

# Random Amplified Polymorphic DNA Analysis of *Heterodera cruciferae* and *H. schachtii* Populations<sup>1</sup>

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**Abstract:** *Heterodera schachtii* and *H. cruciferae* are sympatric in California and frequently occur in the same field upon the same host. We have investigated the use of polymerase chain reaction (PCR) amplification of nematode DNA sequences to differentiate *H. schachtii* and *H. cruciferae* and to assess genetic variability within each species. Single, random oligodeoxyribonucleotide primers were used to generate PCR-amplified fragments, termed RAPD (random amplified polymorphic DNA) markers, from genomic DNA of each species. Each of 19 different random primers yielded from 2 to 12 fragments whose size ranged from 200 to 1,500 bp. Reproducible differences in fragment patterns allowed differentiation of the two species with each primer. Similarities and differences among six different geographic populations of *H. schachtii* were detected. The potential application of RAPD analysis to relationships among nematode populations was assessed through cluster analysis of these six different populations, with 78 scorable markers from 10 different random primers. DNA from single cysts was successfully amplified, and genetic variability was revealed within geographic populations. The use of RAPD markers to assess genetic variability is a simple, reproducible technique that does not require radioisotopes. This powerful new technique can be used as a diagnostic tool and should have broad application in nematology.

**Key words:** cyst nematode, diagnostic, DNA, *Heterodera cruciferae*, *H. schachtii*, nematode, RAPD marker, PCR, population genetics.

DNA markers have been useful for identification and phylogenetic comparison of plant-parasitic nematodes (6,21). Nematode genomic DNA has been investigated primarily through restriction fragment length polymorphisms (RFLPs). These RFLPs can differentiate species and, in some cases, races and populations of plant-parasitic nematodes (2,6,11). Mitochondrial DNA (mtDNA) has been more extensively investigated than genomic DNA because mtDNA evolves at a rapid rate, is a relatively simple molecule, and is abundant in eukaryotic cells (10,15). The sensitivity of DNA analysis has been extended to allow identification of individual *Meloidogyne* juveniles through the use of the polymerase chain reaction (PCR) (9). Despite these advances, each currently available technique suffers from one or

more limitations, such as requirement for radioisotope, intensity of labor, or the lack of sufficient sensitivity or specificity to distinguish populations.

A DNA polymorphism assay based on PCR amplification of DNA segments using single primers of arbitrary nucleotide sequence has recently been described (20). The polymorphisms detected are inherited in a Mendelian fashion, have been used as genetic markers in a variety of organisms (20), and have been used to assess genetic variability in a fungus (8). Cyst nematodes (*Heterodera* spp.) are excellent candidates for testing the applicability of this technique to plant-parasitic nematodes because they are easily cultured in the laboratory and greenhouse, adult females are large and easy to manipulate, and several geographic populations are available. Interspecific crosses between cyst nematodes have been carried out but genetic analysis has been limited because of a lack of genetic markers (7,12), and this technique potentially will allow such analyses.

*Heterodera schachtii* Schmidt and *H. cruciferae* Franklin are closely related sympatric congeners that frequently occur within the same fields in coastal California. *Heterodera schachtii* is a member of the *schachtii*

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group, whereas *H. cruciferae* is a member of the *goettingiana* group; the two are distinguishable primarily by vulval cone morphology and host range (1,13).

Genetic variability, as indicated by host-range variation, exists among geographic populations of *H. schachtii* (17). The genetic variability within and between populations of plant-parasitic nematodes is poorly understood yet is important for understanding nematode evolution, ecology, and management. The recent emphasis on nonnematicidal approaches to nematode management has also increased the desirability of assessing genetic variability within nematode populations (3). If *H. schachtii* and *H. cruciferae* are to be managed through resistance or crop rotations, genetic variation within and among populations must be characterized. Molecular markers have potential for differentiating the two species and for measuring population dynamics of interacting species.

We report the use of the polymerase chain reaction (PCR) and random amplified polymorphic-DNA (RAPD) markers to distinguish *H. schachtii* from *H. cruciferae*. In addition, we used this technique to assess the genetic variation among and within different geographic populations of *H. schachtii*.

