Journal of Nematology 20(4):532–538. 1988.

DNA Restriction Fragment Length Polymorphism in Races of the Soybean Cyst Nematode, *Heterodera glycines*[1]

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Abstract: Restriction endonuclease digests of total DNA from races 3, 4, and 5 of the soybean cyst nematode, *Heterodera glycines,* have been analyzed on agarose gels. DNA fragment patterns of race 4 were completely different from those patterns obtained for races 3 and 5 by all eight restriction enzymes tested. Differences in long and short restriction DNA fragments generated by the enzyme Msp I or its isoschizomer, Hpa II, were detected between race 3 and 5 digestion profiles. Rapid DNA isolation followed by its digestion with either Msp I or Hpa II enzymes and visualization of repetitive DNA fragments in agarose gels provided a diagnostic assay for the populations of the three races examined in this study.

Key words: DNA electrophoresis, genetic divergence, *Glycine max, Heterodera glycines,* nematode genome, restriction fragment, soybean, soybean cyst nematode.

In recent years some authors have focused their attention on direct analysis of the nematode genome. The genetic variation in genomic DNA has been studied between species within the genera *Trichinella, Caenorhabditis, Romanomermis, Steinernema,* and *Meloidogyne* and within the species *Bursaphelenchus xylophilus* by using restriction endonucleases and quantitation of specific DNA sequences by nucleic acid hybridization (1–3,9,10). These studies strongly suggest that comparisons of DNA cleaved into defined fragments by sequence specific restriction enzymes should allow discrimination among species of nematodes. An advantage of repetitive DNA fragment comparisons is that the DNA sequence differences may aid in resolving questions regarding race designations in economically important plant-parasitic nematodes. Furthermore, these comparisons may provide both growers and taxonomists with a sensitive and rapid method of race identification.

In the work described here, we examined and compared restriction DNA fragments obtained by digestions with selected restriction endonucleases isolated from races 3, 4, and 5 of *Heterodera glycines,* the soybean cyst nematode (SCN). Laboratory populations of these three races have been established as "isofemale lines" for ca. 20 generations on root-explant cultures. Estimations of genetic divergence of DNA based on a direct analysis of known SCN nematode genomes are necessary in developing a method to distinguish the races of this species.

MATERIALS AND METHODS

Populations of each race of *Heterodera glycines* Ichinohe were obtained from USDA, Jackson, Tennessee. Races 3, 4, and 5 were cultured on root explants as described by Lauritis et al. (7). Once established in culture, each population was redefined to race by its ability to reproduce on root-explants of five resistant and five susceptible cultivars of soybean, *Glycine max* (L.) Merr. Race 3 was evaluated as described by Lauritis et al. (8). Race 4 was evaluated in vitro on Pickett 71, Centennial, Forrest, PI 88788, and Bedford; Bedford and PI 88788 were resistant. Race 5 was evaluated in vitro on Pickett 71, Centennial, Forrest, Bedford, and PI 88788 and reproduced on all of these cultivars.

All three races were increased on Kent or Essex soybean root explant cultures. Nematodes were maintained on excised roots on Gamborg's B-5 medium (4,5). Approximately 4,000–5,000 10–12-day-old white females (no visible eggs) were hand picked into sterile vials and stored at −80 C for each DNA isolation.

Received for publication 2 October 1987.

