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Isolation of a Repeated DNA Probe Showing Polymorphism among *Meloidogyne incognita* Populations

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Abstract: Several *Meloidogyne incognita* geographic populations were characterized by analysis of the restriction fragment length polymorphisms (RFLP) obtained after digestion of their total DNA and hybridization with a [^{32}P]-labeled probe. The probe consisted of a 1.7-kb-repeated DNA sequence, isolated from a *M. incognita* genomic library, that hybridized to multiple BamH I fragments in the genome of each isolate. The patterns showed sufficient polymorphism to enable the accurate differentiation of all the populations tested.

Key words: DNA, hybridization, intraspecific differentiation, *Meloidogyne incognita,* repetitive DNA.

Although characterization of the main *Meloidogyne* species has been successfully realized by electrophoresis of their proteins (7,12) or isozymes (6), this technique cannot distinguish populations of one individual species. The limited discriminatory power of proteins is supported by the fact that they are produced via the expression of genes often highly conserved between closely related taxa and representing only a minor fraction of the total genome, whereas noncoding genomic regions are more abundant and subjected to greater evolutionary change (14). In this connection, recombinant DNA technology is a promising tool for differentiating *Meloidogyne* at interspecific and intraspecific levels. Restriction fragment length polymorphisms (RFLP) obtained by digestion of total DNA with restriction endonucleases have been observed with *M. incognita* populations (4,5), but no results are available on the accurate identification of this species using cloned probes.

The objective of this study was to isolate a DNA hybridization probe that could differentiate geographic isolates of *M. incognita.* Because variation in repeated sequences frequently occurs in sibling species (8,17), our experimental approach was to hybridize repetitive sequences isolated from a genomic library constructed from *M. incognita* juveniles to genomic DNA from other strains of this species. Such molecular studies may be helpful for identifying markers correlated with host range and pathogenicity.

Materials and Methods

Nematode isolates: Six nematode populations, each obtained from a single egg mass, were maintained on tomato (*Lycopersicon esculentum* cv. Saint Pierre) in a greenhouse at 20–25 C. They were identified as *M. incognita* and were indistinguishable by their perineal patterns and isoesterase electrophoretograms (6). The name and geographical origin of each population are as follows: 1) Côte d'Ivoire, Ivory Coast; 2) Taïwan, Taïwan; 3) Valbonne, France; 4) Guadeloupe, French West Indies; 5) NCSU E1135, USA; 6) Calissane, France.

Eggs were extracted from infested roots (13), resuspended in 0.3 M NaCl and 0.7% streptomycin sulfate solution, and placed on a 10-μm-pore sieve at 20 C for a week. The previous solution was replaced by a 0.7% streptomycin sulfate solution, and egg hatch started 3 days later for about 2 weeks. Every 3 days, juveniles were harvested by repeated washing on a 0.5-μm-pore sieve, concentrated by centrifugation at 2,000 *g* for 2 minutes in a 30% sucrose solution, washed in distilled water, pelleted in a microcentrifuge, and stored at −80 C until use.

DNA isolation: From 100 to 200 μl juveniles of each population were frozen in liquid nitrogen and ground by mortar and

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pestle. The DNA was extracted from the resulting powder with a phenol-chloroform procedure (15). After ethanol precipitation, DNA was resuspended in 0.01 M Tris (pH 8.0) and 0.001 M EDTA and stored at −20 C. Average DNA amounts ranging from 50 to 100 μg were routinely obtained for each isolate.

Construction of the M. incognita genomic library: A λ EMBL4 phage library of the Côte d'Ivoire population was prepared as follows. One milligram of λ EMBL4 DNA arms was precipitated together with 0.4 mg of 15–25-kb-selected DNA fragments produced by partial digestion of nematode genomic DNA with Sau3A. Establishment of appropriate conditions for partial digestion was performed by electrophoretic analysis of samples digested during varying times. Ligation was done at room temperature for 4 hours. After ethanol precipitation, ligated DNA was packaged using a λ DNA in vitro packaging kit (Amersham N. 334) according to the manufacturer's suggestions, and the resulting phages were plated on *Escherichia coli* strains Q 359 and C 600.

Electrophoresis, Southern blot, labeling, and hybridization: Genomic and bacteriophage DNA were digested with restriction endonucleases (Boehringer Mannheim) used as recommended by the manufacturer. Electrophoresis of digested DNA samples and processing for transfer to nylon membranes were done following standard procedures (18). The DNA were [^{32}P]-labeled by nick-translation (16) or by the random oligonucleotide primer method (10). All hybridizations were conducted overnight at high stringency (6× SSC, 5× Denhart's, 0.5% SDS at 65 C for 16 hours). After post-hybridization washes, filters were air dried, baked for 2 hours at 80 C, and exposed to X-ray film with an intensifying screen at −80 C.

