Characterization and Phylogenetic Analysis of Four Root-Knot Nematode Species Using DNA Amplification Fingerprinting and Automated Polyacrylamide Gel Electrophoresis

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Species identification and quantification of genotypic diversity in root-knot nematodes were achieved by DNA amplification fingerprinting (DAF). Purified DNA from isolates of *Meloidogyne arenaria*, *M. incognita*, *M. javanica*, and *M. hapla* was amplified by low-stringency amplification reactions with 20 single-octamer primers of arbitrary sequence. Amplification products were separated by electrophoresis in precast polyacrylamide minigels and visualized by silver staining with an automated electrophoresis and staining unit (PhastSystem). Fingerprinting data were analyzed with PAUP 3.1.1 using the Wagner parsimony method. Fourteen primers successfully amplified nematode DNA. Five primers revealed very conspicuous polymorphisms between species, while the remaining primers showed moderate to small differences. Polymorphisms between populations of the four species from the southeastern United States could also be detected. Phylogenetic analyses for the four species showed closer relatedness between *M. arenaria* and *M. javanica* than between *M. arenaria* and *M. incognita*. *M. hapla* was most distant from *M. arenaria*, *M. javanica*, and *M. incognita*. These results are in agreement with phylogenetic trees derived from different characters. DAF utilizing automated polyacrylamide gel electrophoresis and silver staining is highly reproducible and offers excellent resolution, optimal sensitivity, unequaled speed, and the simplicity of automation.

Root-knot nematodes are sedentary endoparasitic plant parasites of a wide variety of agronomic crops. They are distributed worldwide and, in conjunction with secondary invading pathogens, are responsible for substantial crop losses. Average yearly losses are on the order of 5%, but destruction in developing countries greatly exceeds this level (Sasser and Carter 1985). Of the more than 50 described species, *Meloidogyne arenaria*, *M. javanica*, *M. incognita*, and *M. hapla* are responsible for more than 90% of this destruction (Eisenback *et al.* 1981). Infection is initiated by second-stage juveniles (J2), which hatch from eggs in the soil and penetrate roots near the growing tips. With the onset of feeding, an elaborate change in host physiology is elicited, leading to the formation of specialized feeding cells (Hussey 1985). These structures, termed giant cells, sustain further development and reproduction of the now sedentary nematodes. The females swell and become globular, deposit eggs in gelatinous sacs on the root surface, and eventually die. Reproduction in the economically important species is by mitotic parthenogenesis, except for *M. hapla*, which predominantly reproduces by facultative meiotic parthenogenesis, but cross-fertilization can occur.

Root-knot nematodes are controlled by chemical means, host resistance, and crop rotations in combination with sanitary methods. The decreased availability of suitable chemical compounds and increased environmental awareness make nonchemical control strategies more desirable. The implementation of crop rotations and the use of resistant cultivars require timely identification of species and host races (populations within species that differ in their host ranges). Root-knot nematode species are mainly identified by cumbersome and often unreliable analysis of the perineal patterns of adult female nematodes (Eisenback 1985). Race designation is exclusively by time-consuming and extensive host range tests (Eisenback *et al.* 1981). To date, practical procedures for preplanting identification of root-knot nematode species and races are not available.

New biochemical and molecular methods to identify *Meloidogyne* species are emerging. The analysis of isozymes allows species identification of single adult females (Esbenshade and Triantaphyllou 1990), but it is unable to separate races. Restriction fragment length polymorphism (RFLP) procedures have been developed to separate and characterize important species and populations within species (Carpenter *et al.* 1992; Castagnone-Sereno *et al.* 1993; Curran *et al.* 1986; Garate *et al.* 1991; Piotte *et al.* 1992; Powers and Sandall 1988; Powers *et al.* 1986; Xue *et al.* 1992). Polymerase chain reaction (PCR) strategies, however, offer increased sensitivity and speed for identification and characterization of species, populations, and host races. Recently, Powers and Harris (1993) designed PCR primers to polymorphic regions of the mitochondrial genome to discriminate five root-knot nematode species. DNA fingerprinting by random amplified polymorphic DNA (RAPD) analysis and assessment of amplifica-

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tion patterns by agarose gel electrophoresis has been employed to distinguish between the cyst nematodes *Heterodera cruciferae* and *H. schachtii* (Caswell-Chen *et al.* 1992) and four root-knot nematode species (Cenis 1993). Furthermore, the data were used to evaluate phylogenetic relationships of *H. schachtii* populations. Evolutionary relationships in *Meloidogyne* have not been completely resolved; cytological characteristics (Triantaphyllou 1985), isozymes (Dickson *et al.* 1971; Esbenshade and Triantaphyllou 1987), and RFLP data (Castagnone-Sereno *et al.* 1993; Garate *et al.* 1991; Powers and Sandall 1988; Xue *et al.* 1992) have been used for phylogenetic constructions.

In this study, we employed a sensitive DNA fingerprinting technique to investigate genotypic diversity in species and populations of the four common root-knot nematodes *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla*. This strategy, termed DNA amplification fingerprinting (DAF) (Caetano-Anollés *et al.* 1991a,b), relies on PCR-like amplification of DNA regions directed by a single arbitrary primer. Amplified products are visualized in polyacrylamide gels by silver staining. In comparison to RAPD approaches (Welsh and McClelland 1990; Williams *et al.* 1990), DAF uses shorter primers, less stringent reaction conditions, polyacrylamide gel electrophoresis (PAGE), and silver staining, leading to increased production and visualization of amplification products.

Obtained "fingerprints" were used to distinguish species and populations of the four major root-knot nematode species and to construct phylogenetic trees for these four species as well as for populations within *M. arenaria* and *M. incognita*. To simplify and speed up analyses, we developed an automated electrophoresis and staining procedure, using the Pharmacia PhastSystem workstation.

RESULTS

Meloidogyne DNA is reproducibly amplified.

