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Genetic variability among and within races of *Heterodera glycines* Ichinohe assessed by RAPD markers*

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Abstract

Heterodera glycines Ichinohe, the soybean cyst nematode (SCN) was detected in Brazil in 1992 and since then it has been causing losses to Brazilian soybean crop yields. SCN populations have great genetic diversity which makes it difficult to manage this disease. The objectives of this research were to investigate the genetic variation of 16 SCN populations sampled in middle western and southeastern Brazil, utilizing random amplified polymorphic DNA (RAPD) techniques, and to establish useful and specific RAPD markers for SCN race 3. RAPD markers demonstrated genetic variability among and within SCN populations, and they could be used for monitoring nematode population dynamics. The OPA-07 primer was a reliable molecular marker for race 3, while electrophoretic profile analysis of DNA fragments amplified with OPA-10 primer detected slight variation within those populations identified as race 3. The SCN population from Chapadão do Céu, GO - sample 2 - was the most genetically distant from the other populations.

INTRODUCTION

Soybean, *Glycine max* (L) Merrill, is a crop of paramount importance in Brazil, which is the second leading producer of this crop in the world. The country produced about 23 million tons of soybeans on 10 million hectares in 1996 (EMBRAPA, 1996).

Heterodera glycines Ichinohe, the soybean cyst nematode (SCN), is today the most important pathogen of the crop in the United States and eastern Asia (Baldwin and Mundo-Ocampo, 1991; Anand *et al.*, 1994). During the 1991/1992 Brazilian growing season, this nematode was found for the first time in the States of Goiás, Mato Grosso, Mato Grosso do Sul and Minas Gerais (Mendes and Dickson, 1993).

Populations of SCN normally have great genetic diversity, which can be determined by their ability in reproducing on selected soybean genotypes (differential hosts) (proposed by Golden *et al.* (1970) and expanded by Riggs and Schmitt (1988)). Aiba *et al*. (1995), for example, demonstrated genetic variability within and among populations of *H. glycines* based on soybean differentials. Although several biochemical techniques have been attempted for race identification of this nematode, they have not been useful (Griffith *et al*., 1982; Pozdol and Noel, 1984). However, analysis of DNA polymorphisms, either as random amplified polymorphic DNA (RAPD) (Vasconcelos *et al*., 1994) or restriction fragment length polymorphisms (RFLP) (Kalinski and Huettel, 1988), have been successfully used.

Plant-parasitic nematode genomic DNA has been investigated primarily through RFLP, which can differentiate species and, in some cases, races and populations of nematodes (Curran *et al.*, 1986; Kalinski and Huettel, 1988). Nevertheless, the RFLP technique has some limitations, such as the requirement for a large amount of DNA, high labor input, use of radioisotopes, and lack of sufficient sensitivity to distinguish races (Curran *et al*., 1986; Kalinski and Huettel, 1988; Castagnone-Sereno, 1992).

DNA polymorphism assays based on polymerase chain reaction (PCR) amplifications of DNA segments using single primers of arbitrary nucleotide sequence (RAPD) analysis (Welsh and Mcclelland, 1990; Williams *et al*., 1990) have been used for genetic markers in a variety of plants (Demeke *et al*., 1992; Mackill, 1995; Orozco-Castilho *et al*., 1996), fungi (Assigbetse *et al*., 1994; Woo *et al*., 1996) and nematodes (Caswell-Chen *et al*., 1992; Folkertsma *et al*., 1994; Guirao *et al*., 1995). The ability of RAPD markers to detect genetic variation among cyst nematodes was reported by Caswell-Chen *et al*. (1992), Folkertsma *et al*. (1994), and Vasconcelos *et al*. (1994).

This research has the objectives of evaluating the application of RAPD analysis to assess genetic variability among 16 *H. glycines* populations from several locations in Brazil, establishing useful RAPD markers specific for race 3, and determining variation within populations of race 3 (through differential genotypes) of this nematode.

