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Diversity Among a *Heterodera glycines* Field Isolate and Derived Inbreds Based on RAPD Analysis and Reproduction on Soybean Genotypes[1]

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Abstract: A field population of *Heterodera glycines* was inbred by a combination of controlled male-female matings and inoculation of soybean with second-stage juveniles (J2) from single cysts. The initial and four F_6 inbred populations were subjected to random amplified polymorphic DNA analysis and were also tested for their ability to reproduce on race differentials. The RAPD patterns of the inbred populations had a lower number of total bands and a lower percentage of polymorphic bands among individual cysts than the initial population. The estimated number of polymorphic loci detected by RAPD analysis was about 25% for the initial population and 4% to 7% for the inbred lines. Reproduction of *H. glycines* decreased for 6 of 24 inbred-soybean combinations. In particular, reproduction of three inbred populations on PI 90763 was greatly reduced. Inbreeding did not decrease variance of cyst number on soybean genotypes. The inbreeding coefficient calculated from RAPD data was greater than that derived from the known inbreeding pedigree.

Key words: cyst, diversity, genetics, *Glycines max, Heterodera glycines,* inbreeding, isolate, nematode, PCR, population, RAPD, resistance, soybean, soybean cyst.

The interaction between *Heterodera glycines* Ichinohe, the soybean cyst nematode, and *Glycine max* (L.) Merr, soybean, is being used as a model system for the study of resistance genes and the mechanisms of nematode plant-parasitism (Opperman et al., 1994). Field populations of *H. glycines* exhibit remarkable variation in reproductive ability on different soybean cultivars. Sixteen races of *H. glycines* were described based on reproduction on four soybean genotypes (Riggs and Schmitt, 1988). Inbreeding has been used to develop more homogeneous isolates for the study of the parasitic ability of *H. glycines*. Different methods, including full-sib (Dropkin and Halbrendt, 1986) and half-sib matings (Leudders, 1985; Opperman et al., 1994) have been used. After a number of generations of inbreeding, the inbreds are screened for reproduction on selected soybean genotypes and are considered highly homozygous if a distinct parasitic phenotype (i.e., no cyst) is observed (Leudders, 1989; Opperman et al., 1994). Crosses using inbred isolates have allowed postulations of the numbers and dominance of genes for parasitism (Opperman et al., 1994; Dong and Opperman, 1997).

Our objectives were to (i) establish inbred lines of *Heterodera glycines,* (ii) compare the pathogenicity of the initial population and derived inbred isolates, and (iii) characterize individual cysts from the initial population and inbreds to estimate the level of genetic variation in the inbreds using PCR-based Random Amplified Polymorphic DNA analysis (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990). This assessment would then be compared with the theoretical calculated inbreeding coefficient (F) (Falconer, 1989). Because reproduction on soybean genotypes is highly variable (Riggs and Schmitt, 1991; Riggs et al., 1988; Triantaphyllou, 1975) and many factors can mitigate the expression of a genotypic trait, direct assay of genotypes through the use of DNA-based markers should provide a more reliable and convenient way to evaluate the inbreds.

Materials And Methods

Sources of nematode population and soybean seeds: The original field isolate was collected from the Clemson University Edisto Re-

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search and Education Center, Blackville, South Carolina. Nematodes were cultured on soybean cultivar Essex or Hutcheson grown in washed sand in the greenhouse, and were used as the initial isolate for inbreeding. Seeds of cultivars Essex, Hutcheson, Peking, Pickett, PI 88788, PI 90763, and PI 437654 were obtained from Emerson R. Shipe, Department of Crop and Soil Environmental Science, Clemson University, Clemson, South Carolina.

Inbreeding: Inbreeding was accomplished by controlled male-female matings followed by transfer of second-stage juveniles (J2) from single cysts. Controlled male-female mating was performed as described (Dropkin and Halbrendt, 1986) except that one or more random mating generations were produced until enough inoculum was available to initiate the next round of inbreeding. Single male-female mating was performed until F_3. In the transfer-of-J2 procedure, J2 from single cysts were used as inoculum to produce the next generation (Fig. 1).