The value of DAF for species identification and phylogenetic analyses depends largely on its reproducibility. In order to apply DAF to root-knot nematodes, the optimum conditions for DNA amplification were determined. Under the chosen conditions (see Materials and Methods), DNA amplification bands ranging from 100 bp through several kilobase pairs were produced. When more than one sample of the same DNA preparation was amplified in a particular experiment, the banding patterns obtained were identical. Varying the template DNA concentration from 0.1 to 10 ng per 10-µl reaction did not affect the amplification products. Moreover, DAF was also reproducible when runs performed on different days were compared: even minor bands were preserved in separate amplifications. Figure 1 shows the products of two amplification experiments performed on different days. Results of similar quality were obtained with all other primers that yielded amplification products. Amplification of three different DNA preparations from the same *M. arenaria* population also produced identical amplification patterns. These DNA extracts were prepared by different workers with modified protocols involving CsCl gradient purification, extracted on different days (over a 2-yr period), and extracted from cultures maintained in different pots and in two separate greenhouses. Furthermore, the condition of the DNA in these preparations ranged from high-quality to extensively degraded. The degraded DNA preparation also contained substantial amounts of RNA contamination.

DAF readily discriminates *Meloidogyne* species and populations.

A total of 36 *Meloidogyne* populations, shown in Table 1, were included in this study. Fingerprints were obtained with 14 of the 20 primers tested (Table 2). These allowed different degrees of species discrimination. Most primers revealed between 10 and 20 major bands per species. Five primers (8.5b, 8.6c, 8.6d, 8.6j, and 8.6k) yielded amplification products with very conspicuous differences between the four species. Figures 1 and 2 illustrate the quality of species discrimination achieved. For example, primer 8.6d (Fig. 1) produced a double band at approximately 310 bp with DNA from *M. arenaria*, thereby discriminating this species from the others, although *M. javanica* patterns were relatively similar to those of *M. arenaria*. *M. incognita* and *M. hapla* could be readily distinguished by bands of approximately 350 and 500 bp, respectively. Primer 8.6c (Fig. 2) produced a pattern of three bands between approximately 300 and 370 bp unique to *M. arenaria*. *M. incognita* yielded three bands between approximately 200 and 290 bp and none between approximately 300 and 600 bp. A pattern of three bands between approximately 240 and 360 bp was diagnostic for *M. javanica*. Amplification patterns for *M. hapla* showed two very conspicuous products of approximately 430 and 610 bp. Five other primers (8.6i, 8.7g, 8.7h, 8.7 l, and 8.7m) produced patterns with varying numbers of polymorphic bands between species, and four (8.6b, 8.7c, 8.7i, and 8.7j) revealed only few species polymorphisms. Patterns produced by primer 8.7i are shown in Figure 3.

Polymorphisms within species were also detected. Primer 8.6i revealed a strong intraspecific polymorphism with limited specificity for *M. arenaria* host race 2: a band of approximately 160 bp was present in all five *M. arenaria* race 2 isolates tested and was absent in 53% (eight out of 15) of *M. arenaria* race 1 isolates tested. A partial data set is shown in Figure 4. Populations of *M. incognita* were generally very homogeneous, although some conspicuous polymorphisms were present among isolates. Isolate MI3-GA, with about 24% nonshared bands, showed a high degree of difference from other *M. incognita* isolates. Reproducible differences between the two *M. javanica* populations were also revealed by primers 8.6b, 8.6j, and 8.7h. The two *M. hapla* populations yielded DAF patterns very different from those of the other species (Figs. 1–3). They could be readily discriminated from one another with primers 8.5b, 8.6d (Fig. 1), 8.6i, 8.6j, 8.7c, 8.7i (Fig. 3), and 8.7m, but were very similar or identical with the other primers. Primers 8.6b, 8.6c, 8.6d, 8.6i, 8.6j, and 8.7h were chosen to fingerprint the total set of 20 *M. arenaria* and 12 *M. incognita* isolates. Fingerprint patterns were subjected to phylogenetic analysis.

Phylogenetic analysis of DAF patterns.

Scoring the presence or absence of bands in DAF gels was used to create three distinct data matrices. Data set 1 was derived from fingerprinting data from four *M. arenaria*, two *M. incognita*, two *M. javanica*, and two *M. hapla* isolates, pro-

