Isolation, Characterization, and Application of DNA Probes Specific to *Meloidogyne arenaria*

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ABSTRACT

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Approximately 1,100 clones of a genomic *Eco*RI plasmid library of *Meloidogyne arenaria* were differentially screened with ^{32}P-labeled genomic DNA from *M. arenaria* and *M. incognita*. One clone (pRIMA17) hybridized preferentially to *M. arenaria*, and two clones (pRIMA2, pRIMA9) hybridized preferentially to *M. arenaria* and *M. javanica*. Restriction mapping and differential screening of pRIMA17 revealed an internal 900-bp *Sca*I fragment (pSCIMA17) that was specific and produced a strong hybridization signal with DNA from *M. arenaria* only. Neighboring restriction fragments within pRIMA17 were also species-specific but the hybridization signals were less strong. Fragments of pRIMA17 hybridized to polymorphic interspersed repeats in *M. arenaria* populations; however, not all fragments produced the same banding patterns. Sequence analysis showed that pSCIMA17 contained a tandem repeat region of 28-bp subunits repeated 19 times extending from the left border. The tandem repeat was followed to the right by a region rich in A and T nucleotides. A dot blot method to identify root-knot nematode species with species-specific DNA probes using egg masses was developed. High sensitivity as a result of internal tandem repeat sequences and hybridization to a high copy number interspersed repeat make pSCIMA17 particularly suitable for diagnostic applications.

Additional keywords: differential hybridization, diagnosis.

Root-knot nematodes (*Meloidogyne* spp.) are parasites of a multitude of host plants. Estimated yearly crop losses are approximately 5% worldwide, although the damage inflicted in certain regions of developing countries exceeds this level (32). Of the more than 50 recognized *Meloidogyne* species, the vast majority of agronomic damage is attributable to *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla* (13). *M. arenaria*, referred to as the peanut root-knot nematode, is a serious pest in the southeastern United States. Peanut and soybean are severely damaged by this species each year. Tobacco is also becoming increasingly affected in wide areas (20). The North Carolina differential host test (23,34) distinguishes two races within this species, differentiated by peanut cv. Florunner, which is parasitized by race 1 but not by race 2. *M. arenaria* race 1 populations are predominantly encountered in the peanut-growing regions of Alabama, Georgia, and Florida. Race 2 populations are most frequent in the flue-cured tobacco region of the Carolinas and other southeastern states. In South Carolina, *M. arenaria* is often found in mixed populations with the Southern root-knot nematode, *M. incognita*, and occasionally also with *M. javanica* (20). Different *Meloidogyne* species and races require specific cultural control measures, such as choice of crop and/or resistant cultivar, and also exhibit varied susceptibility to nematicidal compounds (3,26,27). Therefore, it is important to rapidly identify field populations to species and, if applicable, also to host race. At present, this need is not being met. Species identification is widely based on tedious and potentially inaccurate analysis of perineal patterns of adult females. Races can only be determined by time-consuming host range tests (23,34).

Several approaches for more rapid and accurate nematode identification have been pursued. Protein analysis (11,12,16–18, 25) culminated in species identification of single adult females by isozyme patterns (18). However, race identification is not yet possible. DNA-based techniques for species identification include restriction fragment length polymorphism (RFLP) (6,10,21,28, 30,35) and procedures utilizing the polymerase chain reaction (PCR) (8,22,29). RFLP approaches, although discriminating species and isolates, require the isolation of considerable amounts of DNA and may not be practical for large numbers of samples. PCR applications are very sensitive and enable rapid processing of large numbers of samples. However, other promising and sensitive techniques are being studied.

We developed an identification technique using a species-specific hybridization probe for *M. arenaria*, which was identified through differential screening. This probe is targeted to a repeated DNA sequence, thus providing a high level of sensitivity. The specificity allows species identification by dot blot experiments with crude DNA extracts. The probe reliably identifies *M. arenaria* in hybridization experiments and is useful as a practical, inexpensive, and rapid diagnostic tool that can be used for large numbers of samples.