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MATERIAL AND METHODS

Genetic material

Sixteen SCN populations from Brazil were used, 10 of which were collected in several sites in Minas Gerais State (Table I). Nine of the populations had their corresponding races previously determined. Therefore, populations from Coromandel, Monte Carmelo, Pedrinópolis, Santa Juliana, Uberaba and Uberlândia were characterized as composed of race 3 (Silva, 1997), while the ones from Primavera do Leste (MT), Campo Verde (MT) and Água Clara (MS) were obtained at Empresa Brasileira de Pesquisa Agropecuária/Centro Nacional de Pesquisa de Soja (EMBRAPA/CNPSo - Londrina, PR) and identified as races 1, 2 and 9, respectively. The remaining SCN populations have not been identified as to their race constitution.

Cyst and egg extraction

Two hundred viable cysts were extracted from each population using the centrifugal-flotation method, with slight modifications (Jenkins, 1964, cited by Mendes, 1986). After extraction, cysts were placed into 1.5-ml Eppendorf tubes and stored at -80ºC. Females and cysts were treated with 0.5% sodium hypochloride for one minute before extraction, following crushing on a 100-mesh sieve. Eggs and second-stage juveniles were washed and collected on a 500-mesh sieve, then placed in 1.5-ml Eppendorf tubes and centrifuged at 3,000 rpm (about 650 *g*) for five minutes. The remaining 100 ml suspension of eggs and juveniles was stored in a refrigerator until DNA extraction.

DNA extraction

Eggs and juveniles were powdered in liquid nitrogen in 1.5-ml Eppendorf tubes and 700 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA and 1% β-mercaptoethanol added at the time of utilization) was added. The homogenate was incubated in a water bath for two hours at 65ºC, cooled on ice, and spun at 1,000 rpm for one minute at 4ºC in a microcentrifuge. The supernatant was transferred to a new tube and an equal volume of chloroform + isoamyl alcohol (24:1) added and extracted twice. After centrifugation at 13,000 rpm (about 12,000 *g*) for 15 min, approximately 500 µl of the aqueous phase was transferred to a new tube, and DNA was precipitated by adding 2 volumes of isopropanol. The suspension was maintained at 4ºC overnight. After a new centrifugation at 13,000 rpm for 10 min, the supernatant was discarded, and the precipitate was rinsed once with 500 µl of 70% ethanol, air dried for two hours, and resuspended in 100 µl TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) plus 40 µg/ml of RNAase. The solution was maintained at 37ºC for 30 min and incubated in a water bath for 20 min at 60ºC. Nucleic acid concentration was determined spectrophotometrically at 260 nm (Sambrook *et al.*, 1989).

DNA amplification

DNA amplification reactions were performed in a thermocycler and consisted of 45 cycles, each consisting of the following steps: denaturation (94ºC for 1 min), annealing (34ºC for 1 min), and elongation (72ºC for 2 min). Each reaction mixture (25 µl) contained: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM of each deoxyribonucleoside triphosphate (dATP, dTTP, dCTP, and dGTP), 1.5 units of Taq DNA polymerase, 20 ng of genomic DNA, and 0.4 µM of a single primer (Operon Technologies, Inc.; Table II). Control reactions, containing all components except template DNA, were included to avoid misinterpretations of RAPD patterns due to PCR artifacts. Samples were overlaid with mineral oil to prevent evaporation. After amplification, DNA products were separated electrophoretically on 8% polyacrylamide gels prepared according to Sambrook *et al*. (1989) in TBE buffer 1X (0.09 M Tris-borate, and 2 mM EDTA). The "1-kb DNA ladder" was used as the molecular weight standard. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed with polaroid 667 film under ultraviolet light.

Data analysis

DNA bands were scored as 1 (presence) or 0 (absence). Only intense and reproducible bands were considered. These data were used to determine the pairwise genetic distances among the SCN populations using percent disagreement (PD) (Skroch *et al.*, 1992). Dendrograms were constructed from these PD values using the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS

The 12 primers (Table II) resolved 68 different DNA fragments. The number of fragments produced per primer varied from two to eight and ranged in size from 0.38 to 5.40 kb.

