Two Microsatellite Markers That Flank the Major Soybean Cyst Nematode Resistance Locus

J. Mudge, P. B. Cregan, J. P. Kenworthy, W. J. Kenworthy, J. H. Orf, and N. D. Young*

ABSTRACT

The use of resistant cultivars is the most effective method for controlling soybean cyst nematode (*Heterodera glycines* Ichinoe; SCN) on soybean [*Glycine max* (L.) Merrill]. However, resistance to SCN is oligogenic, making inheritance patterns complex and breeding difficult. One major partial-resistance locus for SCN resistance is located on molecular linkage group (MLG) 'G'. This locus controls more than 50% of variation associated with response to SCN and resistant alleles are present in many important sources of SCN resistance, including PI 209332, PI 88788, PI 90763, PI 437654, and 'Peking'. Restriction fragment length polymorphisms (RFLPs) linked to the major SCN resistance alleles on MLG G have proven effective in tracking the alleles and predicting SCN response. These RFLPs are much more efficient in terms of time and labor than greenhouse assays for SCN. Nevertheless, more efficient DNA markers are needed to screen the many lines required for marker-assisted selection. Polymerase chain reaction-based markers, such as microsatellites (simple sequence repeats), have been sought because they are faster, less expensive, more polymorphic, and require less labor than RFLPs. In this study, we report two microsatellites, BARC-Satt038 and BARC-Satt130, that flank the major SCN resistance locus on MLG G. These microsatellites efficiently identify the chromosome fragment carrying the resistance allele and are also good predictors of SCN phenotype response.

THE SOYBEAN CYST NEMATODE is one of the most economically destructive pathogens of soybean (Noel, 1992). Breeding for SCN resistance is difficult because the trait is controlled by multiple genes (Caviness, 1992) and nematode populations are genetically heterogeneous (Niblack, 1992). However, one major partial-resistance allele at a locus on molecular linkage group (MLG) G is present in several resistance sources, including PI 209332, PI 88788, PI 90763, PI 437654, and Peking. This locus controls more than 50% of total variation for resistance and the resistance allele is effective against several races of the nematode (Concibido et al., 1994, 1996a, 1997; Webb et al., 1995).

Molecular markers can be used to resolve quantitative traits, including SCN resistance, into their underlying Mendelian factors (Paterson et al., 1988). Markers can also accelerate selection and eliminate the effects of environmental variation during selection (Tanksley et al., 1989). RFLP markers linked to the SCN resistance locus on MLG G have proven useful in identifying genomic regions associated with SCN resistance (Webb et al., 1995; Concibido et al., 1996a). These markers have been especially useful in monitoring alleles at the resistance locus on MLG G (Concibido et al., 1994, 1996a; Denny et al., 1996). Results from a cross between the cultivar Evans and PI 209332, also indicate that selection for this genomic region is as accurate as greenhouse assays in predicting SCN disease response (Concibido et al., 1996a).

DNA markers have great potential in SCN resistance breeding, but RFLP analysis is relatively complicated and time-consuming to perform. Therefore, we have sought new DNA markers based on the polymerase chain reaction (PCR). Microsatellites (also known as simple sequence repeats) are based on PCR amplification of di-, tri-, tetra-, or penta-nucleotide repeats and have several advantages over RFLPs. They are faster, less expensive, and require less labor than RFLP markers (Denny et al., 1996). Microsatellites also tend to show much higher levels of sequence polymorphism in soybean (Akkaya et al., 1992, 1995; Rongwen et al., 1995) than do RFLP markers (Keim et al., 1989, 1992), increasing the probability that marker alleles will vary in populations of interest. Microsatellites are also preferable to other PCR-based markers, such as random amplified polymorphic DNAs (RAPDs), because microsatellites are codominant and highly reproducible. For marker-assisted selection to be practical, DNA markers that are inexpensive, reliable, and suitable for screening thousands of genotypes quickly are necessary. Microsatellites, together with a high-throughput DNA extraction method that we have developed (Lange et al., 1998), meet these standards. In the present study, we report on two microsatellites that flank the major SCN resistance locus on MLG G. These markers can be used to screen rapidly for the presence of the SCN resistance allele and effectively track SCN resistance phenotype during marker-assisted selection.

MATERIALS AND METHODS

Plant Materials

The alleles present at the two microsatellite loci were determined in the following genotypes: 'Amsoy', 'Bedford', 'Bragg', 'Clark', 'Evans', 'Essex', 'Forrest', 'Harosoy', 'Hartwig', 'Jackson', 'Williams', 'Fiskeby V', 'Minsoy', 'Noir I', Peking, 'Pickett 71', and 'Tokyo' and the Plant Introductions (PI) 438497, PI 88788, PI 90763, PI 209332, and PI 437654. Seeds of the soybean cultivars were obtained from Dr. Randall Nelson (USDA-ARS, Univ. of Illinois, Urbana, IL).