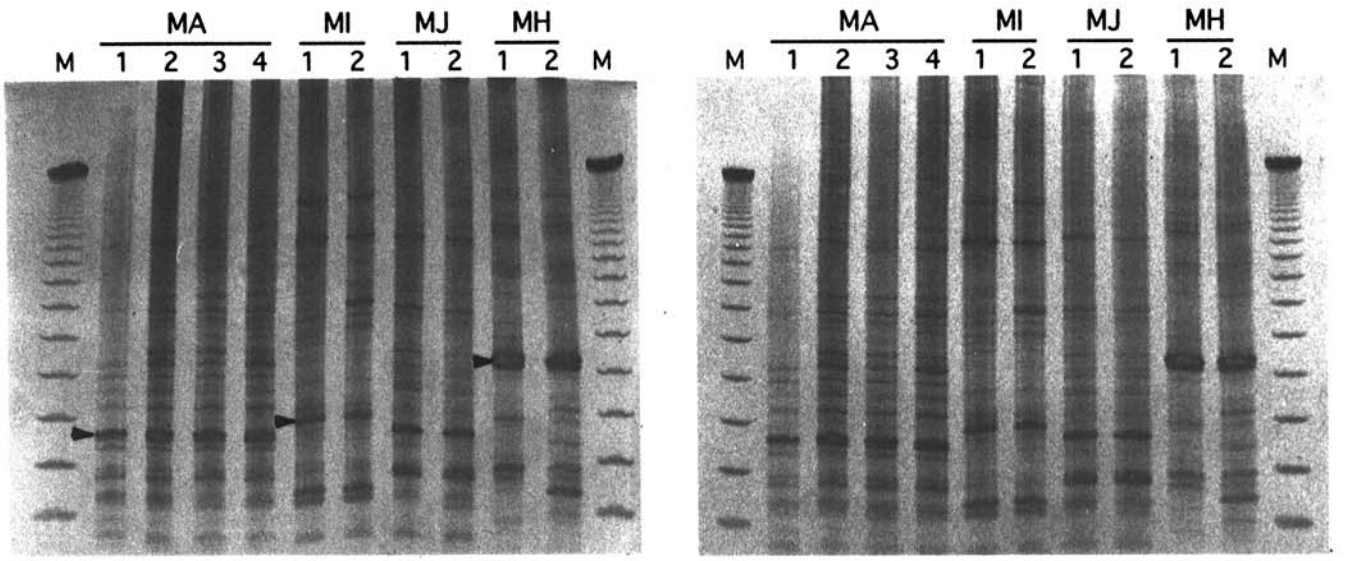

Fig. 1. Two silver-stained gels of DNA amplification products repeated on different days. Identical banding patterns are produced for individual populations. The primer is 8.6d. The isolates are as follows: in the *Meloidogyne arenaria* (MA) group, 1 = MA1-NC1, 2 = MA1-SC, 3 = MA2-SC1, and 4 = MA2-SC2; in the *M. incognita* (MI) group, 1 = MI3-SC and 2 = MI4-NC2; in the *M. javanica* (MJ) group, 1 = MJ1 and 2 = MJ2; and in the *M. hapla* (MH) group, 1 = MH1 and 2 = MH2. The isolates are described in Table 1. Lane M is the 123-bp DNA ladder. Species-specific amplification products are indicated by arrows (see Results).

Table 1. *Meloidogyne* populations used for DNA amplification fingerprinting

Species	Designation	Culture name	Race	Origin[a]
M. arenaria	MA1-NC1	Bladen	1	Bladen Co., N.C. (1)
	MA1-NC2	MA P	1	Martin Co., N.C. (1)
	MA1-SC	SC1	1	Barnwell Co., S.C. (2)
	MA1-GA1	Baker	1	Baker Co., Ga. (3)
	MA1-GA2	Benson 1	1	Tift Co., Ga. (3)
	MA1-GA3	Benson 2	1	Tift Co., Ga. (3)
	MA1-GA4	Blakley	1	Early Co., Ga. (3)
	MA1-GA5	Decatur	1	Decatur Co., Ga. (3)
	MA1-GA6	Donaldsonville	1	Seminole Co., Ga. (3)
	MA1-GA7	Gibb's Farm	1	Worth Co., Ga. (3)
	MA1-GA8	Gopher	1	Tift Co., Ga. (3)
	MA1-GA9	Headland	1	Headland, Ala. (3)
	MA1-GA10	Hussey	1	Unknown (3)
	MA1-GA11	Mitchell	1	Mitchell Co., Ga. (3)
	MA1-FL	Live Oak	1	Live Oak, Fla. (3)
	MA2-VA	54	2	Virginia (1)
	MA2-NC1	NTP	2	North Carolina (1)
	MA2-NC2	83	2	Cumberland Co., N.C. (1)
	MA2-SC1	Govan	2	Barnwell Co., S.C. (2)
	MA2-SC2	Pelion	2	Barnwell Co., S.C. (2)
M. incognita	MI1-NC1	Avoca36	1	Bertie Co., N.C. (1)
	MI1-NC2	Avoca85-1	1	Bertie Co., N.C. (1)
	MI2-NC	MI2Kng	2	North Carolina (1)
	MI2-SE	MI2EM	2	Southern United States (1)
	MI3-NC1	MI3EM	3	Rocky Mt., N.C. (1)
	MI3-NC2	99COT	3	North Carolina (1)
	MI3-SC	Witcher	3	Pickens Co., S.C. (1)
	MI3-GA	Emanuel	3	Emanuel Co., Ga. (4)
	MI3-FL	JAY	3	Jay Co., Fla. (4)
	MI3-MX	JYE	3	Mix from Florida, Georgia, and South Carolina (4)
	MI4-NC1	MI4Kng	4	North Carolina (1)
	MI4-NC2	SPG28	4	Rockingham Co., N.C. (1)
M. javanica	MJ1	MJUSDA	NA	Unknown (5)
	MJ2	MJNCSU	NA	Rocky Mt., N.C. (1)
M. hapla	MI1	MHNCSU	?	Rocky Mt., N.C. (1)
	MH2	MHUTK	A	Warren Co., Tenn. (6)

[a] Populations were obtained from (1) K. R. Barker, North Carolina State University, Raleigh; (2) Clemson University, Clemson, South Carolina; (3) J. P. Noe, University of Georgia, Athens; (4) R. S. Hussey, University of Georgia, Athens; (5) R. N. Huettel, U.S. Department of Agriculture, Hyattsville, Maryland; and (6) E. C. Bernard, University of Tennessee, Knoxville.

duced by the 14 successful primers. Data sets 2 and 3 contained scores from the six selected primers used to fingerprint 20 *M. arenaria* and 12 *M. incognita* isolates, respectively. Data sets 1, 2, and 3 contained 400, 72, and 160 scorable bands, respectively. Phylogenetic analysis of data set 1 by the Wagner parsimony method of PAUP (Phylogenetic Analysis Using Parsimony) 3.1.1 resulted in two very similar, equally parsimonious minimal phylogenetic trees (Fig. 5). The trees differ in the arrangement of the two *M. arenaria* race 2 isolates, but are otherwise identical. Table 3 shows the observed character differences (the number of actual nonshared bands) and the homoplasy values (the number of additional character state changes necessary for the observed character differences to conform with a phylogenetic tree) for the four *M. arenaria*, two *M. incognita*, two *M. javanica*, and two *M. hapla* populations. *M. arenaria* appeared more closely related to *M. javanica* than to *M. incognita* and *M. hapla*. *M. hapla* was least related to the three other species. The absolute distance values of *M. hapla* with respect to the other populations were in general greater than 200 nonshared bands (>50%) (Table 3). Distances from *M. arenaria* to *M. javanica* averaged 130 (32.5%) nonshared bands and from *M. arenaria* to *M. incognita* averaged 194 (48.5%) nonshared bands. Bootstrap confidence levels were 100% for all interspecific branch points, thus strongly supporting these topologies. Lower confidence values, between 50 and 75%, were obtained for the nodes within the *M. arenaria* branch. This was not surprising, because the two minimal trees exhibited differences in this region.

