A DNA Hybridization Probe for Detection of Soybean Cyst Nematode

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ABSTRACT

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A DNA hybridization probe, constructed from mitochondrial DNA (mtDNA), was developed for the detection of the soybean cyst nematode (SCN) *Heterodera glycines*. The probe can detect a single female nematode applied as an unpurified homogenate to nitrocellulose filters. mtDNA was extracted from nematode eggs of an Arkansas race 3 population. *Mbo*I-digested mtDNA was inserted into the *Bam*HI site of the molecular cloning vector pBR322. Transformants were screened by colony hybridization with intact ^{32}P-nick-translated SCN mtDNA. Potential probes were evaluated for species specificity by hybridization to homogenized cysts immobilized on nitrocellulose. Several techniques were evaluated for homogenizing and applying cyst DNA to filters. One probe of approximately 500 base pairs hybridized consistently to eight widely geographically divergent SCN populations. An SCN population from Java displayed weak hybridization. No hybridization was observed with *H. leuceilyma*, *H. weissi*, and *Globodera virginiae*. Occasional weak cross-hybridization was observed with *H. trifolii*, and slightly stronger cross-hybridization was observed in three populations of *H. schachtii*. Hybridization at high stringency reduced cross-hybridization to barely detectable levels.

The soybean cyst nematode (SCN) *Heterodera glycines* Ichinohe is a major factor limiting soybean (*Glycine max* (L.) Min.) production in the United States, with current reductions in yield estimated at 1.5 million metric tons (6). Since its initial discovery in the United States in 1954, SCN has been found in 24 states.

Control strategies for SCN include sanitation, crop rotation, nematicides, and the use of resistant cultivars. Sanitation efforts, including federal and state quarantines, have been unsuccessful in limiting the nematode's spread, presumably because SCN can be dispersed by a number of methods, including contaminated machinery or seed, birds, and surface water. Crop rotation is effective, although weed hosts may compromise control efforts. Resistant cultivars can provide adequate yields, but their use is dependent on the ability to identify biotypes or races of SCN present in the field.

Presently, the standard method of race identification is through the evaluation of SCN reproduction on a differential series of soybean cultivars. Morphometric (11), biochemical (3,9), and serological (11) methods have been employed in an attempt to improve differentiation of SCN populations and closely related species.

We chose to investigate the feasibility of using DNA-DNA hybridization to rapidly distinguish between closely related *Heterodera* species and races of *H. glycines*. As a source of DNA for diagnostic probe construction, we selected mitochondrial DNA (mtDNA) because it is highly repeated in animal genomes, is relatively easy to extract, and appears to be evolving rapidly (1). This report describes the development of a DNA hybridization probe for the detection of SCN.

MATERIALS AND METHODS

Nematode culture. Ten isolates of SCN and five other cyst-forming species were used in this study. SCN isolates were from China, Java, Colombia, Japan, North Carolina, Wisconsin, South Carolina, Virginia, Arkansas, and Nebraska. Other cyst-forming species examined include *Heterodera schachtii* Schmidt (isolates from Kansas, Nebraska, and California); *H. trifolii* (Goffart) Raski and Hart (from Pennsylvania); *H. leuceilyma* DiEdwardo and Perry (from Louisiana); *H. weissi* Steiner (from Indiana); and *Globodera virginiae* (Miller and Gray) Behrens (from Virginia).

SCN isolates were cultured by inoculating soybean (*Glycine max* 'Lee' or 'Essex') plants with eggs and second-stage juveniles of each isolate. Other cyst-forming species were cultured by similarly inoculating red or white clover (*Trifolium repens* L. 'Kenland' or 'Ladino') with *H. trifolii*; cabbage (*Brassica oleracea* L. 'Early Marvel') with *H. schachtii*; knotweed (*Polygonum douglasi* Greene) with *H. weissi*; horse nettle (*Solanum carolinense* L.) with *G. virginiae*; and St. Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze 'Bitter Blue') with *H. leuceilyma*.

Plants were grown in greenhouses at the University of Arkansas, Fayetteville, at ambient temperatures of 21–28 C. After 4–10 wk, cysts were collected from plant roots and soil by using the sugar flotation method (2) and stored in water at 15 C until used.