#### MATERIALS AND METHODS

Nematode populations included *H. schachtii* and *H. cruciferae* originating from the same field in Half Moon Bay, California, *H. schachtii* populations from Salinas (C1 from L. I. Miller), Lodi, Clarksburg (2 locations), and the Imperial Valley (2 locations), California, and an inbred line of *H. schachtii* (5027-5-2 from P. R. Esbenshade). The identities of the species were confirmed by vulval cone morphology (13) before RAPD analysis. All populations were maintained on cabbage (*Brassica oleracea* cv. Copenhagen Market) in the greenhouse. Gravid, brown cysts were hand picked from cabbage roots and stored at 5 C in 0.8% NaCl for less than 7 days before DNA extraction.

**DNA extraction:** Brown cysts (ca. 150) of *H. schachtii* or *H. cruciferae* were crushed in 100  $\mu$ l of cold extraction buffer (100 mM EDTA, 0.5% SDS, 50  $\mu$ g/ml Proteinase K, pH 8.0) in a tissue homogenizer. The homogenate was transferred to a 1.5-ml microcentrifuge tube, and extraction buffer was added to a final volume of 500  $\mu$ l. Tubes were incubated in a water bath for 2 hours at 50 C, cooled on ice, and spun at 1,000 rpm for 1 minute in a microcentrifuge at 6 C. The supernatant was transferred to a new tube and extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation at 14,000 rpm for 15 minutes, approximately 400  $\mu$ l of aqueous phase was transferred to a new tube, and DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.5) and three volumes of 95% ethanol. The solution was vortexed, placed at -20 C for 1 hour, and centrifuged at 14,000 rpm for 15 minutes at 4 C with a microcentrifuge. The precipitate was rinsed once with 70% ethanol, dried under vacuum overnight, and resuspended in 100  $\mu$ l TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0). Nucleic acid concentration was determined spectrophotometrically at 260 nm. Yield from 150 cysts was typically 300–850  $\mu$ g of nucleic acid.

Single gravid, brown cysts were crushed in 10  $\mu$ l of cold, sterile, distilled water with a glass homogenizer (25  $\mu$ l size, Radnoti Glass, Arcadia, GA). The homogenate was transferred to a 0.5-ml Eppendorf tube and centrifuged at 14,000 rpm for 3 minutes at 4 C. The supernatant was discarded, and the pellet was resuspended in 10  $\mu$ l of 5% Chelex 100 resin in water (100–200 mesh, sodium form; Bio-Rad, Richmond, CA) and mixed for 10 seconds (18). Tubes were incubated at 56 C for 30 minutes in a water bath, mixed vigorously for 10 seconds, and transferred to a 100 C water bath for 8 minutes. The tube contents were mixed vigorously, centrifuged at 14,000 rpm for 3 minutes in a microcentrifuge, and the supernatant was collected.

**RAPD analyses:** Eighteen different random, decamer primers (Primer Kit A, Op-

eron Technologies, Alameda, CA) were prepared following manufacturer's directions. Primer sequences (5' to 3') were as follows: A-1, CAGGCCCTTC; A-2, TGC-CGAGCTG; A-3, AGTCAGCCAC; A-4, AATCGGGCTG; A-5, AGGGGTCTTG; A-6, GGTCCTGAC; A-7, GAAAC-GGGTG; A-8, GTGACGTAGG; A-9, GGGTAAACGCC; A-10, GTGATCGCAG; A-11, CAATCGCCGT; A-12, TCGGC-GATAG; A-13, CAGCACCCAC; A-14, TCTGTGCTGG; A-15, TTCCGAACCC; A-16, AGCCAGCGAA; A-17, GAC-CGCTTGT; A-18, AGGTGACCGT; A-19, CAAACGTCCG.

Two microliters of extract containing approximately 20–30 ng of nucleic acid extracted from multiple cysts, or 10  $\mu$ l of nucleic acid from single cysts (approximately 3  $\mu$ g nucleic acid), was amplified in 25  $\mu$ l reaction volumes with 1.5 U Taq polymerase (Promega Corp., Madison, WI). The PCR was performed in a Perkin-Elmer Cetus thermal cycler for 45 cycles of 1 minute at 94 C; 1 minute at 35 C; and 2 minute at 72 C, as previously optimized for RAPD analysis (20). Approximately 12  $\mu$ l (bulk) or 25  $\mu$ l (single cysts) of the amplification reaction was electrophoresed in a 1.7% agarose gel in TBE buffer (pH 8.0) at 70 V for approximately 1 hour. Products were visualized by ethidium bromide staining (0.5  $\mu$ g/ml) and examined on a UV transilluminator. Hind III-digested lambda and pGEM markers (Promega) were molecular size standards.