For the 20 *M. arenaria* and 12 *M. incognita* populations, phylogenetic analyses were conducted to reveal relationships between populations within the two species. Consensus trees of 90 and 102 equally parsimonious minimal trees were revealed by 5,000 heuristic searches of the *M. arenaria* data set (Fig. 6) and the *M. incognita* data set (Fig. 7), respectively. Within both species only comparatively small differences were observed between populations. The absolute and relative distance values ranged from two to 16 nonshared bands (3–22%) between *M. arenaria* populations and from two to 12 nonshared bands (1–8%) between *M. incognita* populations, excluding MI3-GA, which on average had distance values of 38 nonshared bands (24%) with respect to other *M. incognita* isolates. Phylogenetic analyses of isolates of both species

Table 2. Octamer primers tested for amplification of *Meloidogyne* DNA

Name	Sequence	Amplification
8.5b	CTGGACTA	Yes
8.6b	CCTGTGAG	Yes
8.6c	AACGGGTG	Yes
8.6d	GTAACGCC	Yes
8.6e	GACGTAGG	No
8.6i	GTTACGCC	Yes
8.6j	GTACTGCC	Yes
8.6k	GTAAGGCC	Yes
8.6m	GTAACGGC	No
8.6n	GTAACGCG	No
8.7c	GAGGGTGG	Yes
8.7e	CCTGGTGG	No
8.7f	CGTGGTGG	No
8.7g	CCAGGTGG	Yes
8.7h	CCTCGTGG	Yes
8.7i	CCTGCTGG	Yes
8.7j	CCTGGAGG	Yes
8.7k	CCTGGTCG	No
8.7l	CCTGGTGC	Yes
8.7m	CAGCTCGG	Yes

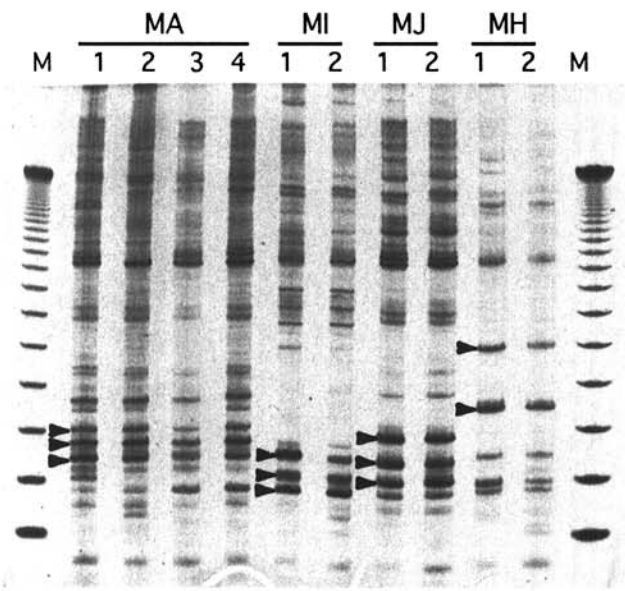

Fig. 2. Amplification using primer 8.6c produces conspicuous polymorphisms between species and *Meloidogyne arenaria* populations. The isolates are as follows: in the *M. arenaria* (MA) group, 1 = MA1-NC1, 2 = MA1-SC, 3 = MA2-SC1, and 4 = MA2-SC2; in the *M. incognita* (MI) group, 1 = MI3-SC and 2 = MI4-NC2; in the *M. javanica* (MJ) group, 1 = MJ1 and 2 = MJ2; and in the *M. hapla* (MH) group, 1 = MH1 and 2 = MH2. The isolate designations are described in Table 1. Lane M is the 123-bp DNA ladder. Species-specific amplification products are indicated by arrows (see Results).

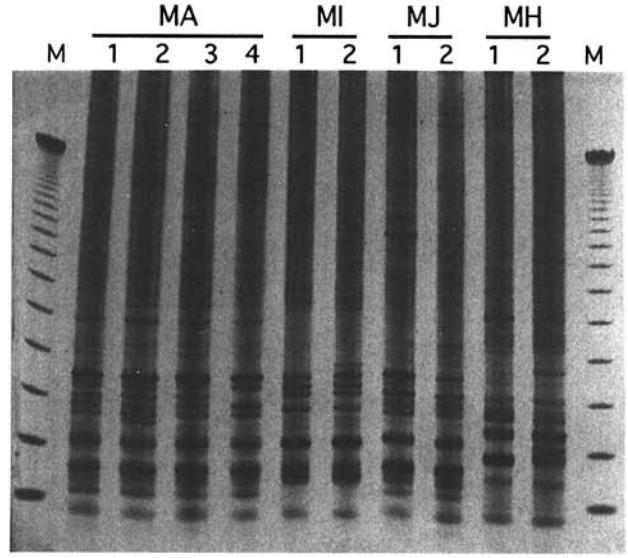

Fig. 3. Amplification using primer 8.7i produces almost identical patterns for *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* and a similar pattern for *M. hapla*. Intraspecific polymorphisms can be seen in *M. incognita*, *M. javanica*, and *M. hapla*. The isolates are as follows: in the *M. arenaria* (MA) group, 1 = MA1-NC1, 2 = MA1-SC, 3 = MA2-SC1, and 4 = MA2-SC2; in the *M. incognita* (MI) group, 1 = MI3-SC and 2 = MI4-NC2; in the *M. javanica* (MJ) group, 1 = MJ1 and 2 = MJ2; and in the *M. hapla* (MH) group, 1 = MH1 and 2 = MH2. The isolates are described in Table 1. Lane M is the 123-bp DNA ladder.

consistently produced clusters of two or more populations, indicating a higher degree of relatedness within these groups.

