Identification of Four Major *Meloidogyne* spp. by Random Amplified Polymorphic DNA (RAPD-PCR)

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ABSTRACT

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The random amplified polymorphic DNA (RAPD) assay is a variant of the polymerase chain reaction (PCR) in which a single primer of random sequence is used at a low-annealing temperature. Twenty-two primers were evaluated for their usefulness in identifying 18 populations of *Meloidogyne incognita, M. arenaria, M. javanica,* and *M. hapla* collected worldwide. Reactions with primer OPA-01 produced amplified DNA bands whose size allowed the separation of the four species. Reactions with another group of 11 primers made possible the separation of one, two, or three species. Numerous polymorphisms were found between one population of race A and one of race B of *M. hapla* as well as between populations of *M. arenaria*. In contrast, the four races of *M. incognita* were indistinguishable.

Additional keywords: root-knot nematodes.

The genus *Meloidogyne* comprises a widely distributed group of plant-parasitic nematodes usually known as root-knot nematodes. Over 55 species have been described, but four account for more than 90% of the estimated damage caused worldwide by root-knot nematodes (6). *Meloidogyne incognita, M. arenaria, M. javanica,* and *M. hapla* are considered the four major species of the genus.

Identification of *Meloidogyne* spp. is important for the design of an efficient control plan. Identification of species within this genus was initially based on morphological characteristics and host preferences (6); however, because these features vary and are difficult to observe, other taxonomic criteria have been developed. Cytogenetic and serological methods have been used, but the most reliable method until now has been isozyme analysis. The comparison of esterase patterns shows great consistency in the separation of the four major species throughout the world, and it is the most economical of the biochemical methods used to classify this genus (7). Isozyme analysis, however, does not detect much intraspecific variation among *Meloidogyne* nematodes.

The appearance of nucleic acids technology has allowed new approaches to nematode identification. Curran et al (5) used the restriction patterns of repetitive DNA to distinguish physiological races within the four major species of *Meloidogyne*. Powers et al (12,13) proposed a phylogeny of the genus based on the restriction patterns of mitochondrial DNA (mtDNA). These approaches are time consuming, however, and, therefore, are not practical for rapid identification.

The polymerase chain reaction (PCR) is a new development that enables the exponential amplification of DNA starting from a single molecule. Using this technique, Harris et al (10) amplified a 1.8-kb fragment of mtDNA from the DNA contained in a single egg or larvae of the root-knot nematode. This fragment contains restriction fragment length polymorphisms (RFLPs) for the enzyme *HinfI* that allow the separation of *M. incognita, M. arenaria,* and the *M. javanica* and *M. hapla* group. Thus, PCR allows the identification of *Meloidogyne* species during a life stage indistinguishable by any other technique. One drawback to this approach is the need to obtain information on nematode DNA sequences for the design of the two primers used in the reaction. This involves a substantial investment of time and resources. Recent developments in PCR technology have made it possible to use a single random primer about 10 nucleotides long (1,18,19). By lowering the annealing temperature during the amplification cycle, the primer anneals at random in the genome, allowing the synthesis of highly polymorphic amplification products. Through the use of random amplified polymorphic DNA (RAPD-PCR) or PCR with arbitrary primers (AP-PCR), species and individual strains of diverse organisms such as bacteria (*Staphylococcus* spp., *Streptococcus pyogenes* [18], and *Escherichia coli* [19]), fungi (*Neurospora crassa* [19], *Leptosphaeria maculans* [9], and *Fusarium solani* [4]), and plant cultivars (*Glycine* spp. [1,19], *Oryza* spp. [18], *Zea mays* [19], and *Brassica oleracea* [11]) were distinguished.

In this work, RAPD-PCR was evaluated for its ability to identify several populations and races of the four major species of *Meloidogyne*. Special attention was devoted to those aspects relevant to practical identification of root-knot nematodes, such as reproducibility of the reaction and simplicity of use.