*Comparison of H. schachtii and H. cruciferae:* Total DNA was extracted from approximately 150 brown, egg-filled cysts of *H. schachtii* and *H. cruciferae* that originated from Half Moon Bay, California. Eighteen different random primers were evaluated for their ability to distinguish *H. schachtii* from *H. cruciferae*.

*Comparison of populations with RAPD markers:* Total DNA was extracted from approximately 150 brown, egg-filled cysts of *H. schachtii* from each of six different geographic populations, including Salinas (C1 from L. I. Miller), Lodi, Clarksburg (2 locations), and the Imperial Valley (2 loca-

tions), California. RAPD markers were obtained for each population using ten primers (A-1, A-2, A-3, A-6, A-8, A-9, A-11, A-12, A-18, and A-19). Markers were scored for all different primers as present or absent, and only amplified markers clearly present were included in the analyses. Jaccard's similarity coefficients (14) were calculated for the six geographic populations using a binomial summary of the 78 markers obtained from the 10 primers. The Jaccard similarity matrix was used to conduct a nearest neighbor, hierarchical cluster analysis (19) for the six different geographic populations.

## RESULTS

*Comparison of H. schachtii and H. cruciferae:* Typically 2 to 12 fragments, from 0.2 to 1.5 kb, were obtained with each primer. All random primers tested distinguished the Half Moon Bay populations of *H. schachtii* from *H. cruciferae*. For example, primer A-1 yielded markers of approximately 540 and 410 bp specific to *H. cruciferae* and markers of 1,850, 1,250, and 460 bp specific to *H. schachtii* (Fig. 1). Primer A-3 yielded fragments of approximately 1,100, 820, and 700 bp, among others, specific to *H. cruciferae*, and fragments of 786 and 312 bp specific to *H. schachtii* (Fig. 1). Some primers, such as A-4 and A-5, yielded complex patterns with several markers common to both species (Fig. 2). In contrast, primer A-6 yielded a simple pattern, with fragments of approximately 1,200 and 900 bp, that differentiated *H. cruciferae* and *H. schachtii* (Fig. 2). Replicate PCR reactions with the same DNA preparations yielded reproducible fragments.

*Comparison of populations with RAPD markers:* RAPD analysis of total DNA extracted from approximately 150 cysts of *H. schachtii* originating from each of six different California populations revealed common markers and differences among the populations (Figs. 3,4). All populations examined with primer A-1 (Fig. 3) yielded complex patterns, with major bands of approximately 1,800, 1,200 and 980 bp com-