The phylogenetic groupings for *M. arenaria* were complex, and many branches could not be resolved. This was evident from obtaining numerous branch-point values less than 100%. The out-group MI4-NC2 as well as the in-group taxa MA1-NC2 and MA2-VA were fully resolved. A cluster containing MA1-GA1, MA1-GA2, MA1-GA3, MA1-GA5, and MA1-GA8 always formed a distinct branch, but only MA1-GA2, MA1-GA3, and MA1-GA8 were then resolved within this group. No race phenotype could be clearly associated with individual clusters of *M. arenaria*. Furthermore, populations did not cluster into clearly defined geographic groups.

Within *M. incognita*, the North Carolina populations MI3-NC1, MI3-NC2, MI4-NC1, and MI4-NC2 and the mixed population MI3-MX, formed an unresolved polytomous node. Only one North Carolina population (MI2-NC) was not contained within this cluster. All other branch points were fully resolved and were present in all minimal trees revealed by the 5,000 heuristic searches. The lack of resolution within the polytomous node was responsible for the overall generation of many equally parsimonious minimal trees. Populations did not cluster into race phenotypes. The two race 1 populations formed a branch distinct from the other races; however, both populations were from the same North Carolina county. The *M. incognita* race 3 populations from Florida (MI3-FL), Georgia (MI3-GA), and South Carolina (MI3-SC) formed three distinct branches in all searches. In amplification patterns, MI3-GA showed major differences from other *M. incognita* populations.

Bootstrap analysis of these two data sets produced poorly resolved consensus trees with confidence values for individual nodes ranging from 3 to 34% for *M. arenaria* topologies and from 9 to 71% for *M. incognita* topologies, excluding MI3-GA, whose branch point was supported by all bootstrap replicates. Eliminating from the *M. incognita* data set taxa that were unresolved (MI3-MX, MI3-NC1, MI3-NC2, MI4-NC1, and MI4-NC2) resulted in bootstrap values ranging from 61 to 96% for the remaining nodes. Data sets were also analyzed under the constraint of forcing monophyletic origin of host races. Heuristic searches identified minimal trees that

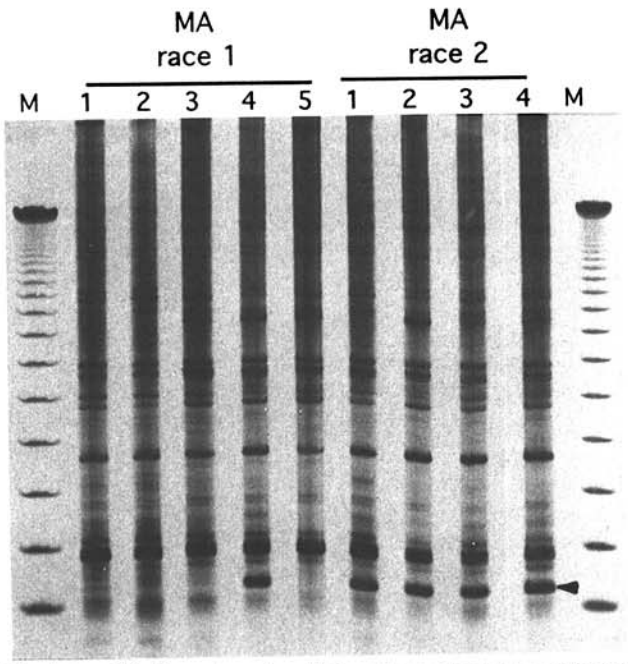

Fig. 4. Amplification using primer 8.6i produces a band at approximately 160 bp (arrow) in all *Meloidogyne arenaria* (MA) race 2 isolates, but not in most *M. arenaria* race 1 populations. The isolates are as follows: in the MA race 1 group, 1 = MA1-GA2, 2 = MA1-GA3, 3 = MA1-NC1, 4 = MA1-GA7, and 5 = MA1-SC; in the MA race 2 group, 1 = MA2-SC1, 2 = MA2-SC2, 3 = MA2-NC1, and 4 = MA2-NC2. The isolates are described in Table 1. Lane M is the 123-bp DNA ladder.

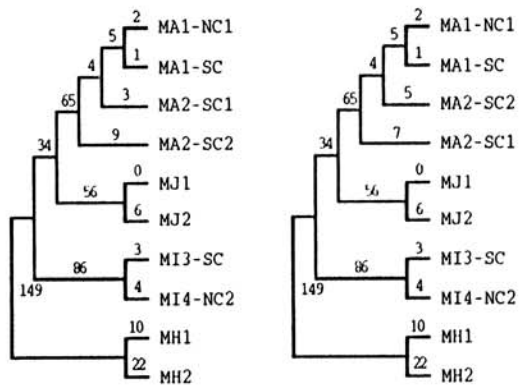

Fig. 5. Exhaustive phylogenetic searches revealed two equally parsimonious minimal trees, which differ only in the arrangement of *Meloidogyne arenaria* isolates MA2-SC1 and MA2-SC2. *M. arenaria* is most closely related to *M. javanica*, less closely related to *M. incognita*, and still less closely related to *M. hapla*. The branch labels indicate the number of character state changes. The isolate designations are as in Table 1.

Table 3. Absolute character differences[a] (below diagonal) and homoplasy values[b] (above diagonal) for the two equally parsimonious minimal trees for isolates of the root-knot nematode species *Meloidogyne arenaria* (MA), *M. javanica* (MJ), *M. incognita* (MI), and *M. hapla* (MH)

Isolate	MA1-NC1	MA1-SC	MA2-SC1	MA2-SC2	MJ1	MJ2	MI3-SC	MI4-NC2	MH1	MH2
MA1-NC1	...	0	0/8[c]	8/0[c]	8	14	38	36	26	30
MA1-SC	3	...	0	8	6	12	36	34	24	28
MA2-SC1	10	9	...	0	2	10	32	30	16	20
MA2-SC2	12	11	12	...	0	4	30	28	18	22
MJ1	130	131	128	128	...	0	0	0	0	4
MJ2	130	131	126	130	6	...	2	2	0	4
MI3-SC	193	194	191	191	165	169	...	0	0	0
MI4-NC2	196	197	194	194	166	170	7	...	0	2
MH1	224	225	225	222	186	190	203	204	...	0
MH2	241	242	243	239	201	207	226	225	22	...