Reproduction of nematode isolates: Four F_6 inbred isolates, IP 1, IP 2, IP 5, and IP 7, were selected for assessment of reproduction on selected soybean genotypes and for the single-cyst RAPD analysis described below. Each inbred isolate was cultured on Hutcheson for one or two generations to increase inoculum. Eggs were extracted on a sucrose gradient (Acedo and Dropkin, 1982). Inoculum density was determined from aliquots placed on a 1-ml eelworm counting slide (Hawksley City, England). For each isolate, 500 eggs and juveniles per plant were prepared as inoculum. Seedlings were selected for uniformity and grown for 5 to 7 days before being transferred into infested soil. Plants were grown at 28 °C in the greenhouse for 35 to 38 days until harvest. Cysts were dislodged from the roots with a jet of water and counted with a dissecting microscope (Halbrendt et al., 1987).

For each test, there were three to five replications for each soybean genotype, depending on the availability of inoculum. All tests for reproduction were repeated once. Because the results were statistically similar, data from the two experiments were combined for analysis. Female index (Golden et al., 1970) and standard error were calculated. All data were subjected to the Student t-test (SAS Institute, Cary, NC).

PCR-based RAPD analysis: Cysts were cleaned with sterile water several times before DNA extraction. DNA was extracted from single cysts or bulked-cyst collection as described (Caswell-Chen et al., 1992). To determine the DNA concentration necessary to ensure reproducible band patterns, 8, 4, 2, and 1 µl from a total extraction volume of 18 µl from single cysts were used as templates for the single-cyst RAPD analysis. Twenty-five microliters of reaction mix contained about 15 ng bulk DNA or 6 to 8 µl of single-cyst DNA extract, 0.15 unit of *Tfl* polymerase (Epicentre Technologies, Madison, WI), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (Promega, Madison, WI), 0.5 µM tenmer primer (University of British Columbia, Vancouver, Canada), 0.05 µg/µl BSA, and 1.28 × reaction buffer (64 mM Tris-HCl pH 9.0 at 25 °C, 25.6 mM ammonium sulfate). The reaction mixture was heated to 94 °C for 5 minutes, followed by 45 cycles of 1 minute at 94 °C, 1 minute at 42 °C, and 2 minutes at 72 °C. The cycle was extended another 5 minutes at 72 °C at the end of the reaction. Fifteen microliters of the amplification reaction was electrophoresed in a 1.4% agarose gel stained with 0.5 µg/ml ethidium bromide with TBE buffer at 1.5 V/cm for approximately 4.5 hours. Products were examined and photographed under UV light. Hind III-digested λ DNA was used as the molecular size standard.

The DNA from four individual cysts from the initial population and from each of the four inbreds were subjected to RAPD analysis. The sequences of the 20 selected polymorphic primers used for RAPD analyses of the inbred isolates are shown in Table 1. Clearly demarcated bands in the gels for each primer were scored according to the following system: 0 = band absent in four cysts; 1 = band present in one cyst, absent in three; 2 = band present in two cysts, absent in two; 3 = band present in three cysts, absent in one; and 4 = band present in four cysts.