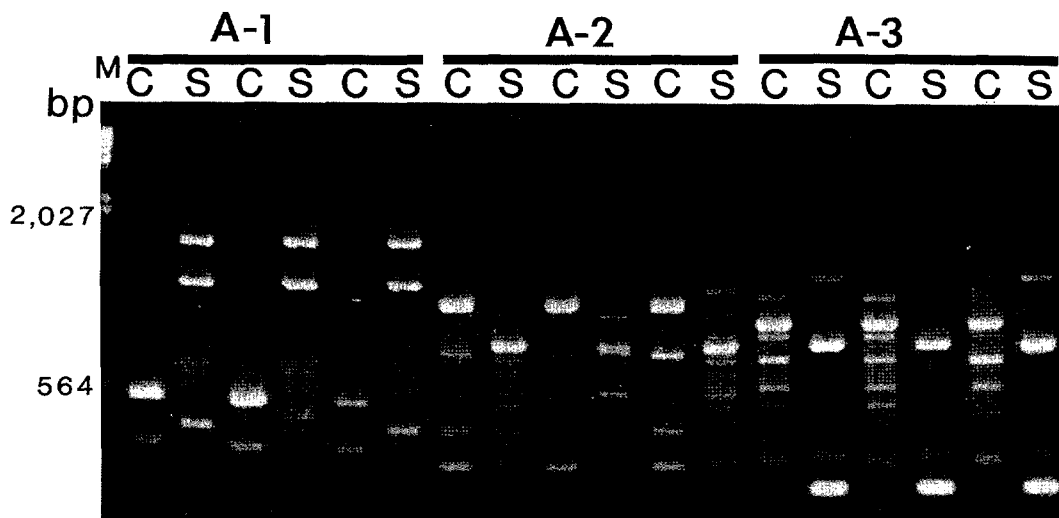


FIG. 1. RAPD markers from ca. 150 brown cysts from Half Moon Bay, California, populations of *Heterodera cruciferae* and *H. schachtii* produced using three random, decamer primers—A-1, A-2, and A-3. Three replicate PCR reaction with the same DNA from *H. cruciferae* (c) and *H. schachtii* (s) are shown in alternating lanes. M = molecular size standards.

mon to all populations examined. Differences occurred among populations in several, smaller bands. Primer A-2 yielded common bands of approximately 1,900, 1,100, and 330 bp with all the populations (not shown). Primer A-3 yielded two major

fragments (approximately 1,100 and 800 bp) common to each population tested and several fragments specific to only a few populations (Fig. 4). Similarity coefficients of pairwise comparisons for the six *H. schachtii* populations ranged from 0.65 to

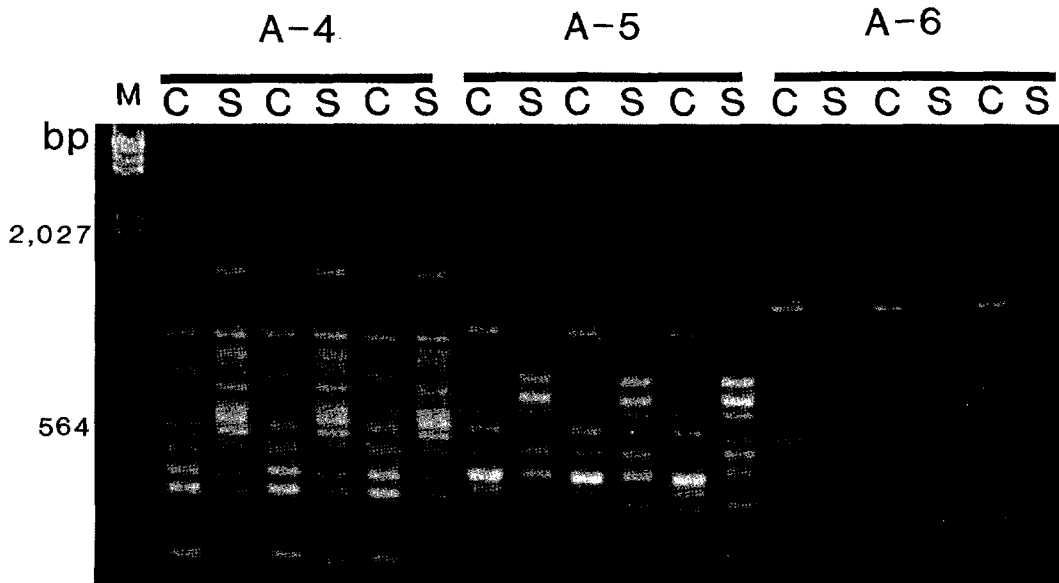


FIG. 2. RAPD markers from ca. 150 brown cysts from Half Moon Bay, California, populations of *Heterodera cruciferae* and *H. schachtii* produced using three random, decamer primers—A-4, A-5, and A-6. Three replicate PCR reactions with the same DNA from *H. cruciferae* (c) and *H. schachtii* (s) are shown in alternating lanes. M = molecular size standards.