The genetic distance matrix, based on band presence versus absence, was estimated as percent disagreement (Table III). The shortest distance was 1%, found between some of the SCN populations from Minas Gerais State. These results are in accordance with reports that characterize all soybean cyst nematode populations from Minas Gerais as race 3 (Arantes *et al*., 1994; Noel *et al*., 1994; Rossi *et al.*, 1994; Wain and Silva, 1996; Silva, 1997). The greatest distance was found between SCN populations from Chapadão do Céu, GO, and Iraí de Minas, MG (Table III).

A dendrogram was constructed, representing the relationships among the 16 populations of the nematode

(Figure 1). There were large divergences among these populations. The SCN populations from Uberlândia and Pedrinópolis were quite similar and were clustered together, followed by populations from Uberaba, Monte Carmelo, Iraí de Minas (sample 1), Iraí de Minas (sample 2), Coromandel, and Santa Juliana (all from Minas Gerais State). These populations were clustered together at 17% disagreement. Six of them (populations 3 and 6 through 10 in Table I) were previously characterized as race 3 through differential varieties (Silva, 1997).

All random primers tested distinguished population 5 (Chapadão do Céu, GO - sample 2) from the others. OPA-07 primer (Figure 2) yielded a similar pattern of DNA amplification products, with a clear marker of approximately 2.5 kb that was common to the majority of the populations that were characterized as race 3 through differential genotypes. The electrophoretic profile obtained with primer OPA-10 detected slight variation within eight SCN populations from Minas Gerais (Figure 3), and six of them were characterized as race 3 through differential varieties.

DISCUSSION

In the dendrogram generated through the use of genetic distances (Figure 1), eight groups of SCN populations were formed from left to right at the 17% genetic distance level: I) Primavera do Leste, MT (race 1); II) Uberlândia, MG, through Santa Juliana, MG; III) Chapadão do Céu, GO (sample 1) and Chapadão do Sul, MS; IV) Água Clara, MS (race 9); V) Campo Verde, MT (race 2); VI) Romaria, MG; VII) Nova Ponte, MG, and VIII) Chapadão do Céu, GO (sample 2).

Cluster II included eight of the 10 populations that were collected in Minas Gerais State, and six of them were

Table I - Populations of *Heterodera glycines* from Brazil used in this study.

SCN Population	Site (State)	Previously determined race
1	Primavera do Leste - Mato Grosso (MT)	1
2	Campo Verde - Mato Grosso (MT)	2
3	Uberlândia - Minas Gerais (MG)	3
4	Água Clara - Mato Grosso do Sul (MS)	9
5	Chapadão do Céu - Goiás (GO) (sample 2)	-
6	Pedrinópolis - Minas Gerais (MG)	3
7	Monte Carmelo - Minas Gerais (MG)	3
8	Coromandel - Minas Gerais (MG)	3
9	Uberaba - Minas Gerais (MG)	3
10	Santa Juliana - Minas Gerais (MG)	3
11	Iraí de Minas - Minas Gerais (MG) (sample 1)	-
12	Iraí de Minas - Minas Gerais (MG) (sample 2)	-
13	Nova Ponte - Minas Gerais (MG)	-
14	Romaria - Minas Gerais (MG)	-
15	Chapadão do Céu - Goiás (GO) (sample 1)	-
16	Chapadão do Sul - Mato Grosso do Sul (MS)	-

Table II - List of primers used in this study, range in size of DNA fragments (kb), and number of monomorphic and polymorphic fragments.

Primer	Sequence (5'→3')	Range in size of DNA fragments (kb)	Number of fragments	
			Monomorphic	Polymorphic
OPA-02	TGC CGA GCT G	0.39-1.25	5	3
OPA-03	AGT CAG CCA C	0.38-1.60	2	6
OPA-05	AGG GGT CTT G	1.50-3.10	-	5
OPA-06	GGT CCC TGA C	1.20-1.60	-	2
OPA-07	GAA ACG GGT G	1.20-5.20	2	6
OPA-08	GTG ACG TAG G	0.89-1.80	2	1
OPA-10	GTG ATC GCA G	1.80-5.40	-	8
OPA-11	CAA TCG CCG T	0.60-2.50	3	1
OPA-13	CAG CAC CCA C	0.50-4.00	6	-
OPA-17	GAC CGC TTG T	0.70-5.00	-	8
OPA-18	AGG TGA CCG T	1.10-1.60	-	4
AT-O4	AAT CGG CTG	0.40-2.00	-	4
Total		-	20	48

Table III - Pairwise genetic distances as percent disagreement among 16 populations of *Heterodera glycines* used in this study.