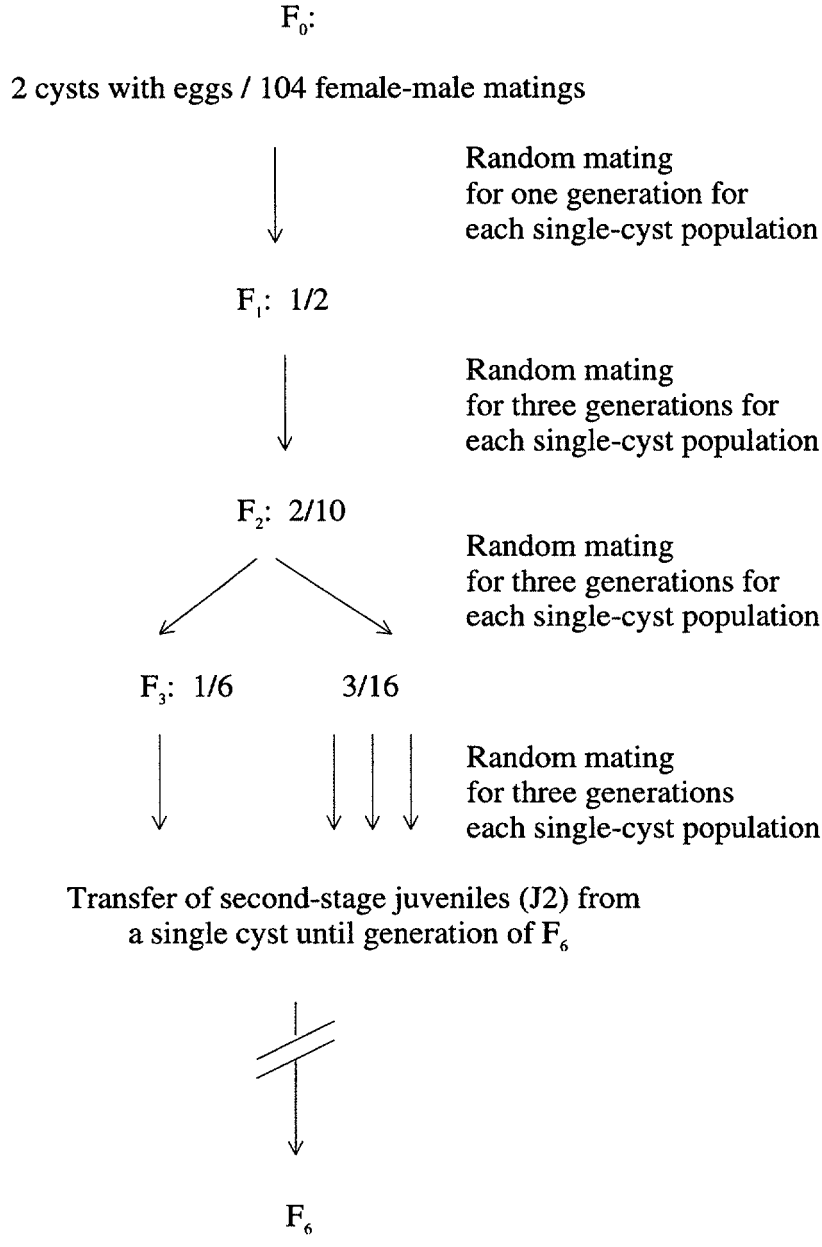

FIG. 1. Diagram of the inbreeding process for *Heterodera glycines*. Denominator, total number of female-male pairings; numerator, number of matings producing eggs.

RESULTS

Inbreeding: Single male-female mating and transfer of J2 from single cysts yielded different numbers of progeny. Generally, the number of males recovered was greater than that of females. The overall mating success rate, determined by the presence of eggs, was around 15% of total pairings. The number of eggs per cyst after 1:1 mating was generally very low (average around 20) and variable. After the F_3 generation, when J2 were transferred from single cysts, progeny number increased greatly (10 to 30 cysts per generation) relative to those from the controlled male-female matings. The inbred iso-

TABLE 1. Sequence of the 20 primers polymorphic in the initial isolate of *Heterodera glycines* from which inbred isolates were derived.

Primer	Sequence									
6	C	C	T	G	G	G	C	C	T	A
13	C	C	T	G	G	G	T	G	G	A
17	C	C	T	G	G	G	C	C	T	C
24	A	C	A	G	G	G	G	T	G	A
35	C	C	G	G	G	G	T	T	A	A
42	T	T	A	A	C	C	C	G	G	C
51	C	T	A	C	C	C	G	T	G	C
53	C	T	C	C	C	T	G	A	G	C
54	G	T	C	C	C	A	G	A	G	C
55	T	C	C	C	T	C	G	T	G	C
58	T	T	C	C	C	G	G	A	G	C
60	T	T	G	G	C	C	G	A	G	C
75	G	A	G	G	T	C	C	A	G	A
78	G	A	G	C	A	C	T	A	G	C
79	G	A	G	C	T	C	G	T	G	T
90	G	G	G	G	G	T	T	A	G	G
92	C	C	T	G	G	G	C	T	T	T
94	G	G	G	G	G	G	A	A	C	C
133	G	G	A	A	A	C	C	T	C	T
134	A	A	C	A	C	A	C	G	A	G

lates reproduced well on the susceptible hosts, although there was a slight reduction in the average number of eggs within the cysts. The number of eggs per cyst for the initial isolate was about 270, compared with 180 to 240 for eight of the nine F_6 inbred isolates, and 140 for one isolate. However, because the average egg number per cyst was highly variable, the reductions were not statistically different.

Reproduction of nematode isolates: The average number of females of the initial population and the derived F_6 inbreds IP 1, IP 2, IP 5, and IP 7 on Essex and the female indices on the race differentials are summarized in Table 2. Except for the level of reproduction on Peking and PI 437654, female indices of *H. glycines* differed from the initial population for at least one of the tested inbreds. In particular, female indices of IP 1, IP 2, and IP 7 on PI 90763 were reduced from an average of 40% for the initial isolate to less than 15% for the inbreds, while that number for IP 5 remained the same. Reproduction on PI 88788 was significantly reduced for IP 7. Female indices on Pickett were lower for IP1 and IP2 than for the initial isolate.