[a] Number of actual nonshared bands.
[b] Number of additional character state changes necessary for the observed character differences to conform with a phylogenetic tree.
[c] Homoplasy values for the two phylogenetic trees.

were longer than those identified by searches without constraints. The number of additional steps was nine (a 5% increase) for *M. arenaria* and 18 (a 14% increase) for *M. incognita*.

DISCUSSION

The results of our phylogenetic analyses of the four species is in agreement with other investigations. Trees deduced from RFLP data (Castagnone-Sereno *et al.* 1993; Garate *et al.* 1991; Xue *et al.* 1992) are similar to our phylograms. Similar results were also obtained from isozyme analysis (Esbenshade and Triantaphyllou 1987; Dickson *et al.* 1971). One exception is the phylogeny derived from RFLP data of mitochondrial DNA by Powers and Sandall (1988), who concluded that a close relationship exists between *M. incognita*, *M. javanica*, and *M. hapla* and that *M. arenaria* was the most divergent. However, this conclusion is not supported by a recent reevaluation of the original data (T. O. Powers, personal communication). Our data are consistent with other findings that *M. hapla* is the most distantly related to the mitotic parthenogenetic species *M. arenaria*, *M. javanica*, and *M. incognita*. This finding has been suggested to indicate that amphimixis is the ancestral reproductive state (Triantaphyllou 1985; Esbenshade and Triantaphyllou 1987). Bootstrap confidence values of 100% for all nodes connecting the four species imply that all groupings are robustly supported. The finding that a DAF-derived tree is supported by established procedures, validates the use of arbitrarily amplified DNA for phylogenetic analyses at the species level.

Differences between populations within *M. arenaria* and *M. incognita* were small, as indicated by low distance matrix values. This is probably due to the mitotic parthenogenetic mode of reproduction, leading to theoretically clonal progeny. Furthermore, all isolates originated in the southeastern United States. Only relatively few markers discriminating between isolates of *M. arenaria* or *M. incognita* were revealed, which did not allow an exhaustive phylogenetic investigation. Many equally parsimonious minimal trees were identified by the Wagner parsimony method of PAUP, and a consensus of all obtained trees had to be calculated to illustrate most likely groupings. Certain populations were always grouped together in clusters or always formed distinct branches. However, others could not be resolved and therefore resulted in the construction of many equally parsimonious trees. Some of the consistent clusters appear to be due to common geographical origin. The *M. incognita* race 3 population from Georgia (MI3-GA) was a clear exception and was very different from the other *M. incognita* populations. An explanation for this observation, besides the notion that populations from that area might be different from other southeastern populations, was not found. Host races within *M. arenaria* and *M. incognita* did not cluster in distinct branches. This finding suggests that host races do not form monophyletic groups, i.e., do not originate from a common ancestor, but rather evolved in a convergent manner. Although the majority of equally parsimonious minimal trees revealed by 5,000 searches supported this hypothesis, bootstrap analyses did not place high confidence values on any of the branch points in the consensus trees for either *M. incognita* or *M. arenaria* isolates. This was not surprising, because few discriminating characters were present in the data sets, and resampling for bootstrap analyses was not likely to produce representative data sets and is of questionable significance (Wendel and Albert 1992). Furthermore, bootstrap values can be influenced by unresolved polytomous nodes (Swofford 1993; Wainright *et al.* 1993).

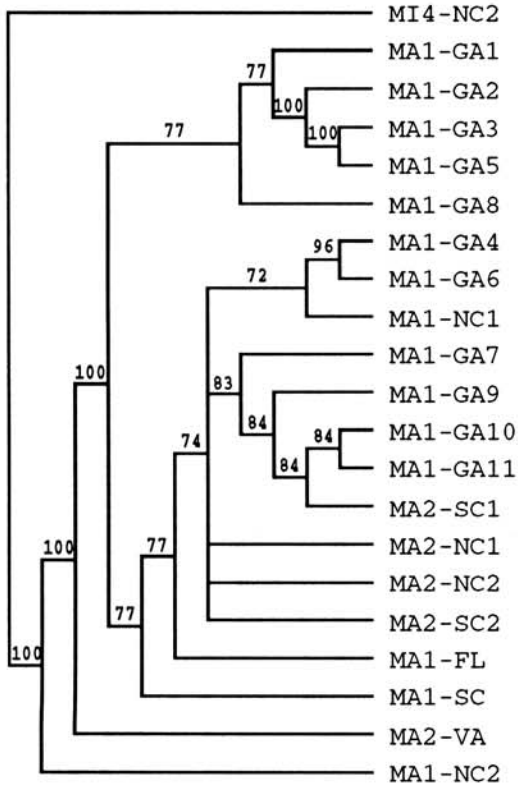

Fig. 6. Consensus tree of 90 equally parsimonious minimal trees for *Meloidogyne arenaria*. The node labels indicate the percentage of trees with the particular branching pattern shown. Individual isolates of *M. arenaria* are incompletely resolved with the available data set. *M. arenaria* isolates do not cluster according to host race designation. The isolate designations are as in Table 1.