Cyst filter preparation. To determine the best methods for applying cysts to nitrocellulose filters, three homogenization solutions and two cell disruption techniques were evaluated. Homogenization solutions included 10× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 20× SSC, and MSB-PK (0.20 M mannitol, 0.07 M sucrose, 0.05 M Tris, pH 7.5, 200 μg/ml of proteinase K). Homogenization techniques included grinding in a microfuge tube with a pellet pestle (Kontes Scientific Glassware, Morton Grove, IL) or disrupting cysts directly on nitrocellulose by using a polypropylene pipette tip.

One, three, five, or 10 washed cysts of SCN Nebraska isolate were transferred to 0.5 ml polypropylene microfuge tubes containing 10 μl of homogenization solution. Cysts were either homogenized immediately (35–50 sec) or stored 12–48 hr in tubes at 4 C. Tube-ground cysts were diluted with an additional 10 μl of solution. Cyst homogenates were spotted onto nitrocellulose filters that had been presoaked in 10× SSC or 20× SSC and immobilized in a minifold vacuum apparatus (Bio-Rad Laboratories, Richmond, CA). For direct filter disruption, cysts were transferred from tubes and disrupted directly on filters. Samples were fixed to filters by soaking in 80 μl of denaturation solution (0.05 M NaOH,

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1.5 M NaCl) 15–20 min, 80 μl of 2× SSC vacuumed through briefly, 80 μl of neutralization solution (0.5 M Tris-A buffer, pH 7.5, 3.0 M NaCl) for 7–10 min, and two 5-min rinses with 2× SSC. Filters were dried at 80 C in a vacuum oven for 3–10 hr.

Filters for interspecific comparisons were prepared as above by using tube homogenization in 10× SSC solution and from one to six cysts of different cyst-forming isolates or species. Variability in DNA content of cysts was examined by applying one homogenized cyst of an SCN race 3 population per well. All filters were fixed and dried as described above.

Cloning strategies. Mitochondrial DNA was extracted from eggs of an SCN Arkansas race 3 population by using techniques of Powers et al (8). An SCN mtDNA library was prepared by digesting SCN mtDNA with *Mbo*I and ligating it into the *Bam*HI site of plasmid pBR322 (4). Transformants were screened by colony hybridization with nick-translated [^{32}P]-labeled SCN mtDNA. Strongly hybridizing colonies were selected for further evaluation.

Probe preparation and hybridization. Plasmids of 12 strongly hybridizing clones were isolated by isopycnic centrifugation in CsCl-ethidium bromide gradients (5). Plasmid and mtDNA was nick-translated and labeled with [^{32}P]dATP (10) or with biotin-streptavidin (Bethesda Research Laboratories, Gaithersburg, MD). Unincorporated nucleotides were separated from labeled probe fractions by spin column centrifugation (5).

Filters were prehybridized in boilable cooking pouches in 4× SSC hybridization solution (4× SSC, 10× Denhardt's [1× Denhardt's = 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone], and 0.1% SDS) with 10 μl/ml of denatured calf thymus DNA at 60 C for 4–12 hr with gentle agitation.

Labeled probes were denatured at 100 C in a water bath for 10 min and added to prehybridized filters with 10–15 ml of 4× SSC hybridization solution for [^{32}P]-labeled probes or with 45% formamide for biotin-labeled probes. Typically, nucleic acid hybridizations contained 5×10^6 cpm of labeled probe. Filters were incubated at 60 C or 65 C (^{32}P) or 42 C (biotin) for 24 hr with gentle agitation.

Filters treated with radiolabeled probes were washed at 60 C or 65 C four times with two washes each of 4× SSC-0.1% sodium dodecyl sulfate (SDS) and 2× SSC-0.1% SDS. Filters were air-dried before autoradiography with X-OMAT AR X-ray film (Kodak, Rochester, NY) and, in some cases, a Cronex intensifying screen (Dupont, Wilmington, DE). Biotin filters were washed, blocked, and treated with indicators, according to manufacturer's specifications (Bethesda).

RESULTS

Homogenization techniques. There appeared to be little difference in effectiveness between homogenization techniques based on evaluation of hybridization signal intensity of cyst dot blots. Tube-homogenized and filter-disrupted cysts gave equivalent results when hybridized with cloned SCN mtDNA probes. There was no difference among homogenization solutions; MSB-PK, 10× SSC and 20× SSC all facilitated release of nematode DNA and permitted fixation of DNA on nitrocellulose filters.