MATERIALS AND METHODS

Nematode populations. Two sets of populations were used in the study. A group of 12 populations, fully characterized with respect to taxonomic, pathogenic, and genetic features, was obtained from the collection of North Carolina State University, Raleigh (Table 1). The group was used in a previous study (3). Forty-five days after inoculation of tomato plants (cv. Rutgers), eggs were collected from roots and stored at −20 C until used. The second group consisted of populations isolated from diseased tomato plants in different fields in northeast Spain and maintained in a collection in the Institut de Recerca i Tecnologia Agroalimentaries (IRTA, Barcelona, Spain) (Table 1). These populations were identified only by observation of perineal patterns, and all except two were grown on transformed tomato roots in axenic cultures started from a single-egg mass (17).

Isolation of DNA. Three methods of DNA extraction were used and compared. Highly pure DNA was obtained according to a modified protocol previously described (15) (referred to as standard method from here on). In brief, 2 ml of frozen eggs was crushed in liquid nitrogen with mortar and pestle and incubated for 1 h at 55 C in 4 ml of extraction buffer (100 mM Tris-HCl, pH 8.5; 50 mM EDTA, pH 7.4; 100 mM NaCl; 1% SDS; and 100 μg, per milliliter, of Proteinase K). The lysate

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was extracted once with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acids were precipitated from the aqueous layer with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volume of absolute ethanol at −20 C. After centrifugation at 8,000 rpm for 15 min, the pellet was washed two times with 70% ethanol and dissolved in 5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). A purification step was added by ultracentrifugation in isopycnic gradient. After adding 0.9 g per milliliter of CsCl and 0.5 μg per milliliter of ethidium bromide (EtBr), the DNA solution was centrifuged at 55,000 rpm for 18 h in a vTi-90 rotor inside a L8-70M Beckman ultracentrifuge (Fullerton, CA). Subsequently, the EtBr was extracted with isoamyl alcohol, and the DNA was dialyzed extensively in TE buffer and was stored at 4 C.

A miniprep for DNA extraction, previously developed for fungi (2), was also tested. Hundreds of juveniles, 10–15 egg masses, or 10 females were collected from culture plates and were placed in a 1.5-ml Eppendorf tube and were disinfested with 500 μl of 0.52% sodium hypochlorite for several minutes. After a brief centrifugation and washing of the pellet with sterile water, 150 μl of extraction buffer (200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA, and 0.5% SDS) was added to the tube. Nematodes were crushed with a conical grinder, which fit the tube exactly, actioned by hand or mechanically (Treff, Degersheim, Switzerland). The lysate was incubated at −20 C for 10 min after the addition of 0.5 volume of 3 M sodium acetate, pH 5.2. After centrifugation and transfer of the supernatant to a new tube, nucleic acids were precipitated with 1 volume of isopropanol at room temperature for 30 min and pelleted by centrifugation at 13,000 rpm for 15 min. The pellet was washed and redissolved in 50 μl of TE buffer as before.

The other method tested involved crushing with a pipet tip a single juvenile in a drop of sterile water added to the reaction mix, as indicated by other workers (10,14).

PCRs. Ten-mer oligonucleotides of random sequence were purchased from Operon Technologies (Alameda, CA) (OPA-01 to OPA-20). Other primers tested included N-3, a highly conserved 20-mer that anneals to the rDNA region of several animals and nematodes, and MT-1, a 20-mer designed from mtDNA-sequence information of *M. incognita* and used in previous studies (3,10).