[1] Mention of a trade name, warranty, proprietary product, or vendor does not constitute a guarantee of a product and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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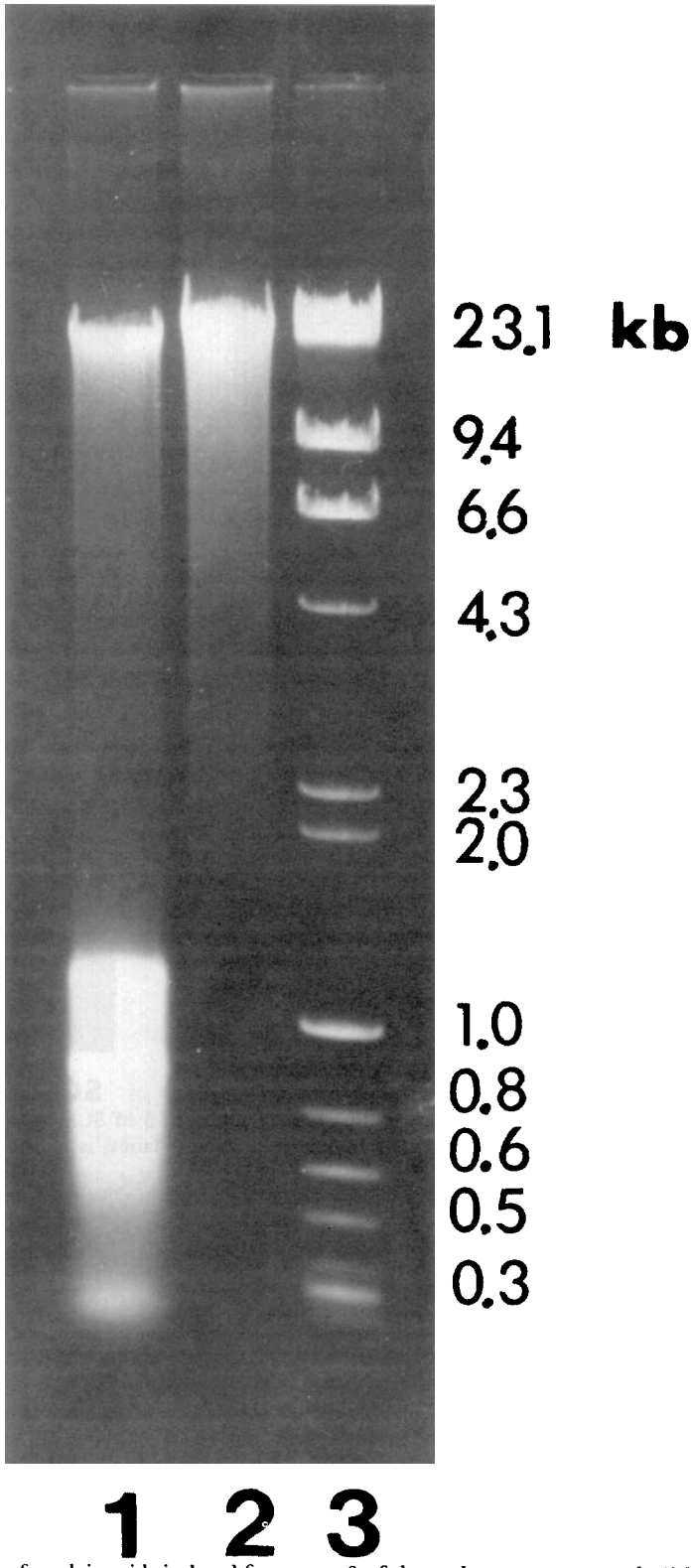

FIG. 1. Analysis of nucleic acids isolated from race 3 of the soybean cyst nematode (SCN). Lane 1) Total cellular nucleic acids (equivalent to 20 nematodes) before treatment with RNase A. Lane 2) Total DNA (0.9 μg, equivalent to 230 nematodes) obtained from nucleic acid preparation after its digestion with RNase A. Lane 3) Molecular weight markers -λDNA-Hind III/ϕX-174 DNA-Hind II digest. Nucleic acids preparations were analyzed on a 0.7% agarose gel. The gel was stained with 0.5 μg/ml ethidium bromide.