MATERIALS AND METHODS

Nematode isolates and DNA extraction. Nematode isolates were grown in greenhouse pot cultures on tomato (*Lycopersicon esculentum* Mill. 'Rutgers') or peanut (*Arachis hypogea* L. 'Florunner'). A list of all isolates is given in Table 1. Eggs were extracted from infected roots (24) and purified by sucrose gradient centrifugation (2). Purified eggs were concentrated by centrifugation in 1.5-ml Eppendorf tubes. After removal of the supernatant, eggs were frozen at −80 C. For further processing, eggs

were suspended in DNA extraction buffer (33) and ground in handheld Tenbroek glass tissue grinders (Baxter, McGaw Park, IL) on ice. After 20 min of incubation at 42 C, homogenates were loaded directly onto a CsCl gradient and processed according to Sambrook et al (31).

DNA blots and hybridizations. Colony blots for differential screening of approximately 1,100 clones of an *Eco*RI genomic *M. arenaria* library from isolate Govan, obtained from A. G. Abbott, Clemson University (6), were prepared in duplicate on Hybond nylon membrane (Amersham International PLC, Amersham, UK) as described in Sambrook et al (31). DNA for Southern blots was digested 15–18 h according to the enzyme manufacturer's (Promega, Madison, WI) guidelines. Digests were separated electrophoretically on 0.8 to 1.0% agarose gels, and blots were prepared by capillary transfer to nylon membranes (31). DNA probes were labeled with ^{32}PαCTP by random priming (70200 random primed DNA labeling kit, USB Corp., Cleveland, OH). The oligonucleotide *M. incognita*-specific probe of Chacon et al (9) was end-labeled using the procedure described in Ausubel et al (1). Hybridizations were conducted in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60 to 65 C for 15 to 20 h. Membranes were washed in 0.2× SSC and 0.1% SDS at 65 C. Autoradiography was performed using X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at −80 C with intensifying screens.

Sequence analysis. Subclones were produced in pBluescript KS$^+$ and sequenced by the dideoxy-chain termination method using the 70770 Sequenase version 2.0 DNA sequencing kit (USB Corp.). Data were processed using the Gene Jockey Sequence Processor program by P. L. Taylor (BIOSOFT, Cambridge, UK) for Macintosh computers. Nucleic acid sequences were used to search the GenBank release 72 and GenBank-NEW 8 databases. Putative protein sequences were compared with the PIR release 33 and Swiss-Prot release 22 databases.

Preparation of DNA dot blots. Root-knot nematode egg masses were collected from infected root tissue and stored individually in 75 μl of sterile distilled water in single wells of 96-well microtiter plates and frozen at −80 C for later analyses. For DNA extraction, 25 μl of 20× SSC was added. Egg masses were then ground for 10 to 20 s using a handheld electric motor (Pellet Pestle Motor, Baxter, McGaw Park, IL) turning a nail head that exactly fit the wells of the microtiter plates. Following this procedure, 100 μl of 20× SSC was added, and the plates were incubated at 52 C for 30 min. Debris was spun down for 10 min at approximately 1,600 *g*. Approximately 180 μl was then pipetted to a dot blot manifold and transferred onto nylon membrane by vacuum. DNA was denatured by application of 200 μl of denaturing solution (1.5 M NaCl, 0.5 N NaOH) to the manifold wells for 5 min and subsequently neutralized by 200 μl of neutralization solution (1 M Tris-HCl pH 8; 1.5 M NaCl). Filters were air-dried, and DNA was fixed by ultraviolet radiation and hybridized with radiolabeled DNA probes as described above.

RESULTS

Isolation of species-specific DNA clones. Differential screening of approximately 1,100 clones of the *M. arenaria* genomic library with radiolabeled genomic DNA from *M. arenaria* (isolate Govan) and *M. incognita* (isolate Witcher) identified clones with apparent preferential hybridization to DNA from *M. arenaria*. Seventeen clones were chosen and rescreened by differential hybridization. Three clones (pRIMA2, pRIMA9, and pRIMA17) hybridized strongly to *M. arenaria* genomic DNA and only very weakly to *M. incognita*. These three clones were used as hybridization probes for Southern blots of *Eco*RI-digested genomic DNA from two isolates each of *M. arenaria* and *M. incognita* and one isolate of *M. javanica* (Fig. 1). Clones pRIMA2 and pRIMA9 hybridized to DNA from *M. arenaria* and *M. javanica*, whereas pRIMA17 produced strong hybridization signals only with DNA from *M. arenaria*. Specificities of all three clones were confirmed by Southern hybridizations to genomic DNA blots of DNA of 20 *M. arenaria*, 12 *M. incognita*, two *M. javanica*, and two *M. hapla* isolates (*data not shown*). Clone pRIMA17 hybridized to a great many restriction fragments, indicative of a high copy interspersed DNA repeat.