TABLE 1. *Meloidogyne* populations used in the study

Population Code	Species	Country	Chromosome Number	Race[a]
Populations from the collection of NCSU[b]				
I-68	*M. incognita*	North Carolina	41–43	Race 1
I-2152	*M. incognita*	Nigeria	45	Race 2
I-2337	*M. incognita*	Argentina	36	Race 3
I-527	*M. incognita*	Texas	42	Race 4
I-RU	*M. incognita*	Spain	42	ND
A-523	*M. arenaria*	Texas	53	Race 1
A-413	*M. arenaria*	Nigeria	53–54	Race 1
J-MB	*M. javanica*	Spain	43	
J-76	*M. javanica*	Georgia	42	
J-93	*M. javanica*	Brazil	42	
H-86	*M. hapla*	Virginia	17	Race A
H-230	*M. hapla*	Chile	48	Race B
Populations from the collection of IRTA[c]				
IRTA-32[d]	*M. incognita*	Spain		
IRTA-35[d]	*M. incognita*	Spain		
IRTA-CA[e]	*M. incognita*	Spain		
IRTA-29[d]	*M. arenaria*	Spain		
IRTA-01[d]	*M. javanica*	Spain		
IRTA-CB[e]	*M. javanica*	Spain		

[a] Race classification according to North Carolina differential test for *Meloidogyne* species and races (6), except races A and B of *M. hapla*, which are cytogenetic races. ND = Not determined.
[b] Code of the nematode collection of North Carolina State University, Raleigh.
[c] Code of the nematode collection from the Institut de Recerca i Tecnologia Agroalimentaries, Barcelona, Spain.
[d] Populations maintained in axenic culture started from a single-egg mass.
[e] Populations taken directly from plants grown in an infested field.

Both were purchased from Bio-Synthesis (Madrid, Spain). PCRs were performed in 25 μl of reaction mix containing about 25 ng of template DNA, 1 unit of Taq polymerase (Pyrostase, Bio-Synthesis, Madrid), 0.2 mM dNTPs, 0.1% Triton X-100, 15 ng of primer, and 1× reaction buffer. This buffer was the same as the one supplied by the enzyme manufacturer (50 mM Tris-HCl, pH 9.0; 20 mM $[NH_4]_2SO_4$; 1.5 mM $MgCl_2$; and 0.005% BSA) but was supplemented with 50 mM ClK, and the concentration of $MgCl_2$ was increased to 2.5 mM. A drop of mineral oil was added on top of the reaction mix. The reaction was run for 35 cycles in a water-cooled thermal cycler (GTC-1, Precision Scientific, Chicago, IL) under the following conditions: denaturation at 94 C for 1 min, annealing at 36 C for 1 min, and extension at 72 C for 1 min. Half of the reaction product was taken from the tubes, loaded in a 1.4% agarose gel, and electrophoresed at 5 V per centimeter for 1 h using 1× TBE buffer. DNA bands were visualized under ultraviolet light after the gel was stained per milliliter with 0.5 μg of ethidium bromide solution. A negative control without DNA was included in all the reactions.

To evaluate the influence of the polymerase source on the reproducibility of the reaction, two additional enzymes were used in several experiments: Replitherm (Epicentre Technologies, Madison, WI) and Amplitaq (Perkin-Elmer Hispania, Madrid, Spain). The enzymes were used according to the manufacturers', instructions. All reactions were repeated at least two times and in the case of primer OPA-01, at least 10 times.

RESULTS

Primer selection. To find a good primer for species identification, several primers were used to amplify DNA from one popu-