PCR-based RAPD analyses: Although the amount of DNA obtained from single cysts was variable, RAPD patterns were reproducible when 8, 4, or 2 µl from a total extraction

TABLE 2. Average numbers of *Heterodera glycines* mature females on Essex soybean and female indices on race differentials plus PI 437654 followed by standard errors.

Nematode isolate	Number of females on Essex soybean	Indices[a]				
		Peking	Pickett	PI 88788	PI 90763	PI 437654
Initial	64.4 ± 14.9	52.8 ± 21.4	71.8 ± 16.9	12.2 ± 5.4	40.9 ± 12.1	1.4 ± 1.2
IP 1	60.5 ± 12.7	44.1 ± 7.1	28.8 ± 10.2[b]	4.3 ± 3.6	14.4 ± 7.3[b]	0.2 ± 0.5
IP 2	62.2 ± 17.9	70.0 ± 15.9	39.6 ± 9.3[b]	5.5 ± 4.3	10.1 ± 3.9[b]	0.3 ± 1.0
IP 5	56 ± 10.7	58.0 ± 15.7	73.8 ± 15.7	3.6 ± 3.1	48.8 ± 15.5	0
IP 7	80.4 ± 15.3	30.4 ± 10.2	47.9 ± 10.8	1.6 ± 1.5[b]	8.3 ± 3.2[b]	0

[a] Female indices are calculated as follows: Number of females on soybean genotype/number of females on susceptible soybean control (Essex) × 100.

[b] Indices in column are significantly reduced ($P = 0.05$) from the initial population according to the Student t-test.

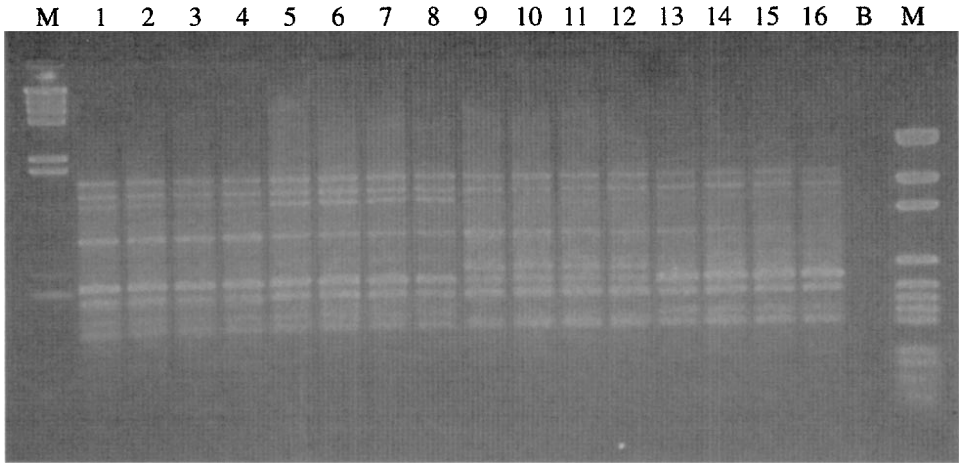

FIG. 2. RAPD patterns of the F_6 IP 7 inbred isolate using different amounts of DNA from four individual *Heterodera glycines* cysts as templates. Primer 6 (Sequence: CCTGGGCCTA) was used. Total extraction from single cysts was 18 µl. Each cyst occupied four adjacent lanes. Lanes 1, 5, 9, 13 = 8 µl DNA; lanes 2, 6, 10, 14 = 4 µl DNA; lanes 3, 7, 11, 15 = 2 µl DNA; lanes 4, 8, 12, 16 = 1µl DNA. Lane M is DNA marker. Lane B is control (without nematode DNA).

of 18 µl were used as the template volumes. Loss of bands was occasionally observed when less than 0.1 volume (approximately 1 µl) of a single-cyst extraction was used as the template (Fig. 2). The PCR-negative controls (without nematode DNA) did not yield detectable amplification products.