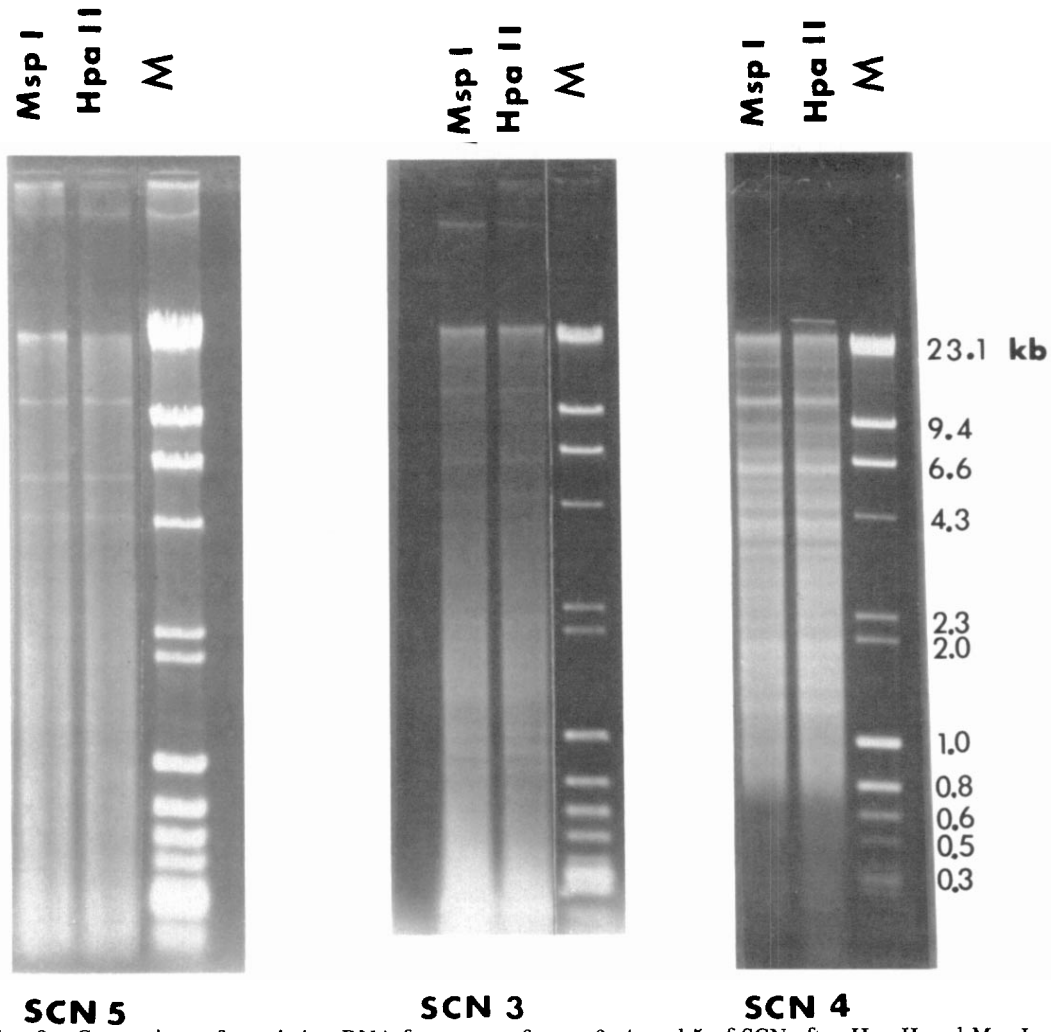

FIG. 2. Comparison of restriction DNA fragments of races 3, 4, and 5 of SCN after Hpa II and Msp I restriction digestions. The migration of molecular weight markers (M lanes) is indicated along the right margin.

Isolation of total cellular DNA: Frozen nematodes (4,000–5,000) were homogenized in a mortar at room temperature with 1 ml 10 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 5 mM EDTA, 1% SDS, and 0.5 ml 10 mg/ml proteinase K (No. P-0390, Sigma Chemical Co., St. Louis, MO) in the presence of sand.