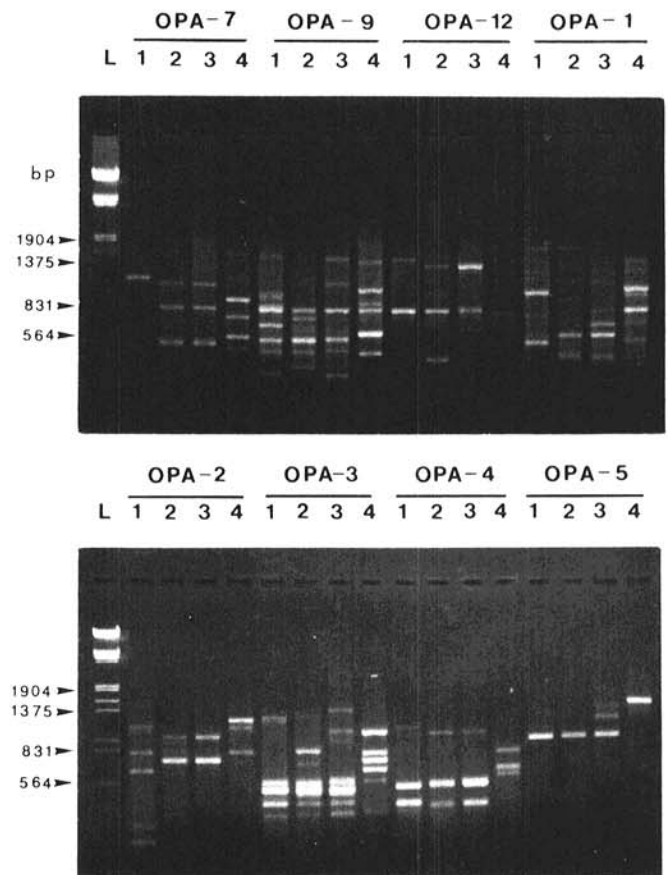

Fig. 1. Polymorphic bands of amplified DNA produced in the four species of *Meloidogyne* with eight primers. Populations from North Carolina State University, Raleigh. Lane L, size marker, lambda DNA cut with *Eco*RI/*Hind*III. Lane 1, *M. incognita*, population I-68. Lane 2, *M. arenaria*, population A-523. Lane 3, *M. javanica*, population J-MB. Lane 4, *M. hapla*, population H-86. Arrows to the left indicate band size in base pairs (bp).

lation of each species (Fig. 1). Three groups of primers were discerned from the amplification. A number of primers produced incomplete or very weak amplification with some species and were discarded as useless (OPA-06, -08, -10, -14, -15, -16, -17, -19, N-3, and MT-1). A second group of primers provided band patterns that allowed the separation of one, two, or three species. *M. javanica* and *M. arenaria* populations usually were grouped together and separated from *M. incognita* and *M. hapla* (OPA-02, -03, -04, -05, -07, -09, -11, -12, -13, -18, and -20). Finally, one primer (OPA-01) produced amplification patterns that allowed the identification of the four species (Fig. 2). After repeated amplification with OPA-01, certain bands were consistent and could be considered diagnostic. The size of these bands was 1,000 and 540 base pair (bp) for *M. incognita*, 580 bp for *M. arenaria*, 660 and 580 bp for *M. javanica*, and 1,550, 1,100, and 850 bp for *M. hapla*. An additional band of 1,020 bp was present in the population from race A of *M. hapla* but was not present in the population from race B. Reactions with primers N-3 and MT-1, which have known homology to the nematode genome, did not produce better amplifications than did completely random primers.

Intraspecific and intrapopulation variation. All of the populations listed in Table 1 were analyzed with the primers that proved useful (Table 2) in detecting intraspecific variation. Most of the primers tested clearly differentiated races A and B of *M. hapla*.

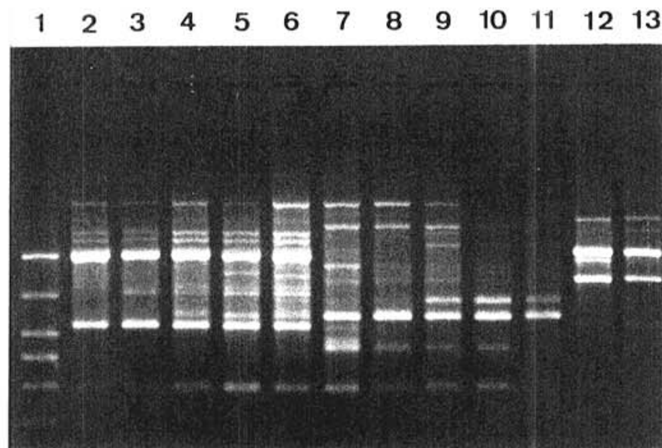

Fig. 2. Polymorphic bands of amplified DNA produced with the primer OPA-01 and *Meloidogyne* populations from North Carolina State University, Raleigh. Lane 1, size marker. Sizes 1,000, 700, 500, 400, 300, and 200 base pairs (bp). Lanes 2–6, *M. incognita*, populations I-68, I-2152, I-2337, I-527, and I-RU, respectively. Lanes 7 and 8, *M. arenaria*, populations A-523 and A-413. Lanes 9–11, *M. javanica*, populations J-76, J-93, and J-MB. Lanes 12 and 13, *M. hapla*, populations H-86 and H-230.