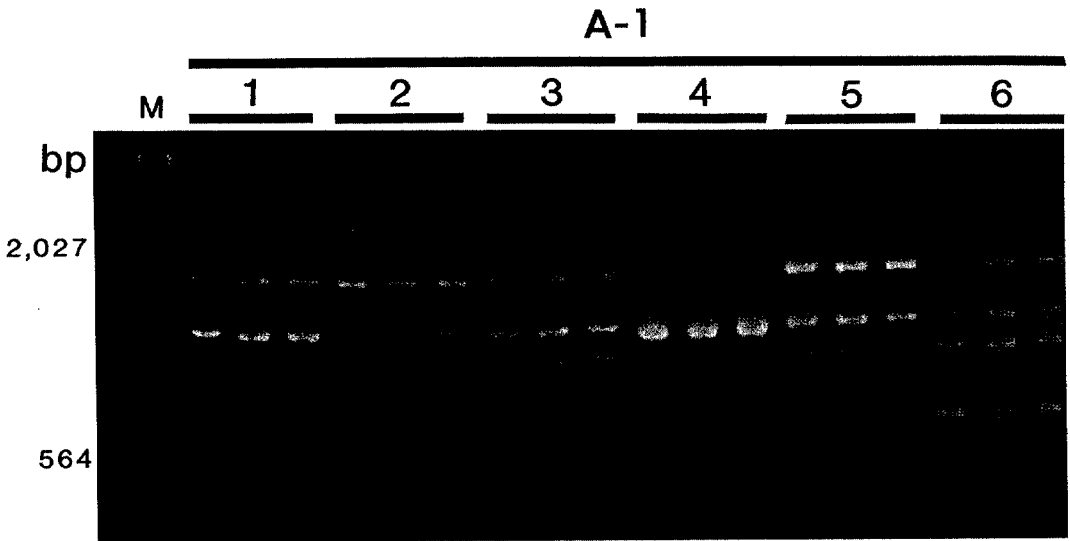


FIG. 3. RAPD markers generated with random decamer primer A-1 and DNA from ca. 150 cysts of *Heterodera schachtii* originating from different geographic locations in California. Each population is represented by three replicate lanes. 1 = Salinas, 2 = Lodi, 3 = Clarksburg-1, 4 = Imperial Valley-1, 5 = Clarksburg-2, and 6 = Imperial Valley-2. M = molecular size standards.

0.45 (Table 1) and were used for nearest neighbor, hierarchical cluster analysis. The cluster analysis revealed that the second Imperial Valley population was included in the final cluster and was considerably different than the other populations (Fig. 5).

*Analysis of single cysts:* RAPD fragments from single cysts of an inbred *H. schachtii* line (5027-5-2) were compared to fragments obtained with DNA prepared from multiple cysts of the same line (Fig. 6). The patterns of markers obtained with primer A-1 from 12 single cysts from the inbred line were very similar, although some differences in banding intensity occurred. In a few instances, little or no amplified product was obtained, presumably due to insufficient template (Fig. 6, lanes 6,13).

*Marker variation within populations:* When RAPD markers were generated from single cysts from Half Moon Bay *H. schachtii* with primer A-3, most major bands were common to all individuals, but some bands were absent in particular individuals (Fig. 7). When RAPD markers were produced using primer A-3 from five individual cysts from Half Moon Bay *H. cruciferae*, patterns among individuals were similar, with only

minor differences (Fig. 8). The patterns obtained for single cysts of *H. cruciferae* were clearly distinguishable from patterns obtained for individual *H. schachtii* from the same field (compare Fig. 7 to Fig. 8). With only minor differences, the patterns obtained from single cysts of *H. schachtii* (Fig. 7) and *H. cruciferae* (Fig. 8) were nearly identical to the patterns obtained from multiple cyst preparations (Fig. 1) for the same populations.

## DISCUSSION

*Heterodera cruciferae* and *H. schachtii* were easily distinguished by differences in fragment patterns with any of nineteen RAPD primers. These distinctive RAPD fragments are candidates for species or race-specific DNA probes for diagnostic applications and for assessing interspecific crosses of *H. cruciferae* and *H. schachtii*. Some primers, e.g., primer A-3, yielded only a few, species-specific bands that enabled differentiation of *H. schachtii* and *H. cruciferae*. Other primers yielded more complex patterns, which were less easily interpreted. Primer A-3 allowed differentiation between individual *H. cruciferae* and

