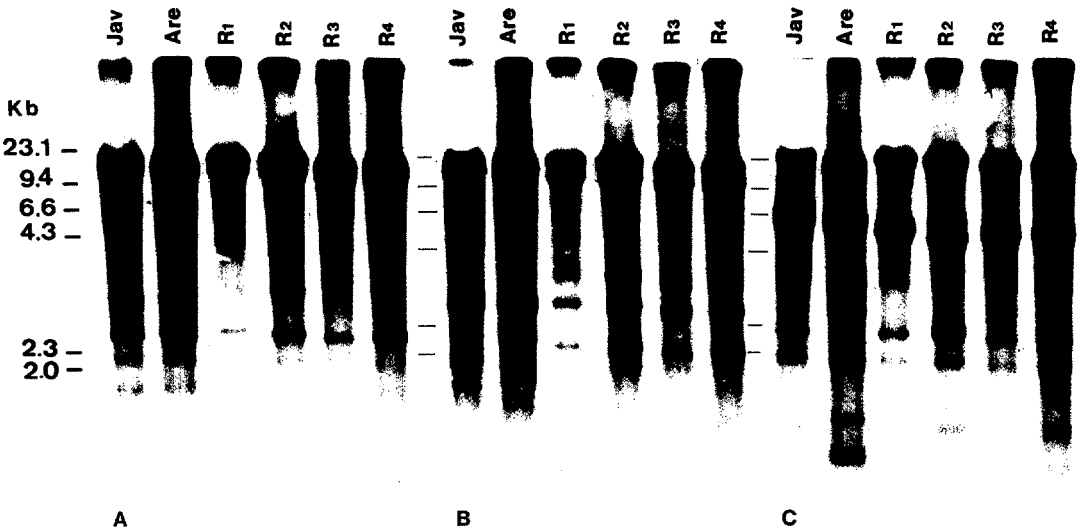


FIG. 2. Southern blot hybridization patterns of *Meloidogyne* spp. DNA with ³²P-labelled *M. incognita* race 1 DNA as a probe and 24-hour exposures following digestion with three restriction endonucleases. A) Cla I. B) Hind III. C) EcoR I. Jav = *M. javanica*. Are = *M. arenaria* race 1. R1-R4 = *M. incognita* races 1-4. Fragment sizes are shown in kilobases.

terns through cluster analysis of the total data (Fig. 5A) depicts the information in a different way and confirms the similarity between races 1, 3, and 4 with a very clear separation of race 2. *Meloidogyne javanica* and *M. arenaria* are more similar to each other than they are to *M. incognita*.

For rapid and clear identification of the species, high molecular weight RFLP (17-25 kb) observed after 6 hours exposure

proved very efficient (Fig. 1). Cluster analysis of the Hind III digest patterns after 6 hours exposure clearly shows separation of *M. arenaria* from *M. javanica* and of these species from *M. incognita*. (Fig. 5B). No race separation was possible at this period of exposure. This technique was used to identify two other populations of *M. incognita* from the Ivory Coast (Robinson and Fargette, data not presented).