TABLE 2. Primers that produced polymorphisms separating one, two, or three species of *Meloidogyne*

Code	Sequence (5′ to 3′)	Separation[a]
OPA-01	CAGGCCCTTC	i, a, j, h (race a, b)
OPA-02	TGCCGAGCTG	i, h (race a, b)
OPA-03	AGTCAGCCAC	h, a (pop 523, 413)
OPA-04	AATCGGGCTG	i, h
OPA-05	AGGGGTCTTG	h
OPA-07	GGTCCCTGAC	i, h (race a, b)
OPA-09	GGGTAACGCC	i, h, a (pop 523, 413)
OPA-11	CAATCGCCGT	i, h, a (pop 523, 413)
OPA-12	TCGGCGATAG	i, a (pop 523, 413)
OPA-13	CAGCACCCAC	i, h
OPA-18	AGGTGACCGT	h (race a, b)
OPA-20	GTTGCGATCC	i, h, a (pop 523, 413)

[a] Indicates the species and races or populations distinguished from other species. i = *M. incognita*; a = *M. arenaria*; j = *M. javanica*; h = *M. hapla*. Populations (A-523 and A-413 from North Carolina State University, Raleigh).

The reactions of several primers that did not differentiate *M. arenaria* from *M. javanica* revealed polymorphisms between the two populations of *M. arenaria* (Table 2). Although there were occasionally some minor polymorphic bands, no primer revealed clear polymorphisms among the four races of *M. incognita* or the three populations of *M. javanica*. Intrapopulation variation was assessed by comparing the amplification products obtained by crushing one juvenile in a drop of sterile water. Six juveniles from a single-egg mass of the field population of *M. incognita* and nine from different egg masses of the field population of *M. javanica* were compared (Fig. 3). No differences were apparent between the individuals, apart from the usual nonreproducible minor bands. Intrapopulation variation was not evaluated in the other populations studied because all of the populations were started from a single-egg mass.

Reproducibility of the method. The reproducibility of the method was assessed by comparing the amplification patterns obtained in different reactions. The reaction products were characterized by the appearance of one to five clearly reproducible, intense bands and many minor bands varying in number and intensity between different runs with the same DNA. Many reactions were made by changing the reagent source and using DNA from different extractions. Although the diagnostic bands, were always present, the presence and abundance of minor bands could not be correlated with any change in the variables of the reaction. Two replications usually were enough to determine which bands were reproducible. Enzyme origin can be a major source of variation, as shown in Figure 4. Three enzymes amplified the diagnostic bands, but in relation to Pyrostase, which was used in most reactions, Amplitaq tended to amplify shorter fragments, while Replitherm amplified longer fragments. The proportion of artifactual bands was negligible.

When different methods of DNA extraction were compared, the miniprep was as efficient as the standard method in producing DNA suitable for amplification (Fig. 4). The amplification product obtained by crushing a juvenile in water showed the diagnostic bands of the species with fewer minor bands (Fig. 4). The extraction of DNA by the standard method yielded about 250 μg of DNA, which is enough for thousands of PCRs, but required the collection of eggs from 20 to 30 inoculated plants. The miniprep extraction yielded DNA for 25–30 reactions and worked equally well with eggs, females, and juveniles. The miniprep could be scaled down to a single-egg mass, producing DNA for about five reactions, but it did not work with a single female or juvenile. The crushing of a single juvenile allowed two reactions. The efficiency of the reaction using this method was reduced, however, and in our hands, about half of the reactions attempted did not yield any amplification product. In contrast, all the reactions with