Nucleic acids were deproteinized by incubating the total homogenate at 65 C for 1 hour and purified by gently extracting twice with an equal volume of a mixture of phenol and chloroform (1:1). Cellular nucleic acids (RNA and DNA) were precipitated by the addition of 2 volumes of ethanol in the presence of 2.5 M ammonium acetate at −20 C. Nucleic acids were collected by centrifugation at 20,000 *g* for 15 minutes, dissolved in 2 ml 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and analyzed on a 0.7% agarose gel (Fig. 1, lane 1). RNA was removed from the nucleic acids preparation by digestion with RNase A (170 μg/ml) free of DNase for 1 hour at 45 C. DNA was extracted with phenol–chloroform (1:1) and then with chloroform–isoamyl alcohol (24:1), precipitated with 2 volumes of ethanol in the presence of 2.5 M ammonium acetate, and redissolved in 60–70 μl 10 mM Tris-HCl (pH

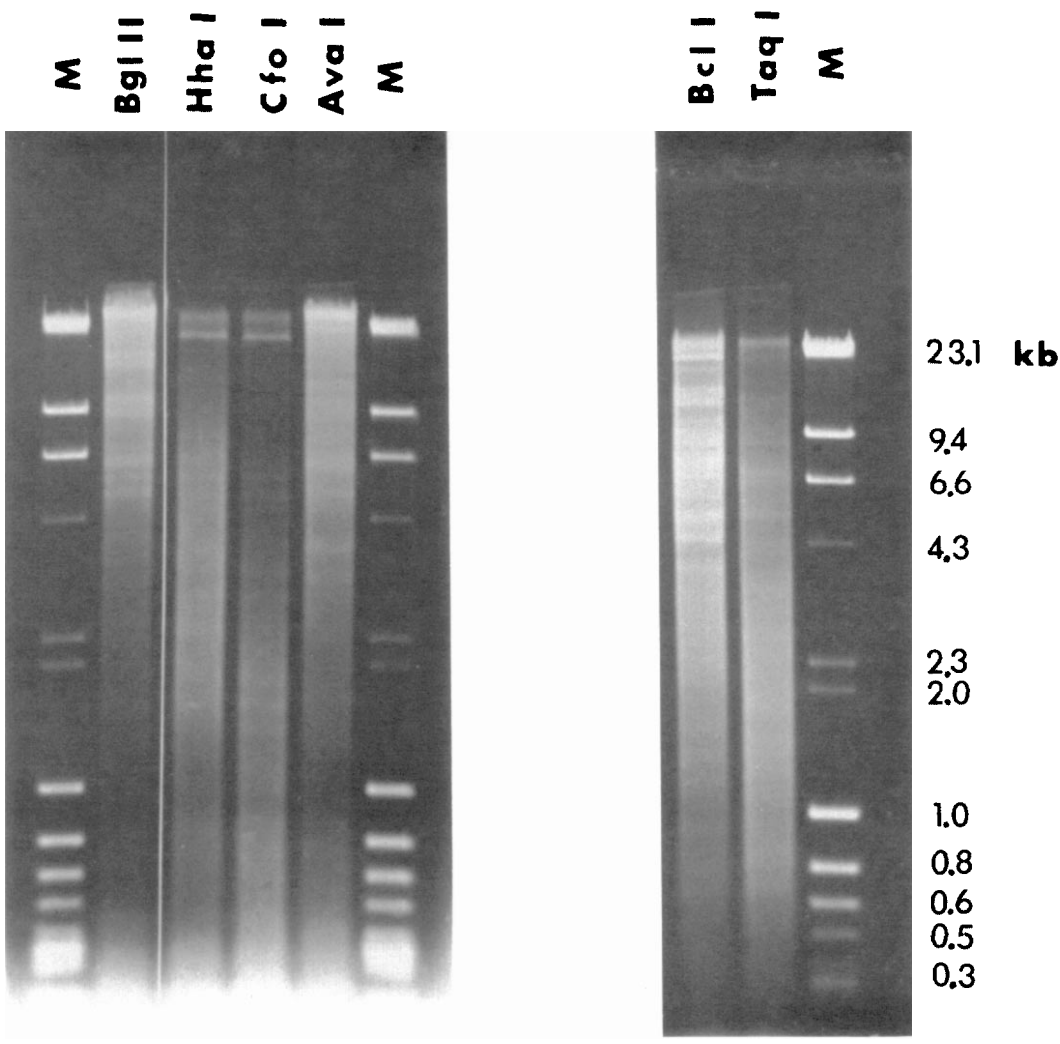

FIG. 3. Cleavage of race 4 DNA of SCN by restriction endonucleases indicated on the top of gels. The migration of molecular weight markers (M lanes) is indicated along the right margin.