Characterization of pRIMA17. Clone pRIMA17 was further characterized to localize the region exhibiting highest *M. arenaria* specificity. The 5.4-kb insert of pRIMA17 was restriction mapped and is shown in Figure 2. Southern analysis revealed that the strongest *M. arenaria*-specific hybridization signal was contained within a 900-bp *Sca*I fragment. This fragment, subcloned to create pSCIMA17, when used as radiolabeled hybridization probe for Southern blots of genomic DNA of the four *Meloidogyne* species, was highly specific to *M. arenaria*. Even long X-ray exposures yielded only negligible background hybridization to *M. incognita* or *M. javanica*. This was true with all 36 *Meloidogyne* isolates tested (*data not shown*). As pictured in Figure 3, Southern blot analyses using the two *Sca*I-*Sph*I fragments of pSCIMA17 (see Fig. 2) demonstrated that both regions hybridized to identical bands in genomic DNA of *M. arenaria* isolates digested with *Sca*I. Similar results were obtained when DNA was digested with *Eco*RI. Figure 4 shows Southern blots of *Eco*RI-digested genomic DNA of one *M. incognita* and two *M. arenaria* isolates probed

TABLE 1. *Meloidogyne* isolates used for hybridization analyses

Species Designation	Race	Culture name	Origin
M. arenaria			
MA1-NC1	1	Bladen	Bladen Co., NC[a]
MA1-NC2	1	MA P	Martin Co., NC[a]
MA1-SC	1	SCI	Barnwell Co., SC[b]
MA1-GA1	1	Baker	Baker Co., GA[c]
MA1-GA2	1	Benson 1	Tift Co., GA[c]
MA1-GA3	1	Benson 2	Tift Co., GA[c]
MA1-GA4	1	Blakley	Early Co., GA[c]
MA1-GA5	1	Decatur	Decatur Co., GA[c]
MA1-GA6	1	Donaldsonville	Seminole Co., GA[c]
MA1-GA7	1	Gibb's Farm	Worth Co., GA[c]
MA1-GA8	1	Gopher	Tift Co., GA[c]
MA1-GA9	1	Headland	Headland, AL[c]
MA1-GA10	1	Hussey	Unknown[c]
MA1-GA11	1	Mitchell	Mitchell Co., GA[c]
MA1-FL	1	Live Oak	Live Oak, FL[c]
MA2-VA	2	54	VA[a]
MA2-NC1	2	NTP	NC[a]
MA2-NC2	2	83	Cumberland Co., NC[a]
MA2-SC1	2	Govan	Barnwell Co., SC[b]
MA2-SC2	2	Pelion	Barnwell Co., SC[b]
M. incognita			
MI1-NC1	1	Avoca36	Bertie Co., NC[a]
MI1-NC2	1	Avoca85-1	Bertie Co., NC[a]
MI2-NC	2	MI2Kng	NC[d]
MI2-SE	2	MI2EM	Southern United States[a]
MI3-NC1	3	MI3EM	Rocky Mt., NC[a]
MI3-NC2	3	99COT	NC[a]
MI3-SC	3	Witcher	Pickens Co., SC[b]
MI3-GA	3	Emmanuel	Emanuel Co., GA[e]
MI3-FL	3	JAY	Jay Co., FL[e]
MI3-MX	3	JYE	Mix from FL, GA, and SC[e]
MI4-NC1	4	MI14Kng	NC[d]
MI4-NC2	4	SPG28	Rockingham Co., NC[a]
M. javanica			
MJ-1	NA	MJUSDA	Unknown[f]
MJ-2	NA	MJNCSU	Rocky Mt., NC[a]
M. hapla			
MH-1	?	MHNCSU	Rocky Mt., NC[a]
MH-2	A	MHUTK	Warren Co., TN[g]

[a] Obtained from K. R. Barker, North Carolina University, Raleigh.
[b] Obtained from Clemson University, Clemson, SC.
[c] Obtained from J. P. Noe, University of Georgia, Athens.
[d] Obtained from S. R. Koenning, North Carolina State University.
[e] Obtained from R. S. Hussey, University of Georgia.
[f] Obtained from R. N. Huettel, U.S. Department of Agriculture, Hyattsville, MD.
[g] Obtained from E. C. Bernard, University of Tennessee, Knoxville.