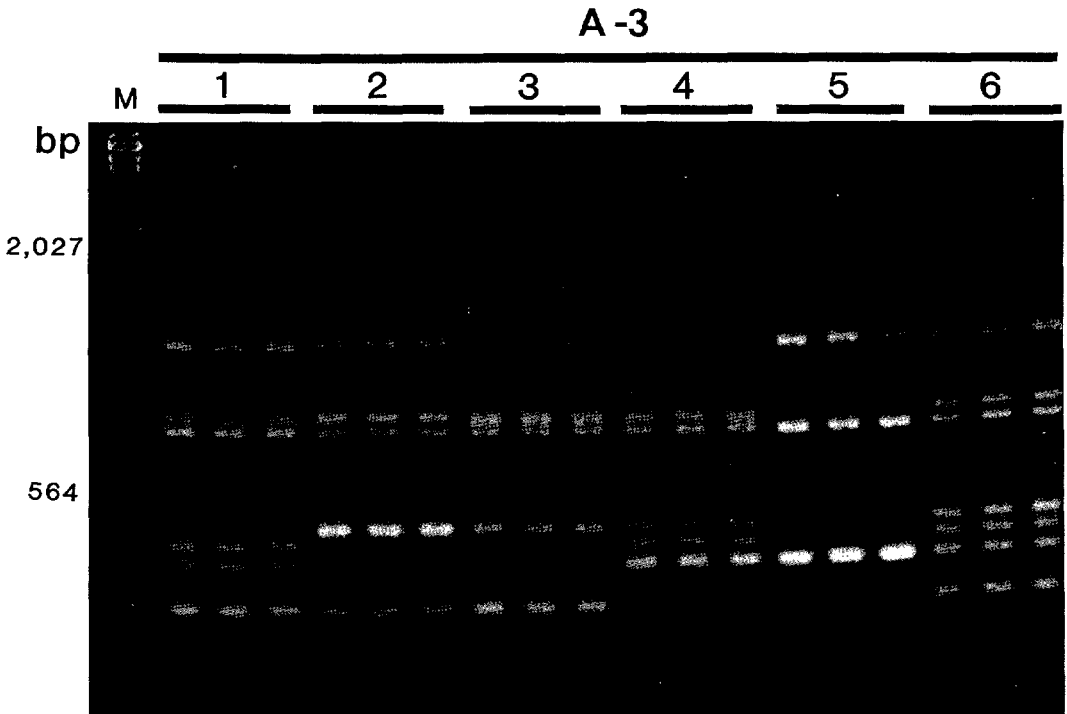


FIG. 4. RAPD markers generated with random, decamer primer A-3 and DNA from *Heterodera schachtii* originating from different geographic locations in California. Each population is represented by three replicate lanes. 1 = Salinas, 2 = Lodi, 3 = Clarksburg-1, 4 = Imperial Valley-1, 5 = Clarksburg-2, and 6 = Imperial Valley-2. M = molecular size standards.

*H. schachtii* cysts, thereby indicating that the RAPD technique is a useful diagnostic tool for nematode species identification. The ability to assay single cysts is of particular value for evaluation of mixed populations of cyst nematodes. The RAPD-PCR technique is quick and easy to use with cyst nematodes, requires a small amount of biomass, and does not require radioisotopes.

We assessed the ability of RAPD mark-

ers to detect genetic variation and relatedness among the six California populations of *H. schachtii*. Similarity coefficients, calculated from pairwise comparisons of shared and nonshared markers for different geographic populations, indicated close affinity, though not identity, among these populations. When RAPD markers from only two or three primers were used, estimates of similarity depended on the particular primers. Including 78 RAPD

TABLE 1. Jaccard's similarity coefficients for different geographic populations of *Heterodera schachtii*.

	SAL	LODI	IMP1	CLR1	IMP2	CLR2
SAL	1.0	0.46	0.52	0.58	0.52	0.55
LODI		1.0	0.62	0.53	0.46	0.65
IMP1			1.0	0.47	0.45	0.52
CLR1				1.0	0.50	0.63
IMP2					1.0	0.52
CLR2						1.0

Coefficients were based on 78 scorable RAPD markers obtained with 10 random decamer primers (A-1, A-2, A-3, A-6, A-8, A-9, A-11, A-12, A-18, A-19). SAL = Salinas, LODI = Lodi, CLR1 = Clarksburg-1, IMP1 = Imperial Valley-1, CLR2 = Clarksburg-2, and IMP2 = Imperial Valley-2, California.