7.5) and 1 mM EDTA. Size of the purified DNA was monitored by electrophoresis on a 0.7% agarose gel (Fig. 1, lane 2). The yield from 4,000–5,000 nematodes was 13–25 μg purified cellular DNA.

Restriction endonuclease digestions and DNA gel electrophoresis: Eight restriction endonucleases were used to digest the nematode DNA: Msp I, Hpa II, Bgl II, Hha I, Cfo I, Ava I, Bcl I, and Taq I (BRL, Life Technologies, Gaithersburg, MD). Enzymatic reactions (15 μl) containing 1.5 μg nematode DNA and 30 units of the enzyme were carried out in the appropriate enzyme buffer (supplied by BRL) for 8 hours at an optimal temperature. The digestion of nematode DNA under these reaction conditions for 16 hours did not change restriction patterns. Agarose gels were run at 60 V for 105 minutes and stained with ethidium bromide at 0.5 μg/ml. DNA was visualized with a TM-36 transilluminator (302 nm) and photographed on Polaroid Type 55 P/N film.

RESULTS

The SCN DNA (nuclear and mitochondrial) represent 15–20% of the isolated nematode nucleic acids (Fig. 1, lane 1). An

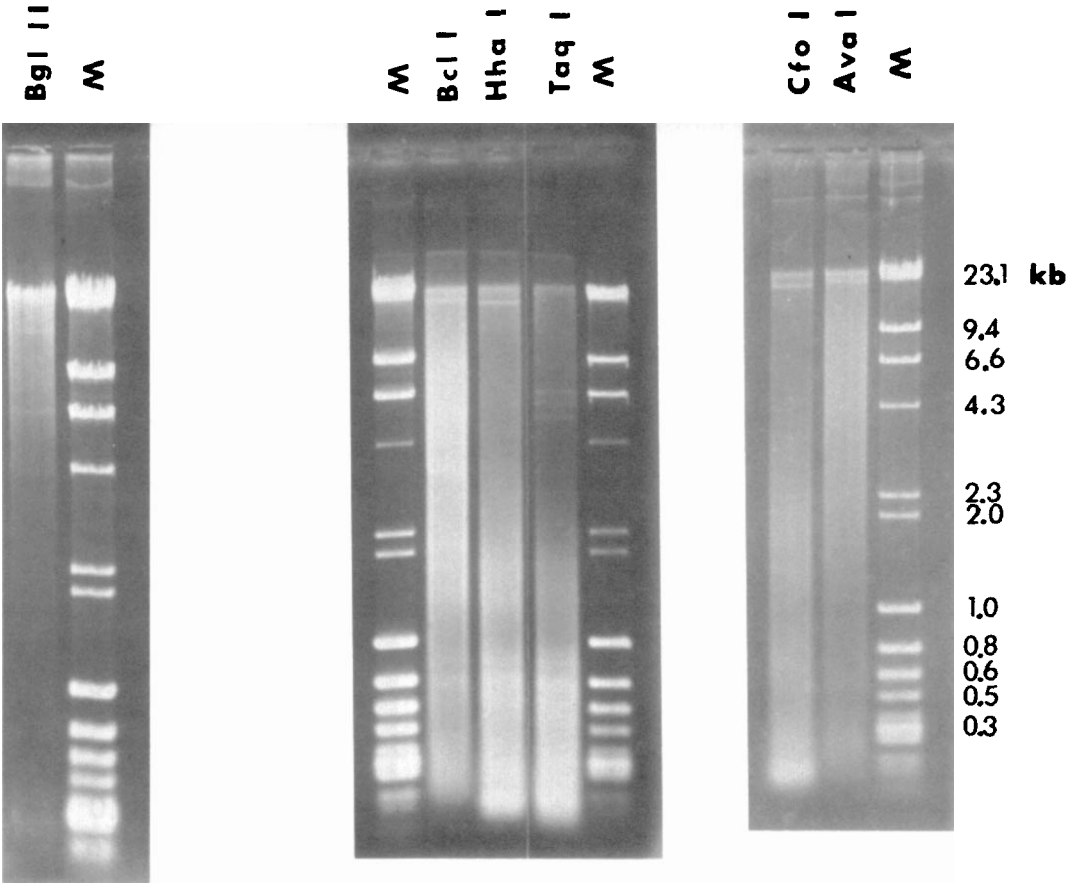

FIG. 4. Cleavage of race 3 DNA of SCN by restriction endonucleases indicated on the top of the gels. The migration of molecular weight markers (M lanes) is indicated along the right margin.

example of high molecular weight DNA purified from race 3 and suitable for restriction analysis is shown in Figure 1, lane 2.

All DNA fragment patterns of race 4 generally differ from those obtained for races 5 and 3 (Fig. 2). Two of the eight restriction enzymes used in these experiments, Hpa II and Msp I, produced the same restriction bands for a given nematode DNA preparation indicating the absence of methylation of the internal cytosine at the sequence (5′) CCGG (3′). These isoschizomers generated more than 12 visible bands in race 4 DNA, whereas only three bands were detected for race 5 DNA and at least five fragments (four high molecular weight fragments and a few short repeats) for race 3 DNA.

Comparisons of the digestion profiles for Bgl II, Hha I, Cfo I, Ava I, Bcl I, and Taq I exhibited some differences between DNA fragments of race 4 (Fig. 3) and races 3 and 5 (Figs. 4, 5). The most striking difference is found for Bgl II and Bcl I restriction patterns. Genomic DNA of races 3 (Fig. 4) and 5 (Fig. 5) appeared the same when digested with these six restriction endonucleases.

Total DNA isolated from race 3, grown on Kent and Essex soybean cultivars, were compared by cleaving with Hpa II, Msp I, and Bgl II. The examined restriction DNA patterns were identical on both hosts (data not shown).