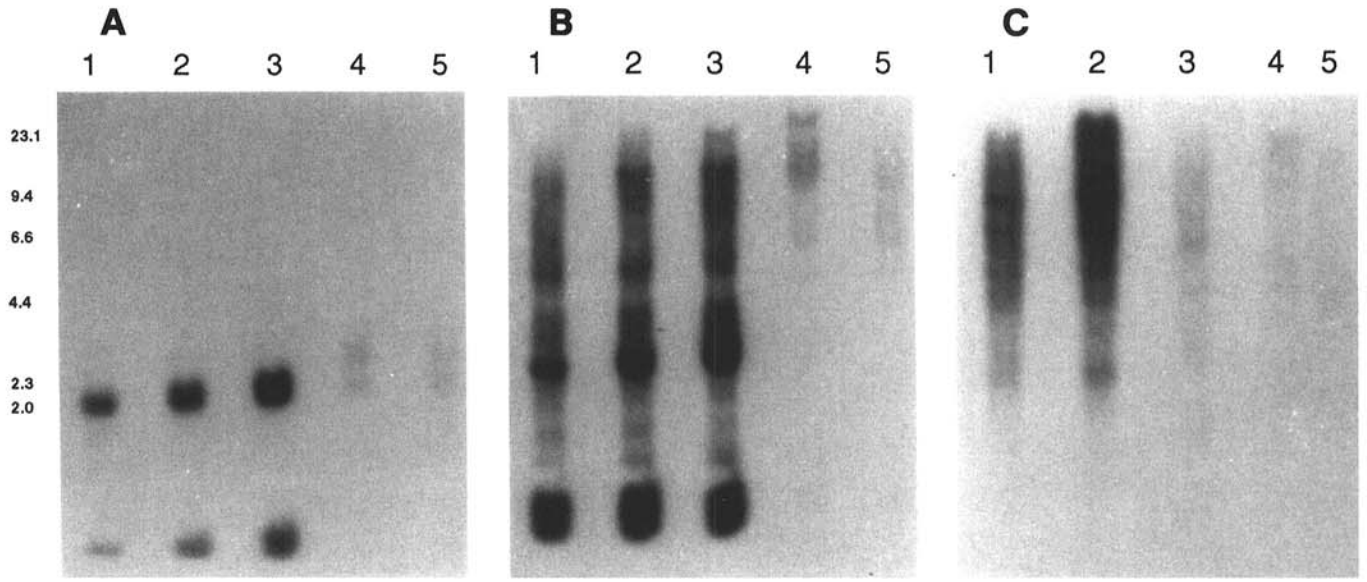

Fig. 1. Southern blots of *Eco*RI-digested total genomic DNA from two *Meloidogyne arenaria,* one *M. javanica*, and two *M. incognita* isolates probed with **A,** pRIMA2, **B,** pRIMA9, and **C,** pRIMA17. Nematode isolates are *M. arenaria* Govan (lane 1), *M. arenaria* SCI (lane 2); *M. javanica* MJUSDA (lane 3); *M. incognita* Witcher (lane 4); and *M. incognita* MI2KNG (lane 5).

with four fragments from pRIMA17 (as depicted in Fig. 2). The 1.1-kb fragment (E/S fragment) to the left of the pSCIMA17 fragment hybridized weakly to an interspersed repeat distinct from the pattern produced by pSCIMA17, although some fragments of similar size hybridized to both probes. The region corresponding to pSCIMA17 (S/S fragment) and the 1.4-kb fragment (S/H fragment) to the right showed identical patterns. Fragment H/E (1.4 kb) following to the right of the S/H fragment yielded a different interspersed repeat pattern. The four regions exhibited different degrees of *M. arenaria*-specificity; the 900 bp *Sca*I fragment (pSCIMA17) hybridized exclusively to DNA from *M. arenaria*, whereas the other three regions also produced weak hybridization signals with *M. incognita* DNA. Clone pSCIMA17 produced much stronger hybridization signals in all experiments and required 10- to 30-fold shorter exposure times than the other probes used. All four hybridization fragments of pRIMA17 produced RFLPs between *M. arenaria* isolates (Fig. 4).