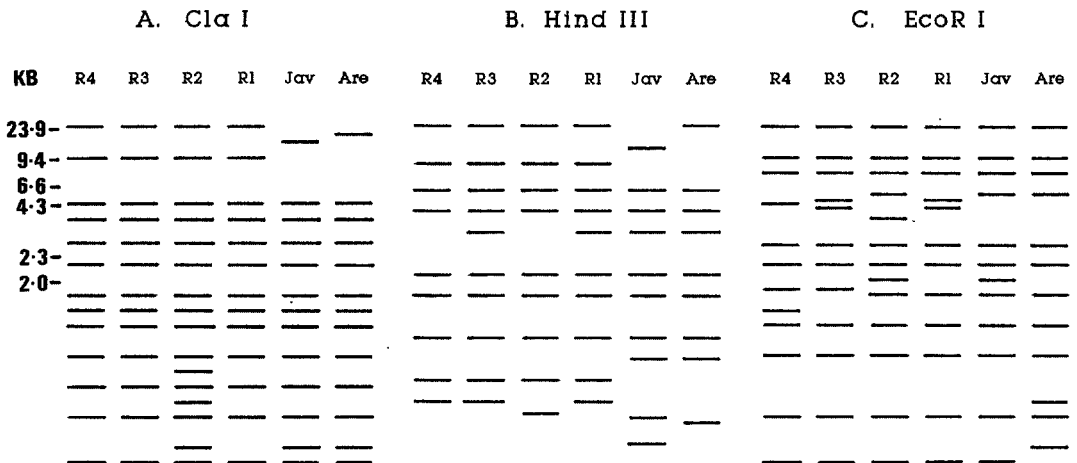


FIG. 3. Combined line drawing of repetitive DNA bands traced from Southern blot hybridization negatives after exposures of 6, 24, and 72 hours following digestion with three restriction endonucleases. A) Cla I. B) Hind III. C) EcoR I. R1-R4 = *M. incognita* races 1-4. Jav = *M. javanica*. Are = *M. arenaria* race 1. Fragment sizes in kilobases are indicated on the left.