For each nematode isolate, different sets of four cysts were used for different primers. Of 55 tested tenmer primers, 25 (45%), produced polymorphic bands for four randomly chosen cysts from the initial isolate. Twenty (out of 25) primers were used for RAPD analyses of the selected inbred isolates. The cysts from the same inbred shared most RAPD bands of the same size (Fig. 3), but a proportion of bands was polymorphic (Fig. 2). Among different inbreds, band patterns were generally different for all tested primers, and some bands appeared to be inbred-specific. Table 3 shows the number of scored bands that appeared in 0, 1, 2, 3, 4 cysts, respectively, each for the initial and derived F_6 inbreds. Twenty-six bands in the initial population have code 0 because they showed up in only some of the inbreds, but not in the tested four cysts from initial populations. However, when bulk DNA of the initial population was used, these bands were detectable. The total number of bands was greatly reduced for the inbred isolates (increased numbers of code 0). Meanwhile, the

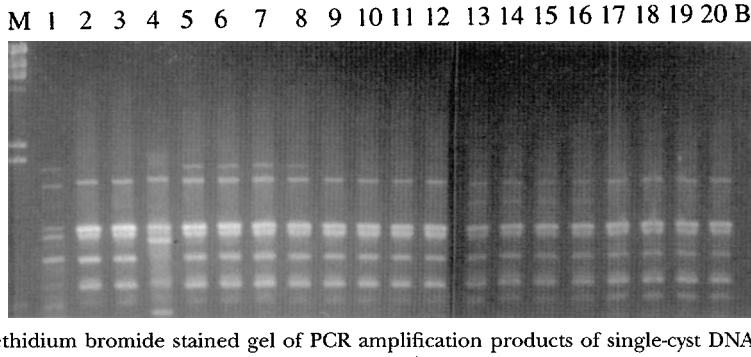

FIG. 3. An ethidium bromide stained gel of PCR amplification products of single-cyst DNA using primer 58 (Sequence: TTCCCGGAGC). Lanes 1–4, 5–8, 9–12, 13–16, 17–20 are DNAs from individual *Heterodera glycines* cysts from the initial and derived inbred isolates IP 1, IP 2, IP 5, and IP 7, respectively. Lane M is the HindIII-digested λ DNA marker. Lane B is blank (without nematode DNA).

TABLE 3. Number of common bands in 0 to 4 tested *Heterodera glycines* cysts and percentage of polymorphic bands among the initial population and derived inbreds as indicated by RAPD markers.[a]

Nematode isolate	Number of common bands in 0 to 4 cysts[b]					Total band number	Percentage of polymorphic bands[c]
	0	1	2	3	4		
Initial	26	73	48	38	131	290	54%
IP-1	110	9	6	4	187	206	9.2%
IP-2	116	10	6	6	178	200	11.0%
IP-5	98	13	8	12	184	217	15.2%
IP-7	109	8	3	9	187	207	9.7%

[a] For each nematode isolate, four cysts were tested.

[b] 0 = number of common bands absent in four cysts (but present in the set of another inbred line(s) or the initial population); 1 = number of common bands present in one cyst, absent in three cysts; 2 = number of common bands present in two cysts, absent in two cysts; 3 = number of common bands present in three cysts, absent in one cyst; and 4 = number of common bands present in four cysts. 0 and 4 indicate the tested loci were homogeneous among tested individuals, and 1, 2, 3 indicate they were heterogeneous.

[c] Percentage of polymorphic bands was calculated as: (Number of common bands in one cyst + number of common bands in two cysts + number of common bands in three cysts)/number of total bands.

number of non-polymorphic bands was increased (increased number of code 4). The number of polymorphic bands was reduced from 54% for the initial isolate to 9% to 15% for the inbreds (Table 3).

Theoretical calculation of inbreeding coefficient: The inbreeding coefficient of an individual is defined as the probability that the pair of alleles carried by the gametes that produced it are identical by descent (Wright, 1933). Equations for regular full-sib mating and half-sib mating have been derived (Falconer, 1989). For full-sib mating,

$$F_t = 1/4\ (1 + 2F_{t-1} + F_{t-2}) \qquad (1)$$

For half-sib mating,

$$F_t = 1/8\ (1 + 6F_{t-1} + F_{t-2}) \qquad (2)$$

In both equations,

F: Inbreeding coefficient,
t: t*th* generation.

To calculate F_1, F_{t-1} and F_{t-2} were both assumed to be zero. It could be an underestimation of the inbreeding coefficient because inbreeding might occur in natural field conditions. Although the inbreeding of *Heterodera glycines* in this study cannot be considered as a regular system (the same mating system is applied in all generations, and all individuals in the same generation have the same inbreeding coefficient) because of the skipped generations of random matings between the controlled full-sib matings, the inbreeding coefficient remains the same during the random mating process (Zhang, 1996). The tested F6 inbreds have an inbreeding coefficient of 0.67 (Table 4).