pSCIMA17 contains a 28-bp tandem repeat. Sequencing of pSCIMA17 revealed a tandem repeat region followed by an AT-rich region, shown in Figures 2 and 5. The tandem repeat consisted of 28 nucleotides repeated 19 times. Repeat unit 15 contained 29 nucleotides. However, the following repeat unit consisted of only 27 bases, presumably due to loss of a G at the fifth or sixth position, thus restoring the overall 28 bases increment of

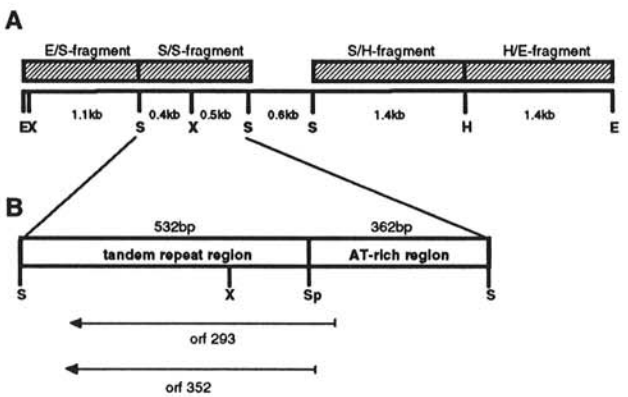

Fig. 2. Restriction map of **A,** pRIMA17 and **B,** sequence characteristics of pSCIMA17. Hatched bars indicate hybridization probes used in Figure 4. Open reading frames (ORF) are symbolized by arrows. Restriction endonuclease sites are E = *Eco*RI, H = *Hin*dIII, S = *Sca*I, Sp = *Sph*I, X = *Xho*I.

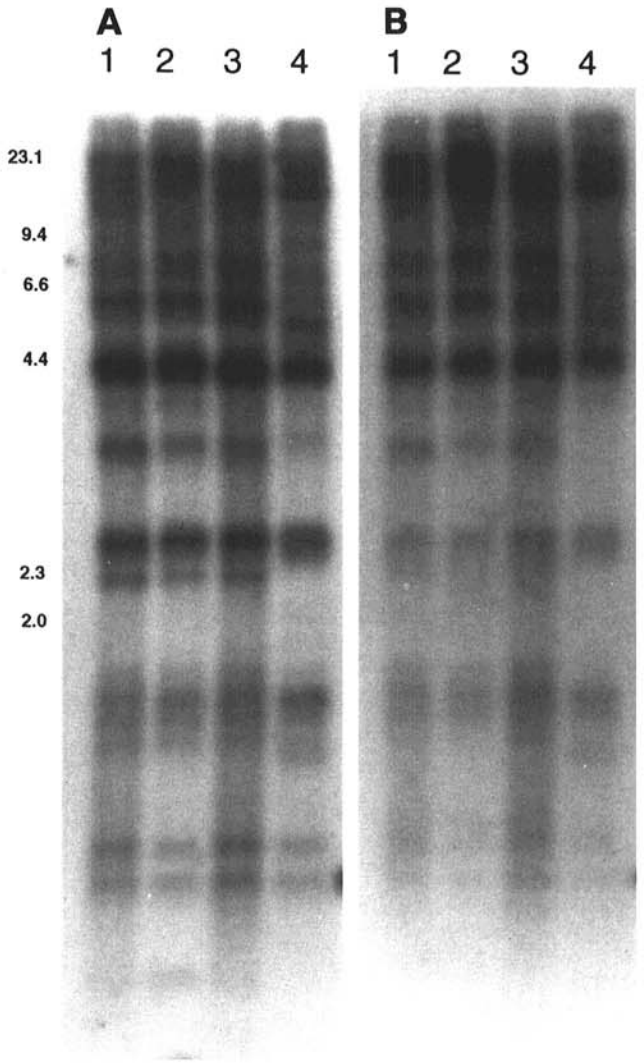

Fig. 3. Southern blots of *Sca*I-digested total genomic DNA from four *Meloidogyne arenaria* isolates probed with the ^{32}P-labeled tandem repeat region (*Sca*I/*Sph*I fragment) of pSCIMA17 in panel **A** and the AT-rich region (*Sph*I/*Sca*I fragment) of pSCIMA17 in panel **B**. The nematode *M. arenaria* isolates are Gibbs Farm (lane 1), Pelion (lane 2), 83 (lane 3), and SCI (lane 4).