SCN populations	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	...	22	16	25	41	18	18	24	19	22	18	21	28	26	22	19
2		...	26	32	37	25	28	28	26	29	28	31	26	19	29	18
3			...	24	49	01	04	13	03	09	01	07	24	22	15	21
4				...	37	22	25	28	24	24	25	31	32	37	21	26
5					...	47	47	50	49	46	47	56	31	38	43	37
6						...	03	12	01	07	03	09	22	21	13	19
7							...	12	04	07	03	12	22	21	13	19
8								...	10	07	12	18	25	26	19	25
9									...	09	04	10	24	22	15	21
10										...	07	13	24	25	18	24
11											...	09	22	21	13	19
12												...	28	24	19	25
13													...	22	32	32
14														...	25	19
15															...	15
16																...

1)-Primavera do Leste-MT (race 1); 2)-Campo Verde-MT (race 2); 3)-Uberlândia-MG (race 3); 4)-Água Clara-MS (race 9); 5)-Chapadão do Céu-GO (sample 2); 6)-Pedrinópolis-MG; 7)-Monte Carmelo-MG; 8)-Coromandel-MG; 9)-Uberaba-MG; 10)-Santa Juliana-MG; 11)-Iraí de Minas-MG (sample 1); 12)-Iraí de Minas-MG (sample 2); 13)-Nova Ponte-MG; 14)-Romaria-MG; 15)-Chapadão do Céu-GO (sample 1); 16)-Chapadão do Sul-MS.