Repetitive sequence isolation: The *M. incognita* genomic library was screened with [^{32}P]-labeled total DNA of the Côte d'Ivoire population by in situ hybridization (1). After autoradiography, 40 clones were selected at random among those that yielded a strong signal, indicating that they should contain a repeated DNA sequence. To identify potential probes, each bacteriophage DNA was digested to completion with several restriction enzymes, fractionated by 1% agarose gel electrophoresis, and transferred onto a nylon filter. After hybridization of the filters with labeled total DNA of the Côte d'Ivoire population, a 1,700 base-pair BamH I sequence was selected because the intensity of its hybridization signal indicated that it consisted of mid-repetitive DNA. This probe, named pMiK4, was recovered from the gel by electrophoresis through a piece of DEAE paper (9).

Each DNA extraction was performed twice, from two distinct juvenile harvests, so as to obtain two Southern blots for the same set of populations. The probe was also prepared twice from the selected clone, and each purified sequence was hybridized with both blots, in order to perform four independent replicates of the experiment.

Results

Southern blots of BamH I-digested genomic DNA probed with pMiK4 revealed a clear polymorphism among the six *M. incognita* isolates (Fig. 1). Multiple BamH I fragments hybridized in each nematode genome, some of them conserved among populations, and some others sufficiently polymorphic to enable their differentiation.

Moreover, a pattern characteristic of each isolate was deduced from comparative analysis. For instance, consistent RFLP with six strong bands occurred at 4.7, 3.6, 3.2, 2.6, 0.45, and 0.3 kb and differed among the digested DNA (Fig. 2). Some of these bands even allowed rapid identification of two populations: The 0.3 kb fragment was absent only for Valbonne, whereas the 2.6 and 3.6 kb ones were observed only with Guadeloupe. Other differences among populations occurred at 8.6, 6.3, and 2.0 kb, but hybridization signals were weak. Eleven bands (at 8.0, 5.5, 5.0, 3.4, 2.5, 2.3, 2.2, 1.7, 1.0, 0.85, and 0.8 kb)

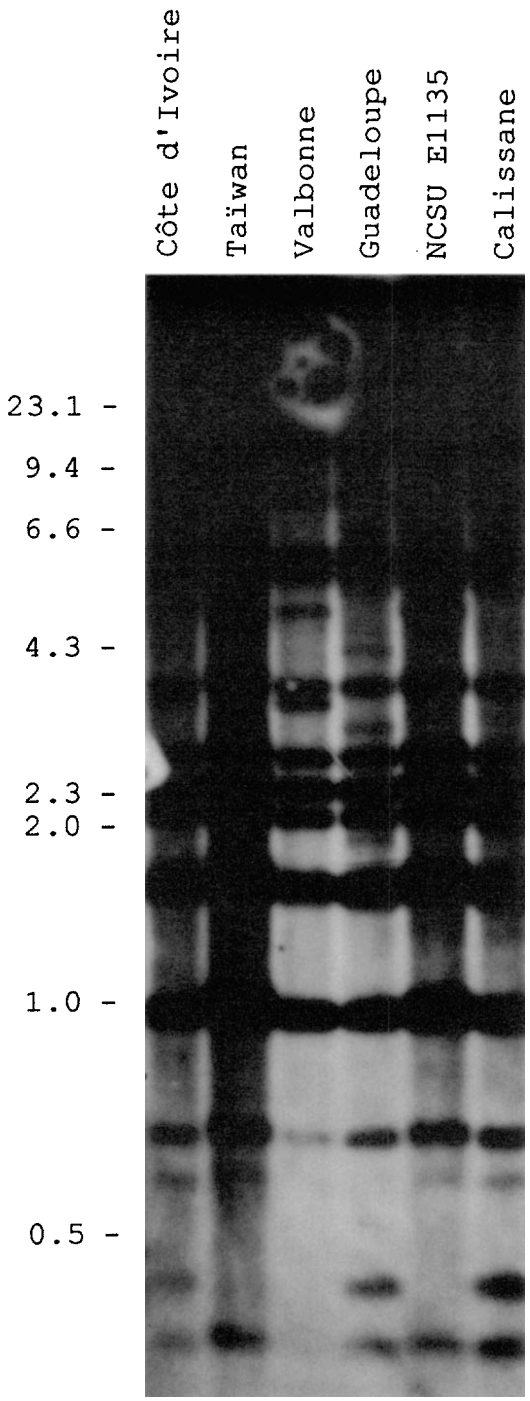

FIG. 1. Comparison of hybridization patterns of BamH I-digested genomic DNA from six *Meloidogyne incognita* populations with probe pMiK4, with an exposure time of a week. Molecular weight markers (λ DNA Hind III digest) and their sizes (in kb) are indicated on the left.