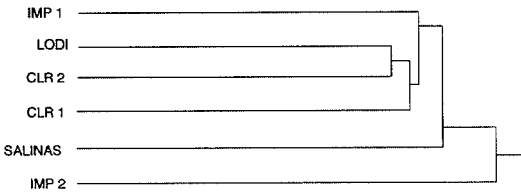


FIG. 5. Nearest neighbor clustering for six different California populations of *Heterodera schachtii*. Clustering was based on Jaccard's similarity coefficient as calculated with 78 RAPD markers generated from 11 different random primers. The horizontal distance between clusters is proportional to similarity. SAL = Salinas, LODI = Lodi, CLR1 = Clarksburg-1, IMP1 = Imperial Valley-1, CLR2 = Clarksburg-2, and IMP2 = Imperial Valley-2.

markers from multiple primers in the analysis provided more robust results. The Jaccard's coefficients and cluster analysis obtained with 10 different primers showed that the nematode population from the second Imperial Valley location was distinct from the nematode populations at the other locations. In addition, the two Imperial Valley populations (approximately 5 km apart) had a similarity coefficient of only 0.45, and the cluster analysis clearly separated the two populations. The two Clarksburg populations were approximately 8 km apart and had Jaccard similarities of 0.63. Cluster analysis grouped

the Clarksburg locations with the Lodi population (34 km distant) in the first and second clusters. Therefore, cluster analysis revealed that geographic proximity of the populations does not necessarily correlate with genetic relatedness and that RAPD analysis may help in defining relatedness among populations and the history of introductions.

The separation of the two Imperial Valley populations obtained by cluster analysis is consistent with earlier observations on the probable origins of *H. schachtii* in the Imperial Valley (4,5). *Heterodera schachtii* was first detected in California in 1907 near Salinas and was discovered in the Imperial Valley in 1957. The introduction of *H. schachtii* to the Imperial Valley was hypothesized to be via contaminated farm machinery originating from nematode-infested areas in northern California (5). The lack of a central focus of infestation in the Imperial Valley has been hypothesized to have resulted from multiple introductions on several different occasions (4). Our RAPD results further substantiate the idea that *H. schachtii* was introduced to the Imperial Valley on several occasions, either from different founder gene pools or from the same gene pool but with subse-

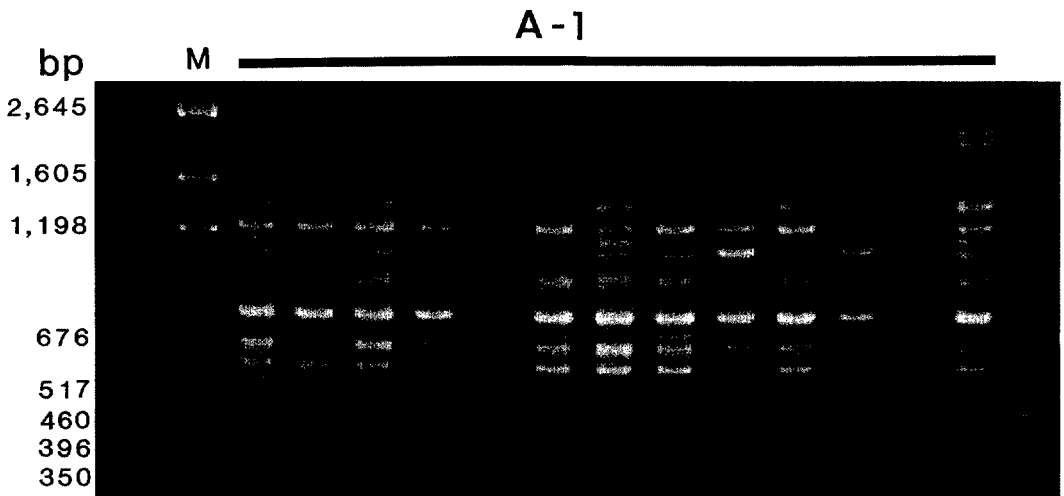


FIG. 6. Comparison of RAPD markers generated with primer A-1 and DNA extracted from single cysts of an inbred line of *Heterodera schachtii* (line 5027-5-2) maintained in the greenhouse (lanes 2–13) through serial transfers of single cysts versus DNA extracted from approximately 150 mature cysts from the same line (lane 14). M = molecular size standards.