the tandem repeat region. Evaluation of the nucleotide sequence identified three variant types of repeat subunits as shown in Figure 6. Most positions within all the repeat subunits were highly conserved. However, nucleotides numbered 2, 5, and 10 through 13 of individual repeat subunits contained a number of concerted changes. For example, T and G at positions 2 and 5, respectively, were always followed by GCGT at positions 10 through 13. Repeat types one, two, and three were present nine, six, and two times, respectively, discounting repeat units 15 and 16. Two type 1 repeat copies deviated by one base each from the basic scheme (Fig. 5). Other nucleotide positions, particularly numbers 20 and 28, changed without apparent rule. Sequence analysis of the E/S fragment (see Fig. 2) revealed that the tandem repeat did not extend into this neighboring fragment. Formation of the internal *Xho*I restriction site (CTCGAG) within pSCIMA17 was due to a change from G to A at the beginning of repeat unit 15 (Fig. 5).

The tandem repeat region was followed to the right by a segment rich in A and T nucleotides. Two open reading frames, ORF 293 and ORF 352, were found extending from the AT-rich region back into the tandem repeat sequence (Figs. 2 and 5). The putative gene products were 172 and 155 amino acids, respectively. Both reading frames started with an ATG start codon. They substantially overlapped but were not in the same reading frame, thus encoding different putative peptides. Database searches with the hypothetical protein sequences detected no convincing similarities.

Searching of databases with the tandem repeat sequence revealed similarity to a 28-base tandem repeat region found downstream of the T24 human bladder carcinoma oncogene (5), a homolog to the oncogene of the Harvey murine sarcoma virus (v-Ha-*ras*). Sequences of individual subunits of this region were between 40 and 60% identical to the subunits found in the tandem repeat region of pSCIMA17.

***M. arenaria*-specific DNA clone as diagnostic tool.** DNA was extracted from egg masses from *M. arenaria* (isolate Pelion) and

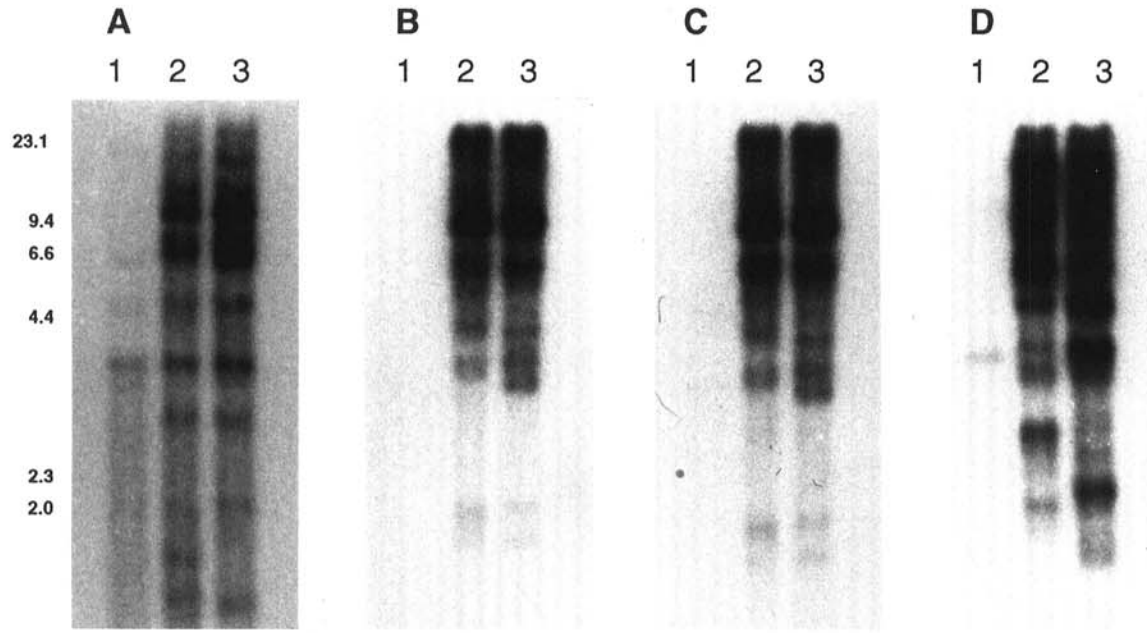

Fig. 4. Southern blots of *Eco*RI-digested total genomic DNA of one *Meloidogyne incognita* and two *M. arenaria* isolates probed with **A,** the ^{32}P-labeled E/S fragment, **B,** the S/S fragment, **C,** the S/H fragment, and **D,** the H/E fragment of pRIMA17 (see Fig. 2). The nematode isolates are *M. incognita* JYE (lane 1), *M. arenaria* SCI (lane 2), and *M. arenaria* Pelion (lane 3). Approximate exposure times were 60 h, 2 h, 20 h, and 20 h for panels A through D, respectively.