[^{32}P]-labeled probes could readily detect the DNA present in a single cyst (Figs. 1, 2). Although the intensity of hybridization from single cysts varied, signals corresponding to 100 pg of purified probe were routinely obtained. The variability in hybridization intensity due to differing amounts of DNA contained in single cysts was minimized by assaying 3–5 cysts per spot (Fig. 3). To test probe sensitivity further, single second-stage juveniles were placed directly on nitrocellulose filters. These filters were processed as above, except that denaturation was extended for 30 min. A positive hybridization signal was detected in less than 50% of the juveniles assayed. In some cases, a 30-min immersion in denaturation solution was insufficent to lyse juvenile nematodes.

Diagnostic probe evaluation. Of the approximately 850 transformants screened by colony hybridization with labeled SCN mtDNA, 12 that elicited intense hybridization signals were selected for further evaluation. One potential probe, pHG23 containing a 500-bp mitochondrial *Mbo*I fragment, showed promise as a diagnostic probe for SCN based on [^{32}P]-hybridization with cyst dot blots. In 12 separate hybridization experiments, this probe consistently hybridized to SCN populations from North Carolina, Wisconsin, South Carolina, Arkansas, Virginia, Nebraska, China, and Colombia, South America, and weakly hybridized to the Java SCN population (Figs. 1, 2). Three other cloned mitochondrial fragments showed hybridization results roughly equivalent to pHG23. All contained mitochondrial *Mbo*I fragments smaller than 1.5 kb. The remaining cloned probes strongly cross-hybridized with one or more cyst species. These probes contained relatively large (1.5 kb) or multiple mitochondrial fragments.

Probe pHG23 did not hybridize to dot blots of *H. leuceilyma*, *H. weissi*, or *G. virginiae*. Occasional weak cross-hybridization was observed with *H. trifolii*. Cross-hybridization was evident with three *H. schachtii* populations, although at a reduced level when compared with SCN controls. Nick-translated pBR322 controls displayed no cross-hybridization with nematode DNA.

Variations in hybridization intensity were observed when SCN DNA from single cysts was hybridized with the same probe (Fig.

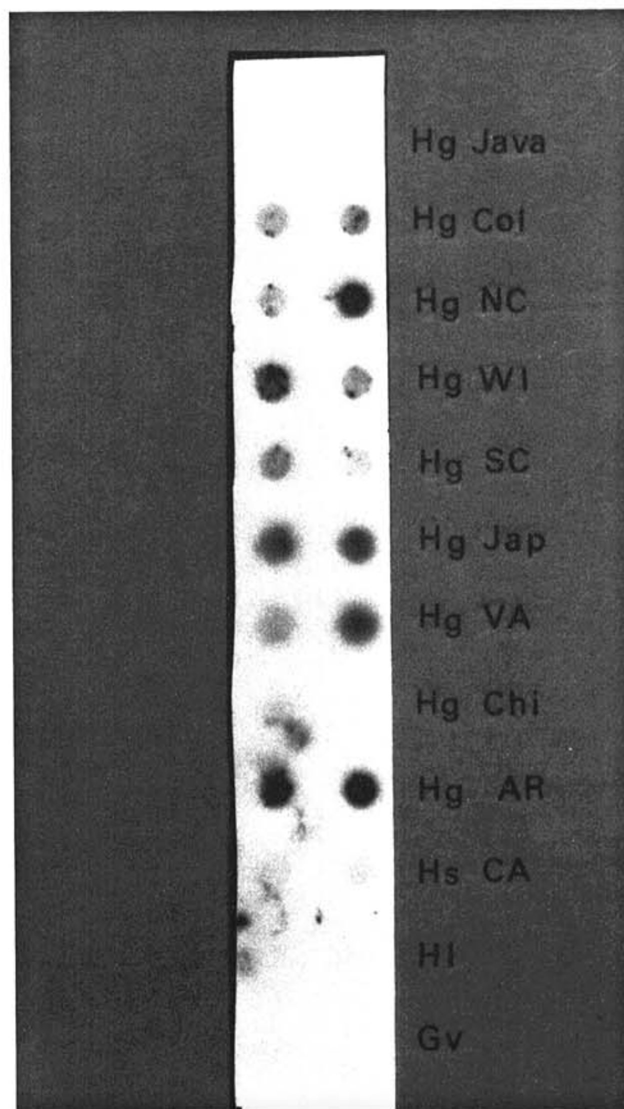

Fig. 1. DNA hybridization of mitochondrial probe pHG23 to single-cyst blots of nine *Heterodera glycines* isolates and three other cyst species. Each spot represents all the DNA from single cyst. Hybridization was at 65 C for 24 hr with two washes each of 2× SSC and 0.2× SSC at 65 C. Filters were exposed to X-ray film for 24 hr. Hg = *H. glycines*; Col = Colombia, South America; WI = Wisconsin; SC = South Carolina; Jap = Japan; VA = Virginia; Chi = China; AR = Arkansas; Hs = *H. schachtii*; CA = California; Hl = *H. leuceilyma*; and Gv = *Globodera virginiae*.