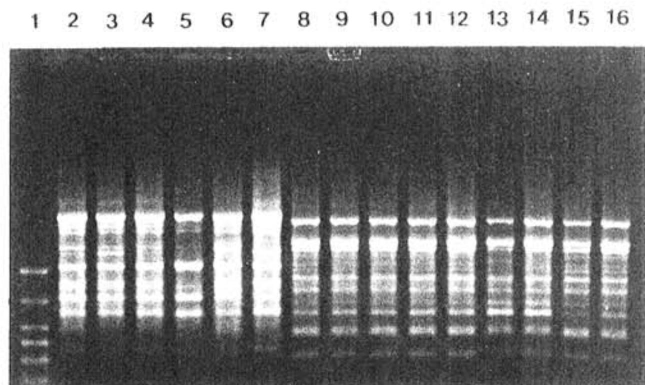

Fig. 3. Intrapopulation variation of two field populations. *Meloidogyne* populations from the Institut de Recerca i Tecnologia Agroalimentaries, Barcelona, Spain. Lane 1, size marker. Sizes 1,000, 700, 500, 400, 300, and 200 base pair (bp). Lanes 2–7, amplification products of six juveniles from the same egg mass of *M. incognita*, population IRTA-CA. Lanes 8–16, amplification product of nine juveniles from different egg masses of *M. javanica*, population IRTA-CB. In all cases, juveniles crushed in a drop of water were added to a reaction primed with OPA-13.

template DNA from the standard method or the miniprep were successful.

DISCUSSION

The results presented here show that it is possible to use RAPD-PCR to identify the four major species of the genus *Meloidogyne*. After we tested 22 primers, one primer annealed to loci with polymorphisms that are diagnostic at the species level. The degree of taxonomic resolution of the technique might be improved by testing more primers. The number of ten-mers of random sequence is virtually unlimited. This is a clear advantage over isozyme studies, in which the number of available enzymatic systems is limited. Through additional testing, primers that act as taxonomic markers for specific populations within species could be found. The set of populations considered here is obviously small and should be enlarged to assess the validity of the data presented; however, the populations included in this study do represent a broad geographic range.

To be useful for diagnostic purposes, RAPD-PCR has to be uncomplicated and reproducible. The technique is simple, rapid (three to six hours depending on the thermal cycler), and safe because it does not involve the use of radioactive isotopes. One notable feature of PCR in relation to RFLP studies, which require microgram amounts of DNA, is its ability to work with crude preparations of DNA in picogram amounts. This makes it possible to analyze as little material as a single juvenile or egg, which is useful in studies of genetic variation in or diagnosis of mixed populations. For in-depth studies of established collections, however, miniprep extraction may be preferable, given its higher reproducibility and higher percentage of successful amplifications.

Reproducibility of results is the most critical point for the taxonomic applications of the technique. The number and, to a lesser extent, the intensity of bands clearly vary from one experiment to another. Usually, one to five bands are very reproducible with each primer. In addition to these, a variable number of less intense bands appears in every reaction. As a result, several replications with the same primer must be made to determine which bands are reproducible in their entirety. It is also essential to work under the same conditions and with the same enzyme. Until an effort toward standardization is made, the results of one laboratory will be difficult to extend directly to others, given the enormous variety of available thermal cyclers and reagent sources.