DISCUSSION

Restriction fragment analyses based on differences in repetitive DNA fragments

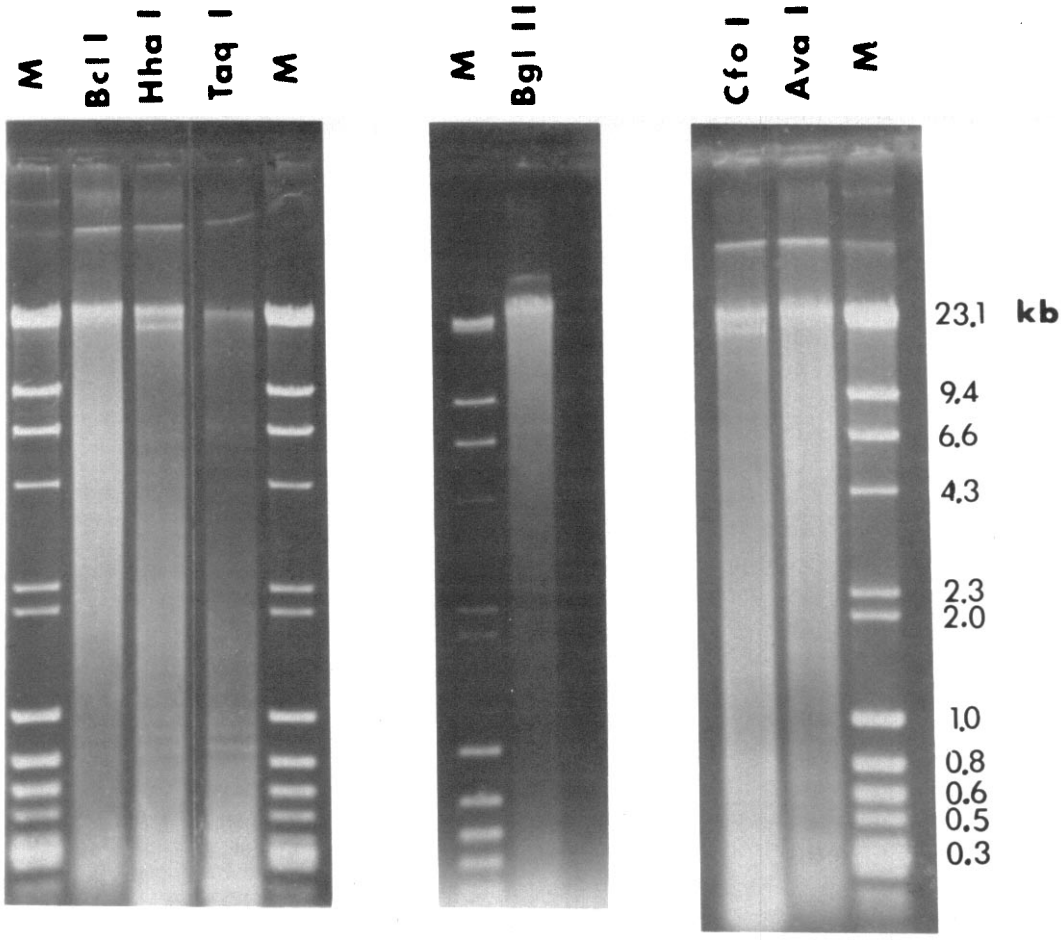

FIG. 5. Cleavage of race 5 DNA of SCN by restriction endonucleases indicated on the top of gels. The migration of molecular weight markers (M lanes) is indicated along the right margin.

were used in this study to identify and distinguish three races of SCN. This information is required before initiating more detailed studies of sequence changes in DNA of this nematode. Estimations of genetic divergence of DNA are also necessary for developing methods for distinguishing these races on the basis of a direct analysis of the nematode genome.

The digestion profiles presented in this study are unique and characteristic of both the nematode DNA and the particular restriction enzyme used. Nematode DNA restriction fragments as visualized in agarose gels stained with ethidium bromide represent multiple copies of repetitive DNA sequences. This class of DNA sequences is characteristic of the eukaryotic genome, which contains numerous repeating elements varying in size from a few hundred to several thousand base pairs. These sequences represent both transcribed and nontranscribed DNA regions and are organized as interspersions of repetitious sequences among unique, or low frequency, sequences (6,11). Races 3, 4, and 5 can be distinguished from one another on the basis of different repetitive DNA sequences appearing as distinct bands on their restriction patterns of total cellular DNA. A striking difference was detected between restriction DNA fragments of race 4 and the two other races. Hpa II and Msp I were the most useful restriction endonucleases