1). It was assumed that this variability was due to differences in amounts of DNA contained in cysts. Attempts were made to minimize this variability by selecting cysts from synchronous cultures that appeared to contain equivalent numbers of eggs. In spite of these precautions, variability in hybridization intensity persisted, although not at a level to confound interspecies comparisons.

Hybridizations in Figure 2 were conducted at a lower stringency than those in Figure 1. By lowering hybridization temperatures from 65 C to 60 C and increasing the salt concentration of the washes, some of the variation in hybridization intensity among SCN cysts was reduced (Fig. 2). However, this lowering of hybridization stringency also increased cross-hybridization with *H. schachtii.*

Biotin-labeled probe pHG23 showed considerable nonspecific binding when hybridized to cyst dot blots. SCN and non-SCN blots displayed equal signal intensity on filters. A proteinase K prewash (0.1 mg/ml in 2× SSC-0.1% SDS) of filters for 1.5 hr did not improve specificity of the probe, but instead appeared to decrease overall signal intensity.

DISCUSSION

We have shown that DNA hybridization probes constructed from mitochondrial DNA can be used to detect soybean cyst nematodes. A [^{32}P]-labeled, cloned hybridization probe containing an approximately 500-base pair mitochondrial fragment was able to distinguish isolates of SCN from closely related cysts species. Some cross-hybridization was observed with three *H. schachtii* isolates, a sibling cyst species that has been shown to interbreed with SCN in the laboratory (7). Hybridization under more stringent conditions reduced this cross-hybridization, although it also accentuated variation in hybridization intensity of SCN DNA from different populations. Currently, we are surveying mitochondrial genomes from these populations for restriction fragment length polymorphisms to determine if this intraspecific variation in hybridization is due to nucleotide sequence divergence among SCN populations.

The variability in the amount of DNA among single cysts is a complicating factor in using DNA hybridization as a diagnostic technique. Generally, a single SCN cyst gives a readily detectable hybridization signal with the probe. The detection of a single second-stage juvenile confirms the sensitivity of the technique. Cysts, however, can vary 50-fold in the number of eggs they contain (unpublished data). Furthermore, the eggs of some cysts may be destroyed by fungal egg parasites (4). For diagnostic purposes the application of 3–5 cysts per dot-blot reduces the problem of variable DNA levels within cysts.

Techniques to apply cyst DNA to nitrocellulose are straightforward. Tube homogenization is the preferred technique because disrupting cysts directly on nitrocellulose can result in tearing of nitrocellulose filters. Also, tube-homogenized cysts can be applied to filters immobilized in a dot-blot apparatus, which simplifies fixing and allows multiple samples to be examined on the same filter without cross-contamination of samples.