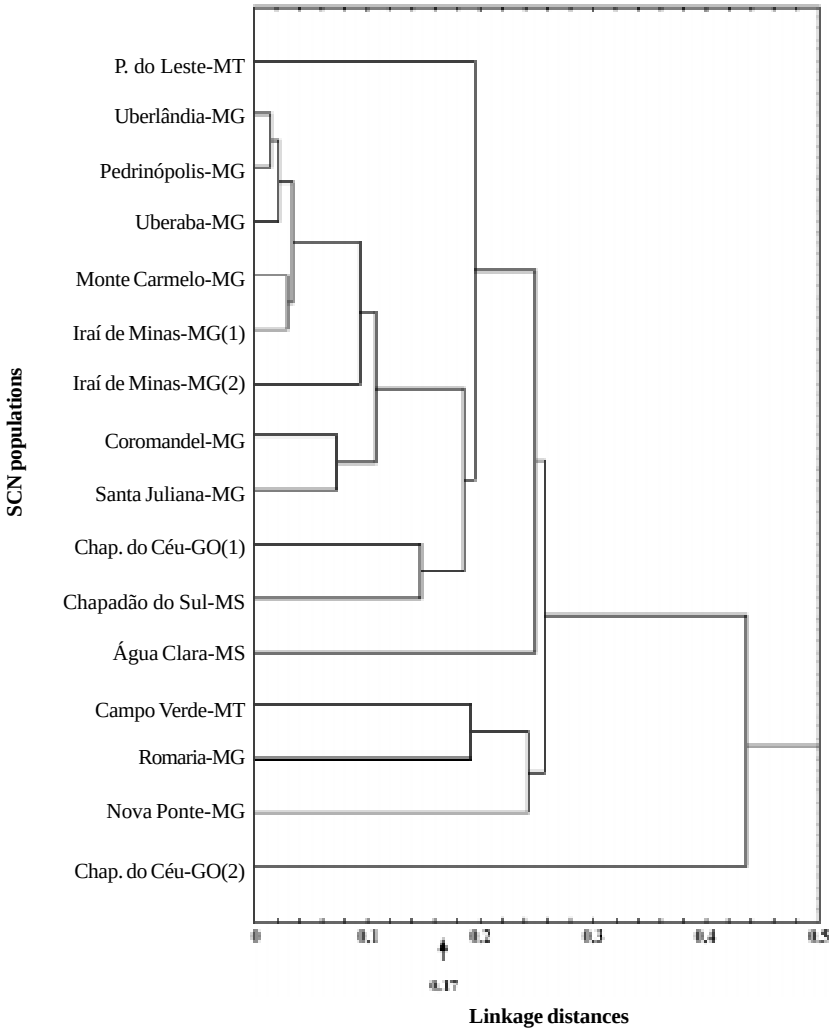

Figure 1 - UPGMA dendrogram generated with genetic distances as percent disagreement for 16 different populations of *Heterodera glycines*. These genetic distances were calculated with 68 RAPD markers obtained with 12 different random primers.

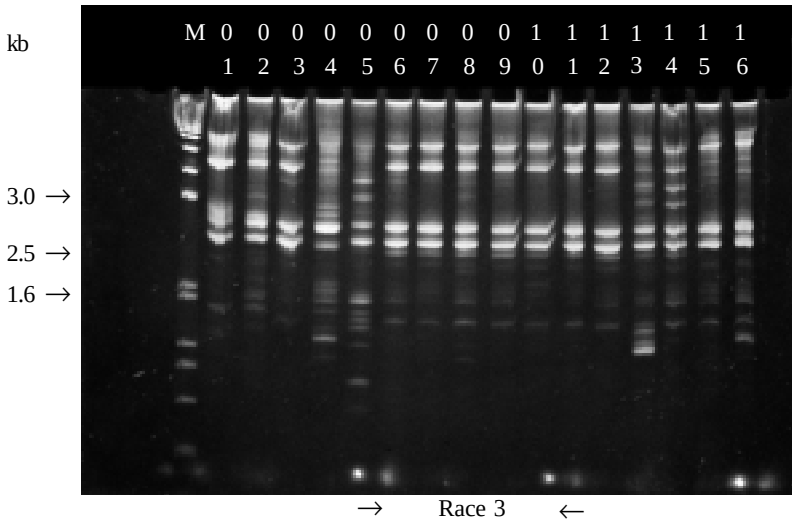

Figure 2 - Electrophoretic analysis of DNA amplification products from *Heterodera glycines* populations obtained on 8% polyacrylamide gel. Amplifications were performed with primer OPA-07. Lane M = Molecular size standard. Lanes 1 through 16 correspond to the amplification products of the 16 SCN populations, as identified in Table I.

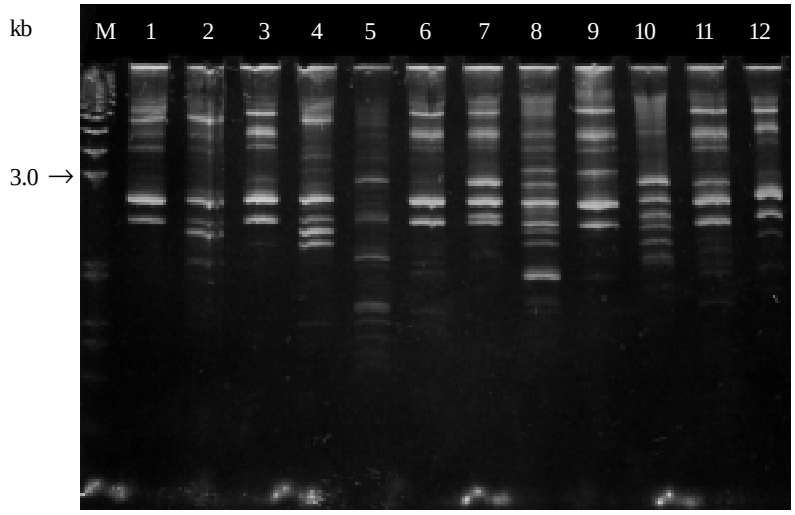

Figure 3 - Electrophoretic analysis of DNA amplification products from *Heterodera glycines* populations obtained on 8% polyacrylamide gel. Amplifications were performed with primer OPA-10. Lane M = Molecular size standard. Lanes 1 through 12 correspond to the amplification products of the first 12 SCN populations, as identified in Table I.