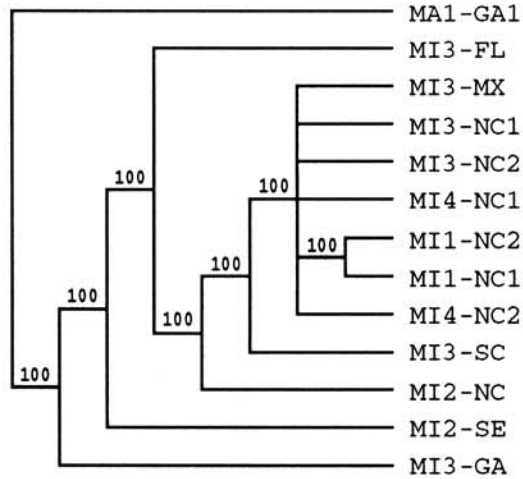

Fig. 7. Consensus tree of 102 equally parsimonious minimal trees for *Meloidogyne incognita*. The node labels indicate the percentage of trees with the particular branching pattern shown. *M. incognita* isolates cluster according to geographic origin. The two isolates of *M. incognita* race 1 (MI1-NC1 and MI1-NC2) are always grouped together. The isolate designations are as in Table 1.

For the *M. incognita* data set, bootstrap values were substantially increased by the exclusion of unresolved taxa. On the other hand, enforcing monophyletic host race origin for the phylogenetic analyses produced trees that were only slightly longer than trees without constraints. Therefore, we conclude that the phylogenetic origin of host races cannot be fully resolved by these data.

Although no race-specific polymorphisms were detected, discrimination between *M. arenaria* populations was observed, as illustrated by the conspicuous approximately 160-bp fragment amplified with primer 8.6i. The use of additional primers may reveal other markers with higher specificities for subspecific taxa, because only 20 primers were screened in this study. Three of the four species analyzed (*M. arenaria*, *M. incognita*, and *M. javanica*) reproduce by mitotic parthenogenesis (apomixis). Since offspring are clonal, it can be argued that mutations will be specific for the particular lineage. This should theoretically enhance the probability of revealing race-specific markers. However, the extensive polyploidy of root-knot nematodes and unclear phylogenetic origin of host races, as discussed above, may reduce the probability of finding race-specific markers. Most *M. hapla* populations (cytological race A) reproduce by facultative meiotic parthenogenesis. The less common but phylogenetically closely related race B is apomictic, like *M. arenaria*, *M. javanica*, and *M. incognita* (Triantaphyllou 1985). The opportunity for sexual reproduction in race A allows meiotic recombination and cross-fertilization. Increased complexity due to sex may explain difficulties in assigning host race status (which has not been done for *M. hapla*), and may decrease chances of revealing molecular markers specific for certain host range phenotypes.

Automation of DAF is likely to enhance other applications that rely on the detection of DNA polymorphisms, such as diagnostics and genome mapping. DAF can be used as a powerful tool to study population dynamics by enabling tracing of genotypes in mixed populations. The development and fate of polymorphisms in various nematode populations maintained under different environmental conditions can be evaluated and thus can reveal valuable insight in genome evolution. In future work with root-knot nematodes, populations from other geographic regions of the United States and from around the world will be subjected to phylogenetic analyses to establish global evolutionary relationships. Species- and race-specific polymorphic bands may be used to design PCR primers to identify root-knot nematodes by amplification of diagnostic DNA from crude extracts from soil or plants under high-stringency conditions. Progress in this regard would allow timely and reliable assessment of *Meloidogyne* species and race infestations before planting. A correct choice of cultivar or crop could be made, thus supporting sustainable agricultural practices.

We have successfully expanded the utility of DAF by employing the PhastSystem electrophoresis and staining unit. Electrophoresis runs were very uniform, which facilitated the comparison of gels from different experiments. The automated silver staining protocol produced high-quality results and allowed reliable scoring of banding patterns by eye or densitometry. The time and work required was significantly reduced. The procedure allowed the analysis of more than 160 samples per day, compared to 60–80 by manual PAGE and staining. The PhastSystem is already widely available and is used for isozyme-based species identification of several root-knot and cyst nematodes (Esbenshade and Triantaphyllou 1990). The versatility and simplicity of this technology is most likely to increase the use of arbitrarily amplified DNA in conjunction with the PhastSystem for a variety of applications.

METHODS

Nematode isolates and sample preparation.

Twenty *M. arenaria*, 12 *M. incognita*, two *M. javanica*, and two *M. hapla* populations were maintained in pasteurized river bottom sand in greenhouse pot cultures on tomato (*Lycopersicon esculentum* Mill.) cultivar Rutgers or peanut (*Arachis hypogea* L.) cultivar Florunner. All cultures were transferred to fresh tomato plants 50–60 days before harvest of eggs to minimize distorting host influence on fingerprinting patterns. Table 1 shows the populations tested, with their species and race designations as determined by morphological or isozyme analyses and host range tests, respectively. All *M. arenaria* race 1 isolates were confirmed by their ability to infect Florunner peanut. This cultivar is resistant to race 2. Eggs were extracted from infected root systems with 0.05% sodium hypochlorite (Hussey and Barker 1973) and purified by sucrose gradient centrifugation (Barker 1985). Eggs were ground in hand-held Tenbroek glass grinders (Baxter, McGaw Park, IL) on ice and suspended in extraction buffer (Schnick *et al.* 1990). After a 20-min incubation at 42° C, the homogenate was applied directly to a CsCl gradient and processed according to Sambrook *et al.* (1989). Purified DNA aliquots were diluted to 1 ng/µl in distilled water as stocks for amplifications.

DNA amplifications.

The reaction volume was 10 µl for all experiments. Reactions were overlaid with two drops of mineral oil for thermocycling. Reaction mixtures contained 1 ng of template DNA, 0.2 mM each nucleotide, 3 µM octamer primer, 3 mM $MgCl_2$, 1 µl of 10× reaction buffer (as supplied with enzyme), and 3 units of Ampli Taq Polymerase, Stoffel Fragment (Perkin-Elmer Cetus, Norwalk, CT). A Perkin-Elmer Cetus Thermocycler was employed for all reactions. The following step cycle file was used: 96° C (1 sec) to 30° C (1 sec) for 35 cycles. The step cycle file was followed by a time delay file (2 min at 72° C) and a soak file (8° C).