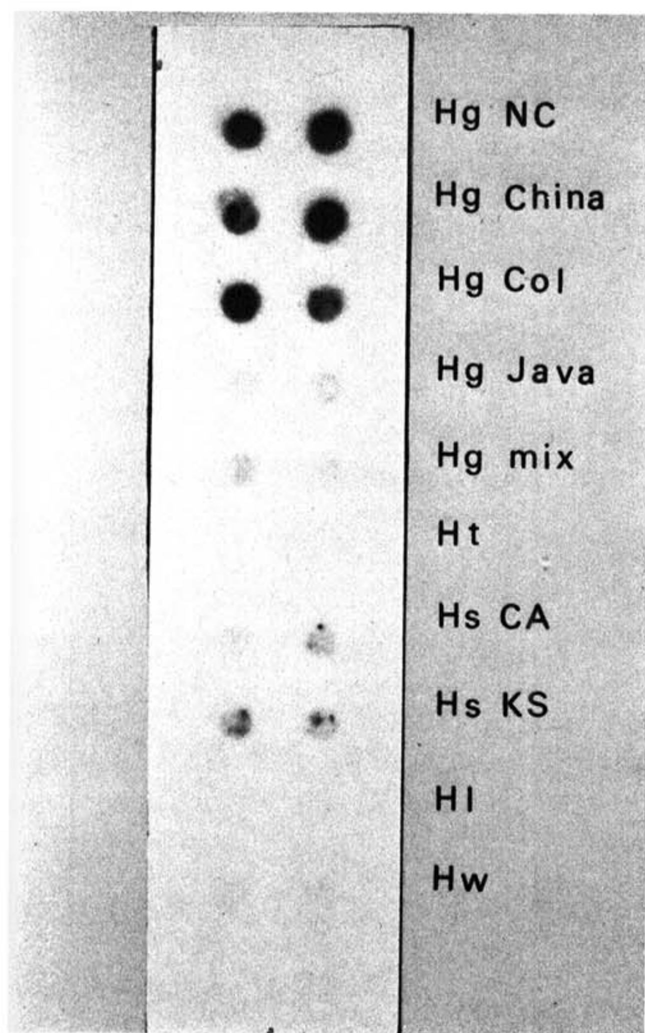

Fig. 2. DNA hybridization of probe pHG23 to single-cyst blots of five *Heterodera glycines* isolates and four other cyst species. Each spot contains DNA from one nematode cyst applied to filter. Hybridization was at 60 C in 4× SSC for 24 hr with two washes each at 60 C of 4× SSC and 2× SSC. X-ray exposure was for 24 hr. Hg = *H. glycines*; NC = North Carolina; Col = Colombia, South America; mix = mixed population; Ht = *H. trifolii*; Hs = *H. schachtii*; CA = California; KS = Kansas; Hl = *H. leuceilyma*; and Hw = *H. weissi*. Cysts from Hg mix population contained few viable eggs.

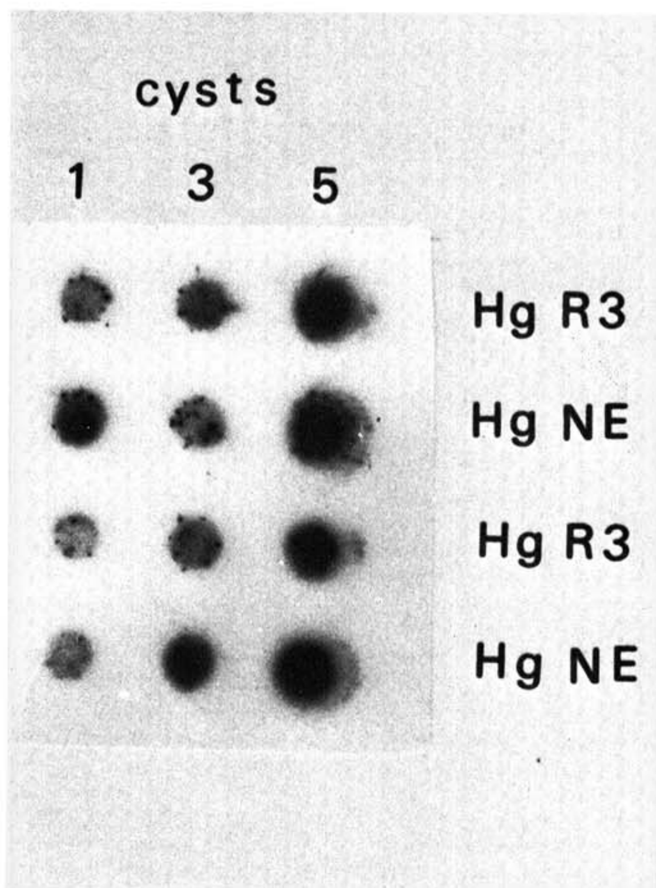

Fig. 3. DNA hybridization of SCN probe pHG23 with dot blots of *Heterodera glycines* race 3 (Hg R3) from Arkansas and *H. glycines* Nebraska isolate (Hg NE). One, three, or five cysts of each isolate were applied to nitrocellulose filters, fixed, and dried as in interspecific comparisons. Prehybridization, hybridization with probe pHG23, washing, and X-ray exposure were as described for Figure 2.

Ultimately, nonradioactive-labeling protocols would be desirable for routine diagnostic applications. Unfortunately, in these studies the biotin-labeled probes produced considerable nonspecific binding with the crude cyst homogenates.

Unacceptable levels of nonspecific binding with biotin-labeled probes have been observed by other researchers (12) and may be due to glycoproteins found in unpurified samples binding with the indicator proteins used in the biotin system. A proteinase K prewash of filters removed some of this binding but also decreased the hybridization signal of positive controls. Future work will investigate the efficacy of other nonradioactive-labeling systems and further refinements of the biotin system.

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