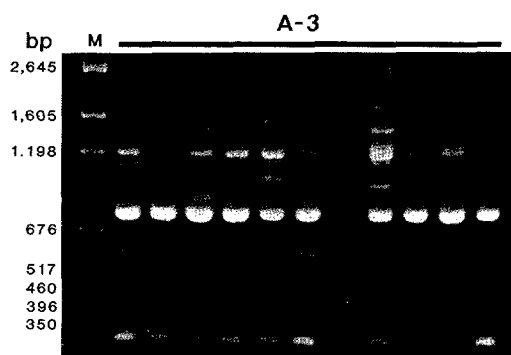


FIG. 7. RAPD markers generated with primer A-3 and DNA extracted from single *Heterodera schachtii* cysts originating from Half Moon Bay, California. M = molecular size standards.

quent selection of different markers in different locations.

Additional studies with outgroups of *H. schachtii* must be conducted to validate the relationships predicted by the cluster analysis. Information on the DNA homology of shared markers is required to refine our similarity estimates and will be obtained by Southern blot analysis. We are attempting to obtain sequence data for specific markers to define primers that will potentially allow us to identify individual juveniles of *H. cruciferae* and *H. schachtii*. The possibil-

ity exists that minor differences in banding patterns were caused by contaminating DNA. However, preliminary experiments using RAPD markers with *Heterorhabditis* DNA revealed that DNA of the nematode's bacterial associate did not affect the RAPD pattern obtained (Gardner and Caswell, unpublished).

Application of RAPD analysis to single cysts was successful, although occasionally some single cysts apparently did not yield sufficient template DNA to allow efficient amplification. Comparison of DNA from individual cysts from the inbred line with bulk DNA extracted from 150 cysts of the same inbred line revealed very similar patterns. The small differences observed among individual cysts from the inbred *H. schachtii* line could result from differential efficiency of DNA recovery from individuals or from variability within individual females from this line. We observed more variability in RAPD markers obtained with single cysts from noninbred populations than from inbred lines, supporting the assumption of high genetic variation within field populations of these nematodes. Therefore, the RAPD technique allows assessment of intra-population genetic variation in the cyst nematodes, although multiple cysts must be examined to define the range of variability in different populations.

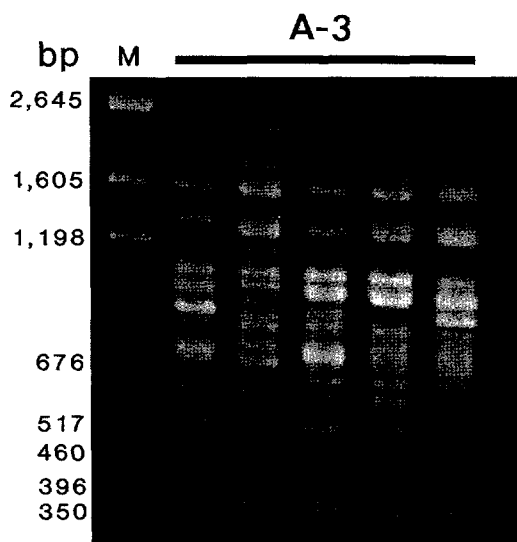


FIG. 8. RAPD markers generated with primer A-3 and DNA extracted from single *Heterodera cruciferae* cysts originating from Half Moon Bay, California. M = molecular size standards.

Applying the RAPD technique to single cysts allowed us to identify genetic variations within populations of *H. cruciferae* and *H. schachtii*. In other organisms, RAPD markers segregate as genetic traits (20) and have allowed the rapid development of genetic maps. *Heterodera* spp. can be used for controlled genetic crosses, but the paucity of genetic markers has limited genetic analysis of such crosses (7,12). The variability documented here indicates that RAPD analysis will be a powerful technique for following controlled genetic crosses and developing genetic maps in *Heterodera* species. RAPD markers will provide a tool for investigating phylogenetic relationships and the genetics of host range and virulence, and will facilitate re-

search on the population dynamics of sympatric congeners.

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