Fig. 5. Nucleotide sequence of pSCIMA17. Tandem repeat units are indicated by horizontal lines with labels indicating repeat subunit number and type (see Fig. 6). Start codons (ATG) and direction of transcription of open reading frames (ORF) are shown. Restriction endonuclease recognition sites are underlined.

M. incognita (isolate Witcher) and transferred to nylon membranes supported in a dot blot apparatus as described above. Figure 7 shows that ^{32}P-labeled pSCIMA17 hybridized only to DNA from *M. arenaria* egg masses, producing conspicuous signals on autoradiographs after 1 to 2 days' exposure. After removing the hybridization probe, DNA isolated from *M. incognita* egg masses was subsequently detected by probing the same membrane with an *M. incognita*-specific oligonucleotide obtained from Chacon et al (9). Observed variation in signal intensity between egg masses was presumably a function of egg mass size. Signals from small egg masses were further diminished because the DNA extraction method was less efficient. This technology was utilized for evaluating greenhouse and field experiments on population dynamics of *M. arenaria* and *M. incognita* in mixed populations. Out of 384 dot blotted egg masses, 93% showed hybridization signals that unambiguously discriminated between both nematode species. The remaining 7% did not exhibit signals with either probe.

DISCUSSION

A clone pRIMA17 containing a 5.4-kb insert with high specificity for *M. arenaria*, which hybridized to a high copy number interspersed repeat, was identified by differential screening of a genomic *M. arenaria* library. The region exhibiting greatest specificity was localized to a 900-bp internal *Sca*I fragment (pSCIMA17). The neighboring regions E/S, S/H, and H/E (Fig. 2), although hybridizing preferentially to *M. arenaria* DNA, produced very weak hybridization signals with DNA from *M. incognita*. The presence of tandem repeat sequences in pSCIMA17 presumably enhanced the number of hybridization fragments annealing and resulted in stronger hybridization signals.

Type 1 -T- -G- - - -GCGT- - - - - -X- - - - - - -X

Type 2 -T- -A- - - -GGAC- - - - - -X- - - - - - -X

Type 3 -A- -A- - - -ATGT- - - - - -X- - - - - - -X

Fig. 6. Three types of tandem repeat subunits. Conserved positions are indicated by dashes, and X represents bases that change without apparent rule. The last position in all subunits is either G or A. The three repeat types are well preserved with only a few exceptions. Repeat units 15 and 16 are not included due to their abnormal sequence characteristics.

Mapping and hybridization experiments indicated that sequences extending from the S/S to the S/H regions of pRIMA17 (Fig. 2) formed an interspersed repeat unit. This is illustrated by the finding that hybridization probes corresponding to these regions produced identical patterns in Southern blots of *Eco*RI-digested DNA (Fig. 4). The E/S and H/E fragments belong to other interspersed repeat structures, as indicated by the different hybridization patterns shown in Figure 4.

The similarity of the *M. arenaria* tandem repeat to a 28-bp tandem repeat region found downstream of the T24 human bladder carcinoma oncogene may be significant, although the repeated DNA region does not appear to be directly involved in oncogenic behavior (5). The T24 oncogene encodes a peptide nearly identical to the gene product of the Harvey murine sarcoma retroviral oncogene (v-Ha-*ras*) (14,19). This may indicate a connection between the *M. arenaria* repeat region and a retrovirus. However, we have no direct evidence to support this hypothesis, and the significance of the repeat region remains uncertain at this point. No other similar repeats with 28-bp subunits were revealed by data base searches.