DISCUSSION

The inbreeding strategy using full- and half-sib selection resulted in successful development of inbred nematode isolates varying in reproductive potential on soybean genotypes. The extraction procedures and PCR conditions utilized allowed successful amplification of DNA from individual nematode cysts using random primers and provided the means to evaluate population homogeneity at the genotypic level. As expected, the RAPD patterns of inbred isolates had fewer total bands and a higher percentage of shared bands, which may be considered as allele fixation. Inbreds are apparently more homogeneous than the initial populations. The percentage of polymor-

TABLE 4. Theoretical inbreeding coefficients of Race 14.

Generation (t)	System of mating	Inbreeding coefficient
1	Full-sib mating	0.25
2	Full-sib mating	0.375
3	Full-sib mating	0.5
4	Full-sib mating	0.59
5	Half-sib mating	0.63
6	Half-sib mating	0.67

phic bands (Table 3) represents only primers polymorphic in the initial nematode isolate, which is 45% of the total tested primers. As a very rough estimation, if we assume that different primers yield same number of bands, we could use the percentage of primers yielding polymorphic banding patterns in the initial isolate as a calibration factor. The polymorphic loci detected by the RAPD analysis of four individuals are about 25% (0.45 × 54%) for the initial population and 4% (0.45 × 9.2%) to 7% (0.45 × 15.2%) for the inbred isolates.

RAPD analysis was performed on the F_6 individual cysts of the inbred isolates, which, according to the pedigrees, would have an inbreeding coefficient of F = 0.67, assuming full-sib mating for the controlled male-female mating and half-sib mating for the transfer of J2 from single cysts. The inbreeding coefficient also can be derived from the RAPD data. Let H_t and H_0 be the frequencies of heterozygotes in inbred and initial isolates, respectively. Then, the average inbreeding coefficient at generation t will be: $F = (H_0 - H_t)/H_0$ (Falconer, 1989). If it is assumed that H_0 is 25% and H_t is 4% to 7%, the average inbreeding coefficient would be 0.72 to 0.84, slightly greater but in reasonable concordance with the value calculated from the known pedigree. The reasons for the small discrepancy could be the large sampling variance associated with sampling size and the dominant nature of RAPD markers. The latter is an unavoidable problem associated with the analysis of population structure using RAPD techniques. Lack of complete genotypic information resulting from dominance enhances the sampling variance associated with a single locus and induces bias in parameter estimation (Lynch and Milligan, 1994). In addition, RAPD analysis of a cyst full of eggs is different from assay of an individual nematode in that it reflects the collection of parental genotypes and polymorphisms may not necessarily show up.

In the nematode reproduction experiments, inbreeding suppressed the level of reproduction of *H. glycines* on certain soybean genotypes. Segregation of the parasitism genes apparently occurred among the inbreds, i.e., on PI 90763, and the female indices of IP 1, IP 2, and IP 7 were reduced greatly. The result suggested the recessive nature for parasitism of PI 90763 in the initial population. Dong and Opperman (1997) also uncovered a recessive gene for parasitism of the same host genotype through cross and backcross analysis. In contrast, the female indices for IP 5 remained unchanged when compared to the initial population.

The variance in cyst numbers was not reduced by inbreeding. Standardized procedures have been developed to reduce variability among race tests, but they are of only limited effectiveness (Riggs and Schmitt, 1991). The variation could be due to test conditions, genetic differences in seeds, or heterogeneous nematode populations. According to our results, variances from sources other than the nematode types, such as environmental conditions, may play an important role in the total observed variation.

Our data also support previous observations that great variation exists in field isolates of *H. glycines*. The observed variation might result from the obligate amphimictic reproduction of the nematode (Triantaphyllou, 1975). In addition, if inbreeding exists in natural field conditions, its effect on the genetic variance is apparent—the gene frequencies in the separate lines tend toward the extreme values of 0 or 1, and the lines become differentiated in gene frequencies. Subpopulational differentiation within field isolates could cause subpopulational isolation and lead to increased phenotypic variation for the total field population. Further studies are needed to evaluate this hypothesis.

In summary, we have applied PCR to single cysts to monitor *H. glycines* genotypes during inbreeding. These results revealed a close match with the inbreeding coefficient predicted on pedigrees alone. The work presented here for single-cyst RAPD analysis will facilitate population genetic studies, as well as the mapping of important genes in the

genome of *H. glycines* in future investigations.

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