among all tested. They generated many more restriction DNA fragments than the other six restriction enzymes used in these experiments. All of these DNA fragments are useful for discriminating these populations of races 3, 4, and 5.

The patterns of restriction DNA fragments were unchanged when nematode DNA were additionally purified in a CsCl gradient (results not shown). Therefore, the preparation of nematode DNA as reported here, without the use of expensive and time-consuming purification in a CsCl gradient, is a good substrate for cleavage with all tested restriction endonucleases. This is very practical for use in analysis of a great number of samples, especially if there is only a small amount of material.

The populations used in this study were characterized in vitro to confirm race identification. It is important to have established isofemale lines that can be maintained under strict monoxenic conditions. This allows for known populations of SCN to be used to generate primary data. Furthermore, the ability to culture the same known populations on different hosts can verify that the sequence of the nematode genome is not influenced by host–parasite interactions. This was demonstrated by race 3 in that it had the same restriction DNA patterns when grown on two different soybean cultivars. We recommend laboratory stock populations be used for comparison with any field populations.

In conclusion, our results show that comparisons of restriction DNA fragments of races 3, 4, and 5 of *H. glycines* allow these races to be distinguished on the basis of analysis of the nematode genome. More experiments are needed to determine whether the high degree of variability of DNA restriction fragment length in the genome of race 4 results from restriction site polymorphism or the presence of new repetitive sequences.

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