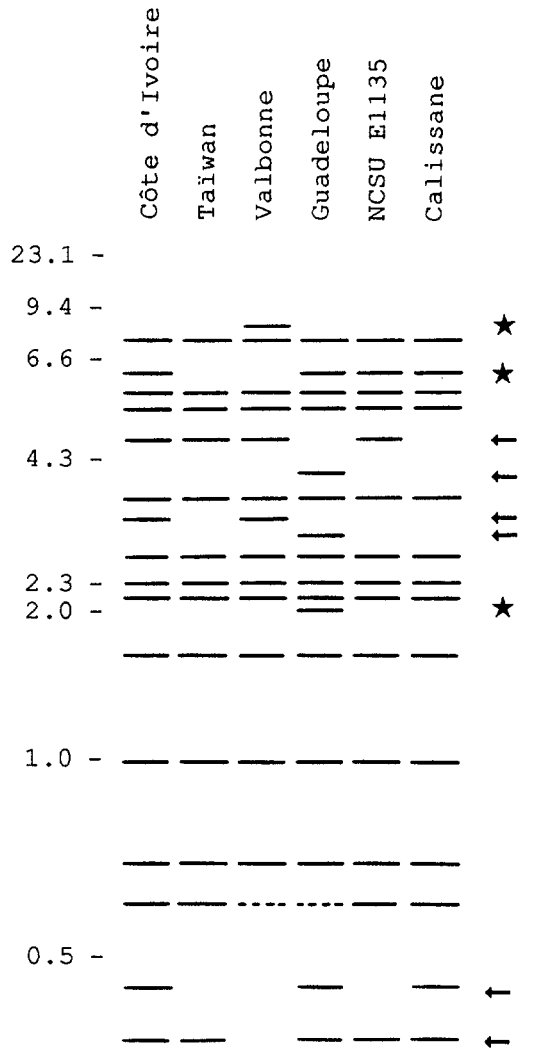

FIG. 2. Line drawings from the autoradiogram shown in Fig. 1. Arrows indicate consistent and stars indicate weak hybridizing bands. Molecular weight markers (λ DNA Hind III digest) and their sizes (in kb) are indicated on the left.

showed no polymorphism at all among the six *M. incognita* isolates.

DISCUSSION

Ethidium bromide staining of restriction digests was used to differentiate *Meloidogyne* populations (5), but the difficulty of visualizing repetitive bands on the smear obtained with a digest of total DNA indicated the weak potential of this technique to separate taxonomically close groups. We report the first clear distinction of *M. in-*

cognita geographic isolates via hybridization with a repetitive DNA probe isolated at random from a *M. incognita* genomic library. Homologous sequences have been utilized to distinguish plant-parasitic nematode species, for example, *Globodera pallida* from *G. rostochiensis* (3), but never populations within a species. Bolla et al. (2) conducted hybridization experiments with *Bursaphelenchus xylophilus,* but their probe consisted of total genomic DNA from a single *B. xylophilus* isolate.

In contrast with morphological and other biochemical analyses, hybridization patterns obtained with the pMiK4 probe exhibit clear polymorphism among all six populations. We considered only consistent differences to show RFLP among the populations. Because of the repetitive nature of the target sequence of the probe, some weak hybridization signals might result from a small number of copies of this sequence in the genome instead of partial digestion of the genomic DNA. The fact that most of these weak signals were repetitively observed in the different experiments definitely demonstrates that they are not artifacts generated by incomplete action of the endonucleases.

As suggested by Hamer et al. (11), who worked on the fungal plant pathogen *Magnaporthe grisea,* time in the evolution of *M. incognita* may account for the strong divergence between isozyme and repeated DNA polymorphisms. The time may have been sufficient to permit modifications in the repetitive DNA of each nematode isolate (exemplified by the distinctive hybridization patterns obtained with pMiK4), but not long enough for development of extensive variations in isozyme electrophoresis patterns (6).

Preliminary results obtained with other *Meloidogyne* species (especially *M. arenaria* and *M. javanica*) suggest that pMiK4 hybridization patterns are not only intraspecific but also allow specific differentiation in this genus, as autoradiograms with two *M. arenaria* isolates display both common and differential bands (data not shown). The 11 bands common to the six populations may comprise a standard pattern for *M. incognita,* whereas differential ones discriminate isolates. In this connection, and until these results have been confirmed through analysis of a large collection of populations for each species, the pMiK4 probe shows promise, in complement with biochemistry and cytogenetics, as a tool for the fine identification and conclusive demonstration of phylogenetic relationships in the genus *Meloidogyne.*

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