previously classified as race 3 (Table I). The two remaining SCN populations (Iraí de Minas, samples 1 and 2) have not been identified thus far through differential genotypes, but presented similar electrophoretic profiles with primer OPA-07 as compared with the profiles obtained for populations identified as race 3.

The clustering at the 17% disagreement level revealed at least three important aspects: I) clear separation of the populations classified previously on the basis of differential varieties, as races 1, 2, 3 and 9. This result is fully consistent with the ones from direct analysis of RFLP carried out by Kalinski and Huettel (1988), who found differences among races 3, 4, and 5; II) the population of soybean cyst nematode from Chapadão do Céu, GO - sample 2 - was considerably different from all others, and III) eight of the 10 SCN populations from Minas Gerais had similar electrophoretic profiles (Figure 2), and were clustered together.

The difference between the Nova Ponte and Romaria nematode populations is obvious, especially when they are compared with others collected in Minas Gerais State. These two populations were exposed to temperatures above 36ºC for nearly one year, and this could have caused an unfavorable selection pressure on the soybean cyst nematode. Slack and Hamblen (1961) demonstrated that soybean cyst nematode larvae failed to emerge from cysts incubated below 16º or above 36ºC. However, research is needed to determine whether high temperature can increase variability in pathogen populations.

The electrophoretic profile obtained with OPA-07 primer (Figure 2) established a similar DNA amplification pattern for SCN populations characterized as race 3 through the host differential technique. This primer yielded a band of approximately 2.5 kb that appeared to be common to the majority of populations characterized as

race 3. Therefore, RAPD markers could be a useful diagnostic tool to distinguish races of soybean cyst nematodes. However, more experiments are needed to determine if the variability within race 3 of the SCN is an indication of a new race, that would result in variable plant symptoms, suggesting nematode adaptation.

There was a slight variation within populations characterized as race 3 in the electrophoretic profile obtained with primer OPA-10 (Figure 3). This variability was consistent with that based on host differentials (Faghihi *et al*., 1986; Rao-Arelli *et al*., 1992; Aiba *et al*., 1995).

In conclusion, RAPD markers are useful for studying genetic variability among SCN populations and within races of this nematode. Variability within nematode races may indicate pathogen adaptation to environment and crop rotation. This variability could cause variable plant symptoms, as observed in genotypes used as host differentials. New RAPD markers would facilitate monitoring nematode population dynamics.

RESUMO

Heterodera glycines Ichinohe, o nematóide de cisto da soja (SCN), foi detectado no Brasil em 1.992 e desde então tem provocado perdas consideráveis à produtividade da soja brasileira. Populações de SCN apresentam grande diversidade genética, o que dificulta o manejo dessa cultura em áreas infestadas. Os objetivos desta pesquisa foram investigar a possível variação genética de dezesseis populações de SCN amostradas nas regiões Centro Oeste e Sudeste do Brasil, utilizando a técnica do RAPD (amplificação casualizada de DNA polimórfico), bem como estabelecer marcadores RAPD específicos para a raça 3 do SCN. Nossos resultados mostraram que os marcadores RAPD podem ser utilizados em estudos de variabilidade genética entre populações de SCN e dentro de raças deste nematóide, bem como podem ser usados no monitoramento de dinâmica populacional deste patógeno. O "primer" OPA-07 foi considerado satisfatório como marcador molecular para a raça 3, enquanto a análise eletroforética dos fragmentos de DNA amplificados com a utilização do "primer" OPA-10 detectou ligeira variação nas populações de SCN identificadas como raça 3. A população do SCN oriunda de Chapadão do Céu, GO - amostra 2 - foi a geneticamente mais distante, quando comparada às demais usadas neste estudo.

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