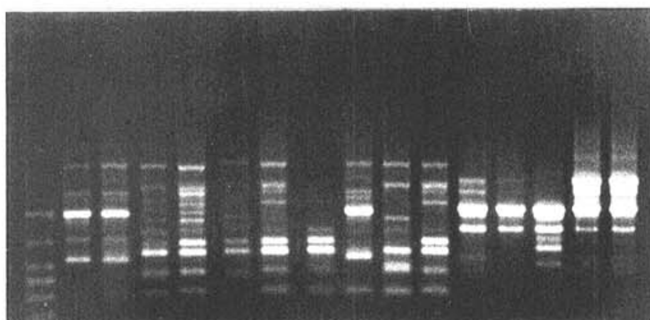

Fig. 4. Comparison of DNA-extraction methods and reproducibility of polymorphic bands of amplified DNA primed with OPA-01. *Meloidogyne* populations from North Carolina State University, Raleigh (designated I, A, J, and H) and from Institut de Recerca i Tecnologia Agroalimentaries, Barcelona, Spain (designated IRTA). Lane 1, size marker. Sizes 1,000, 700, 500, 400, 300, and 200 base pair (bp). Lanes 2–7, DNA extracted with miniprep. Lane 8, DNA extracted from crushed juvenile. Lanes 9–16, DNA extracted with standard method. Lanes 2 and 3, *M incognita*, populations IRTA-32 and IRTA-35. Lane 4, *M. arenaria*, population IRTA-29. Lanes 5–8, *M. javanica*, population IRTA-01 with DNA extracted from eggs, juveniles, females, and a single crushed juvenile, respectively. Lane 9, *M. incognita*, population I-68. Lane 10, *M. arenaria*, population A-523. Lane 11, *M. javanica*, population J-MB. Lanes 12–16, comparison of the effect of enzyme source on the amplification. DNA of *M. hapla*, population H-86, amplified in reactions made with Pyrostase (lanes 12 and 13), Amplitaq (lane 14), and Replitherm (lanes 15 and 16) during different days.

In addition to taxonomic applications, RAPD-PCR clearly is useful for the detection of genetic variability. In this capacity, many polymorphisms were detected between the two races of *M. hapla* and among the three populations of *M. arenaria* that have the same esterase pattern (7). In contrast, the band patterns of the four races of *M. incognita* were very similar. This agrees with the fact that the two races of *M. hapla* genetically differ widely in their mode of reproduction and their chromosome number, while *M. incognita* races, defined by a test of differential hosts, have a highly similar genetic background. A wide variation in *M. arenaria* populations also has been reported (7,8). None of the numerous polymorphisms between the two races of *M. hapla* can be considered diagnostic until more populations of both are studied. In general, *M. arenaria* and *M. javanica* showed the highest degree of band sharing, and *M. incognita* shared some bands with them, while *M. hapla* had very few bands in common with the other three species. This agrees with the currently accepted grouping of these species, deduced from cytogenetic and isozyme analysis (8,16), but there is no current agreement on how RAPD data should be treated for genetic analysis. Some authors (9) have treated RAPD bands and RFLPs similarly, estimating genetic similarities of populations from the proportion of shared to total bands. This approach is sustained by the fact that the source of DNA polymorphisms revealed by RAPDs is somewhat similar to RFLPs, consisting of insertions or deletions between priming sites and single-base changes that cause mismatches in priming sites. However, in contrast with RFLPs, RAPD bands are biased toward sizes under 2 kb, determined by the activity of the enzyme. Moreover, the mismatching allowed by the low-stringency annealing temperature leads to imprecise recognition of priming sites. As a consequence, some caution is necessary before inferring genetic relationships from RAPD data.

No intrapopulation variation was detected in two field populations of *M. incognita* and *M. javanica*. This is common because of the reproduction of these species by mitotic parthenogenesis. Nevertheless, the existence of variation in the form of mixtures of clones originated by mutation cannot be discarded. Its detection would require specific experiments with adequate sampling strategies. More intrapopulation variability is to be expected in field populations of *M. hapla* race A, which reproduces by facultative parthenogenesis. However, no field isolates of race A were available for this study. In any case, the bands proposed as diagnostic appear to be conserved in populations originating from widely different places and are not likely to vary within populations.

The evidence presented here indicates that the RAPD-PCR technique should be a useful tool for diagnosis and for addressing many unresolved questions of genetic variation and population genetics of root-knot nematodes.

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