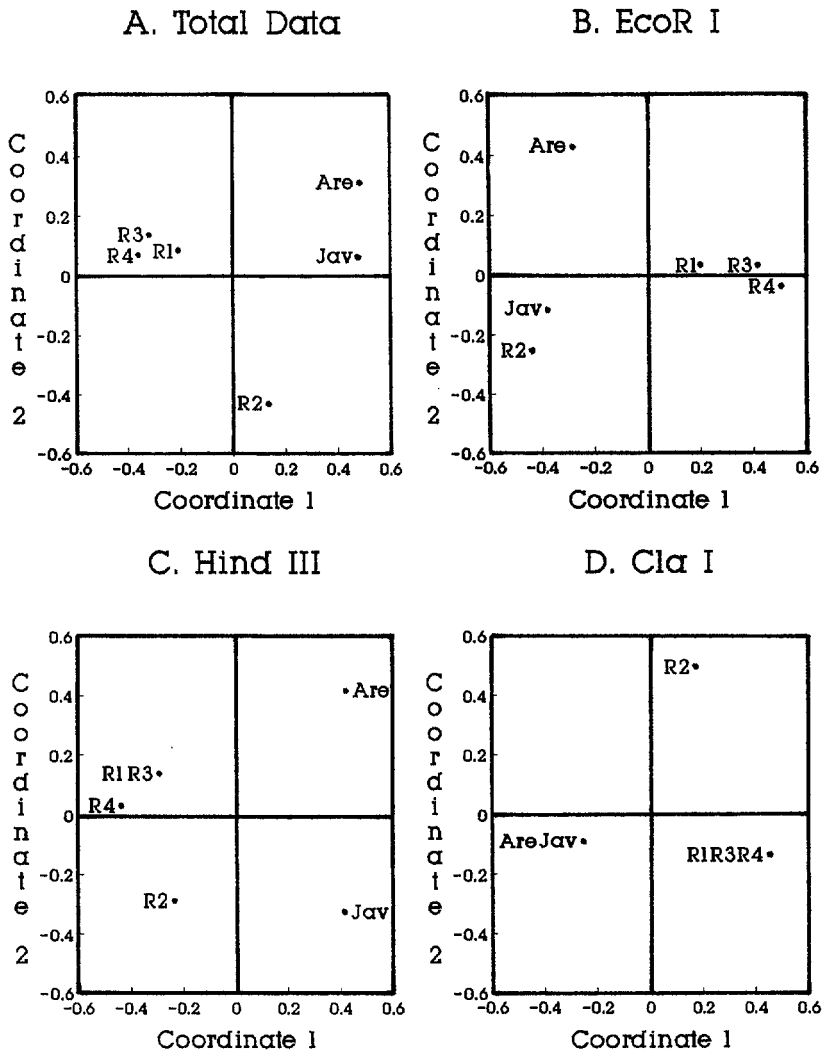


FIG. 4. Principal coordinate analysis showing separation in two dimensions of species and races of *Meloidogyne* spp. based on RFLP analysis. A) Combined restriction enzyme data. B) EcoR I digestion. C) Hind III digestion. D) Cla I digestion. Are = *M. arenaria* race 1. Jav = *M. javanica*. R1-R4 = *M. incognita* races 1-4.

DISCUSSION

The repetitive DNA RFLP patterns revealed in this work appear to be characteristic for the root-knot nematode species and host races examined. They are likely to result from divergence that has occurred in the entire genome. The use of total DNA as a probe was a convenient and efficient method of highlighting both moderately and highly repetitive DNA. This led to greater clarity and an increased number of bands, relative to ethidium bromide staining.

Preliminary work on the choice of probes established that there were no differences in the RFLP patterns obtained when either *M. incognita*, *M. javanica*, or *M. arenaria* DNA was used. Therefore, the polymorphism obtained probably results from relatively few site deletions or additions rather than from extensive sequence divergence. The stability of these patterns with respect to further populations of nematodes has not yet been tested. The pattern of hybridization from one population of *M. incognita* from the Ivory Coast was found

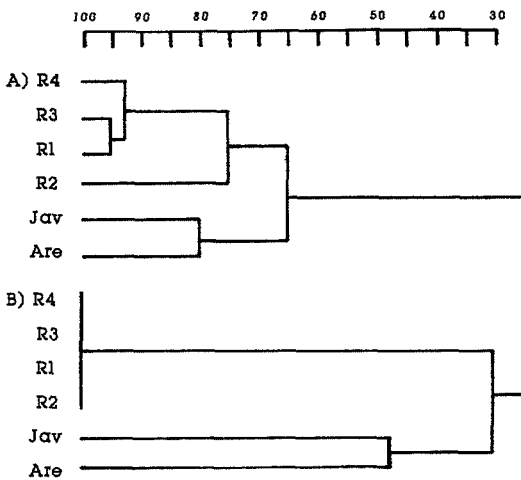


FIG. 5. Cluster analysis dendrograms based on average linkage of RFLP data. A) Combined data from all digestions. B) Six-hour exposure of Hind III restricted DNA. Scale = similarity coefficient. R1-R4 = *M. incognita* races 1-4. Jav = *M. javanica*. Are = *M. arenaria* race 1.

to have the characteristic high molecular weight repetitive bands in the Hind III digest. The diagnostic bands in the EcoR I digest for race 2 of *M. incognita* also agree with the findings of Curran et al. (6). However, studies comparing many populations of the same *Meloidogyne* race are needed urgently for determination of RFLP race specificity.

The information presented in this study will also be useful in assessing the likelihood of isolating diagnostic root-knot nematode molecular probes from a genomic library. There is an indication that there is a greater chance of isolating species-specific probes than race-specific probes from such a library. The use of Cla I or Hind III rather than EcoR I restriction prior to library construction should also increase the chances of finding diagnostic probes. Restriction site polymorphism in nuclear DNA among these populations, although present, is relatively conservative compared to their mitochondrial DNA (15), and even more so compared to species of an animal-parasitic nematode, *Trichinella* spp., in which a DNA probe used in the same way as in this study would not cross-hybridize (5). There is also a striking sim-

ilarity in the base composition observed among four species of *Meloidogyne* (14). Both these facts indicate that the selection of species-specific DNA probes from a genomic library would not be easy. The construction of a library, however, should at least yield cloned probes which could be used in Southern hybridizations to reveal differences in low or single copy DNA between the races or species of *Meloidogyne*.

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