Clone pRIMA17 may represent noncoding spacer regions, commonly found in organisms. Such regions are thought to diverge more rapidly than coding sequences of a genome and may explain why specific regions of pRIMA17 do not hybridize to DNA of other *Meloidogyne* species; spacer regions in other species may have evolved differently than in *M. arenaria* and consequently share little sequence similarity. This hypothesis is supported by observations from *Caenorhabditis elegans* and *C. briggsae*. Almost exclusively, only DNA from coding regions hybridized to DNA from both species, whereas nontranscribed sequences were species-specific (15). Spacer regions of *C. elegans*, as well as of *Meloidogyne* are generally very AT rich (15; A. G. Abbott, *personal communication*) compared with nuclear gene sequences, which usually have more balanced nucleotide proportions. In pRIMA17, the tandem repeat region shows a comparatively low AT content ranging from 33 to 55% among individual repeat units. The adjacent, nontandemly repeated region, is composed of 66% AT nucleotides. It is interesting to note that the two ORFs start in the immediate vicinity of this transition and extend into the low-AT tandem repeat region.

The identification of two clones (pRIMA2 and pRIMA9) that hybridize to *M. arenaria* and *M. javanica* but produce only weak hybridization signals with *M. incognita* or *M. hapla* is consistent with a closer phylogenetic relationship between *M. arenaria* and *M. javanica* than between *M. arenaria* and *M. incognita* and *M. hapla* (4,7,12,17,21,35). An explanation of this observation

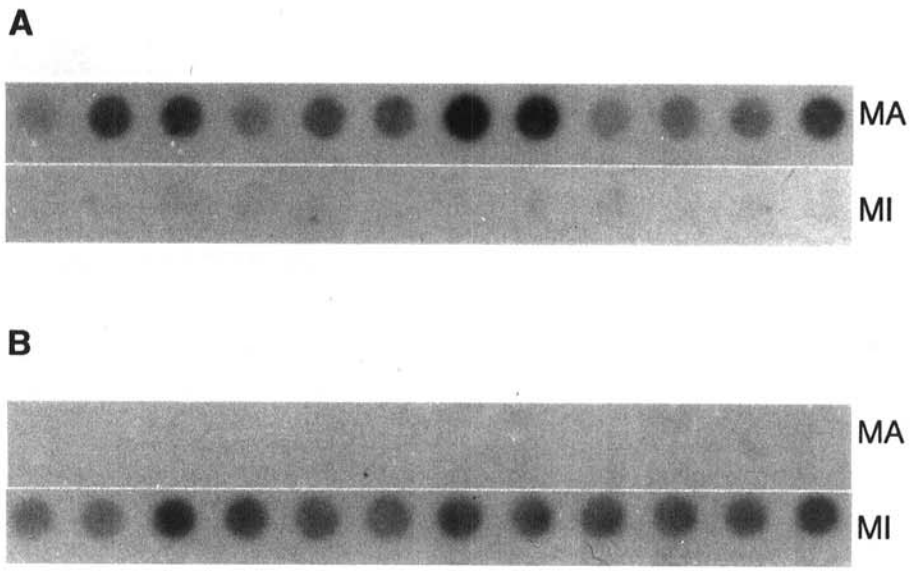

Fig. 7. Dot blots of single egg masses from *Meloidogyne arenaria* isolate Pelion (MA) and *M. incognita* isolate Witcher (MI) probed with **A,** ^{32}P-labeled pSCIMA17 and subsequently with **B,** an *M. incognita*-specific probe.

may involve the evolution of noncoding spacer regions. *M. arenaria* and *M. javanica* apparently diverged only relatively recently, thus sharing sequence motifs that have been lost in the more distantly related species *M. incognita* or *M. hapla*. This hypothesis is supported by the finding that *M. arenaria* and *M. javanica* show similar hybridization patterns when analyzed with these probes (*data not shown*). However, other explanations are possible.

The presented results were obtained from a selection of 20 *M. arenaria*, 12 *M. incognita*, two *M. javanica*, and two *M. hapla* isolates all originating in the southeastern United States. Therefore, extrapolating our findings to other geographical isolates has to be done cautiously and awaits further research.

In this research, DNA from single egg masses was shown to be sufficient to produce strong hybridization signals with species-specific probes. We have developed an easy and inexpensive method for egg mass processing and species identification, which allows a fast turnover of a large number of samples. Freezing of collected egg masses permits interruption of sample processing. Clone pSCIMA17 features 1) high species-specificity, 2) many homologous regions in the *M. arenaria* genome, and 3) tandem repeat sequences, resulting in a very high sensitivity of detection. Hybridization probes for diagnostic purposes are likely to have increased appeal when used with nonradioactive detection systems and when probes for other species and possibly races are isolated.

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