DNA from a set of four *M. arenaria* populations (MA1-NC1, MA1-SC, MA2-SC1, and MA2-SC2), two *M. incognita* populations (MI3-SC and MI4-NC2), two *M. javanica* populations (MJ1 and MJ2), and two *M. hapla* populations (MH1 and MH2) was used to screen the 20 octamer primers shown in Table 2. Primers producing *M. arenaria* or *M. incognita* intraspecific polymorphisms were used to amplify all 20 *M. arenaria* and 12 *M. incognita* populations. Experiments that revealed interesting patterns and polymorphisms were repeated at least once.

Electrophoresis and visualization.

Screening of available precast gradient (4–15, 10–15, and 8–25%) and homogeneous gels (12.5 and 20%) with a 123-bp DNA ladder (GIBCO BRL, Life Technologies, Inc., Gaithers-

burg, MD) revealed the best resolution was obtained in 10–15 and 8–25% gradient PhastGels. The 10–15% gels were used for the remainder of this study for convenience, because these gels are also used for esterase analysis of adult female root-knot nematodes (Esbenshade and Triantaphyllou 1990). Approximately 0.3 µl of each amplification reaction was electrophoresed on the Pharmacia PhastSystem automated electrophoresis unit (Pharmacia LKB, Piscataway, NJ) using precast 10–15% gradient polyacrylamide PhastGels (Pharmacia). The separation program, modified from the Pharmacia application note 18-1022-41, is shown in Table 4. The PhastSystem control unit was programmed according to the owner's manual. Samples were applied with Pharmacia 12-well sample applicators without loading dye to allow the application of sufficient amounts of DNA to each lane. The 123-bp DNA ladder was used as size standard. Gels were silver-stained and developed by a procedure adopted from Bassam and Caetano-Anollés (in press) using the PhastSystem staining unit. The developer solution was held on ice prior to utilization by the staining unit. The staining program is shown in Table 5. The development time (DEV1.07) was set at 3.8 min. The extra alarm during stain development allows customized development extension through pausing of the procedure at this step. After development, the gels were dried between glass plates to prevent wrinkling, photographed, and stored in the dark for permanent record.

Phylogenetic analysis.

For phylogenetic analysis, gel photographs were enlarged, and individual amplification products were scored as present or absent for individual nematode populations. Three data sets were obtained (see Results). Analyses were done with PAUP 3.1.1 (Swofford 1993) for Macintosh computers. All characters were entered as unordered, nondirected, and unweighted (Wagner mode for binary characters). The nature of the first data set allowed performance of exhaustive searches to identify all minimal trees. Exhaustive searches of data from the *M. arenaria* and *M. incognita* data sets exceeded available RAM. Therefore 5,000 heuristic searches, revealing local instead of global most parsimonious trees, were conducted for those sets, and consensus trees were calculated with the 50% majority rule feature of the PAUP program. The settings for the heuristic searches were the following: random addition sequence, no MULPARS option, no collapse of zero-length branches, keep only minimal trees, and TBR branch-swapping mode. Trees enforcing monophyletic origin of host races (constraint trees) were invoked for searches of data sets 2 and 3 to assess length values of trees complying with these assumptions.

For data set 1, trees were rooted by the midpoint rooting mode, since no suitable out-group could be included in the analyses. For analysis of the *M. arenaria* and *M. incognita* data sets one isolate of the corresponding opposite species was included as out-group to allow rooting by the out-group method. Trees were reproduced as cladograms with branches labeled with their corresponding distance value. Distance matrices, showing the absolute and relative number of non-shared bands in pairwise comparisons, were calculated for all data sets. Homoplasy values (low values indicate a good fit of a phylogenetic estimate to the employed data set) were determined for data set 1.

The significance of phylogenetic results was assessed by bootstrap analysis (Felsenstein 1985), featured in the PAUP program. The data set containing isolates of all four species (data set 1), was bootstrapped by 100 replications of branch-and-bound searches (MULPARS option on, "furthest" stepwise addition, and 50% consensus calculation). The *M. arenaria* and *M. incognita* data sets (data set 2 and 3, respectively) were analyzed by 100 bootstrap replications of heuristic searches with stepwise addition sequence (furthest) and TBR branch swapping with MULPARS option. Only 100 trees were saved per replication, and consensus trees were calculated following the 50% majority rule.

Table 4. Separation method file for electrophoresis of DNA amplification fingerprinting samples with the PhastSystem and 10–15% gradient PhastGels

Sample applicator down				@	SEP1.2	0000 Vh
Sample applicator up				@	SEP1.2	0002 Vh
Extra alarm to sound				@	SEP1.1	0097 Vh
SEP1.1	0400 V	10.0 mA	2.5 W	15°C		0100 Vh
SEP1.2	0400 V	01.0 mA	2.5 W	15°C		0002 Vh
SEP1.3	0200 V	10.0 mA	2.5 W	15°C		0090 Vh

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Table 5. Development method file for silver-staining DNA amplification fingerprinting gels with the PhastSystem workstation

DEV1 Ct(5, 30, 40, 50)°C=(1.0, 1.0, 1.0, 1.0)					
Extra alarm to sound @ 1.07 t=03.5min					
DEV1.01	IN=1	OUT=0	t=10.0min	T=15°C	(Fixer)[a]
DEV1.02	IN=2	OUT=0	t=02.0min	T=15°C	(Distilled H_2O)
DEV1.03	IN=2	OUT=0	t=02.0min	T=15°C	(Distilled H_2O)
DEV1.04	IN=2	OUT=0	t=02.0min	T=15°C	(Distilled H_2O)
DEV1.05	IN=3	OUT=6	t=20.0min	T=15°C	(Silver solution)[a]
DEV1.06	IN=2	OUT=0	t=00.1min	T=15°C	(Distilled H_2O)
DEV1.07	IN=4	OUT=0	t=03.8min	T=08°C	(Developer)[a]
DEV1.08	IN=5	OUT=0	t=01.0min	T=04°C	(Stop solution)[a]
DEV1.09	IN=2	OUT=0	t=03.0min	T=15°C	(Distilled H_2O)

[a] Designation of solutions as in Bassam and Caetano-Anollés (1993).

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