The two microsatellites were mapped by means of a popula-

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Abbreviations: cM, centimorgan; MLG, molecular linkage group; PCR, polymerase chain reaction; PI, plant introduction; RFLP, restriction fragment length polymorphism; SCN, soybean cyst nematode.

Although several RFLP markers on the proximal side are closer to the SCN resistance locus than Satt130, the ease of microsatellite analysis makes it much more valuable in marker-assisted selection in combination with Satt038. Both microsatellites can be run on the same lane in a gel, allowing one to analyze Satt038 and Satt130 without much more work than it would take to analyze Satt038 alone. By comparison, use of RFLP markers would be too labor- and time-intensive to be practical in screening the large number of individuals required by marker-assisted selection.

Satt038 and Satt130, together with a high-throughput disk DNA extraction method (Lange et al., 1998), can make marker-assisted selection for the SCN resistance allele on MLG G practical. Satt038 efficiently predicts the allele at the SCN resistance locus and the SCN phenotype (based on a 30% cutoff) with great accuracy. Satt130 can be useful in tracking the resistance allele in crosses in which Satt038 is not polymorphic, in reducing linkage drag around the SCN resistance allele, and/or in confirming the prediction of Satt038 as to the presence or absence of the resistance allele. We are currently looking for additional microsatellites in the MLG G region, as well as in other genomic regions that contribute to SCN resistance.

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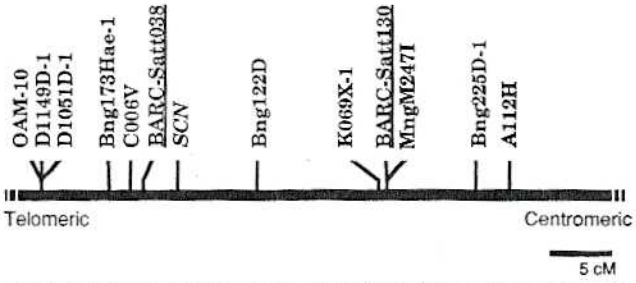

Fig. 3. Molecular linkage group (MLG) 'G' (Shoemaker and Olson, 1993) near the SCN resistance locus (Concibido et al., 1996b), showing the relative positions of the two microsatellites, Satt038 and Satt130 (underlined), the SCN resistance locus, and nearby RFLP loci. Distances are in centimorgans (Kosambi, 1944).

average of 0.30 for 128 RFLPs in soybean (Keim et al. 1992). Microsatellites linked to the SCN resistance gene on MLG G are therefore likely to be polymorphic for a broader range of crosses than are RFLPs.

Several SCN-resistant and -susceptible genotypes analyzed with Satt038 and Satt130 are shown in Fig. 1. Five different allele sizes were observed for each microsatellite locus and the results indicate that Satt038 and/or Satt130 would be polymorphic in many susceptible × resistant crosses. PI 88788 and PI 209332 exhibit Satt038 alleles not present in any of the susceptible genotypes tested. The rest of the SCN resistance sources are distinguishable from one or more susceptible genotypes, including Williams, Clark, Minsoy, and Noir I. Polymorphic alleles of Satt130 distinguish several resistant genotypes (e.g., PI 88788, PI 437654, and PI 209332) from the susceptible genotypes screened. The rest of the sources for SCN resistance are distinguishable from some (e.g., Williams, Essex, Clark, and Minsoy), but not all susceptible genotypes tested. Polymorphism at the Satt130 locus may be valuable for tracking the SCN resistance gene in crosses where the parents are not polymorphic at Satt038. Together the two microsatellites should provide at least one polymorphic, PCR-based marker in most SCN breeding crosses.

Satt038 and Satt130 Flank the SCN Resistance Locus on MLG G

Figure 2 shows the segregation pattern of Satt038 in 98 F_{45} individuals in a mapping population derived from the cross of Evans and PI 209332. Comparisons of the microsatellite segregation patterns with those of the other DNA markers on MLG G and of SCN resistance by Mapmaker linkage analysis demonstrated that Satt038 and Satt130 flank the SCN resistance locus (Fig. 3). Satt038 is approximately 3 centimorgans (cM) on the distal (telomeric) side of the SCN resistance locus while Satt130 is approximately 20 cM on the proximal (centromeric) side. Thus, Satt038 is the closest PCR-based DNA marker to the major SCN resistance locus on linkage group MLG G reported to date.

Predicting SCN Phenotype with Satt038 and Satt130

We compared the accuracy of the microsatellites, singly and as a pair, in distinguishing between SCN-resistant and susceptible lines in the Evans × PI 209332 population. This involved a contingency table analysis of microsatellite genotype versus SCN phenotype, as assayed by the greenhouse assay (Table 2). The chi-squared test null hypotheses, that the given microsatellite genotypes is not associated with the SCN phenotype, were rejected with $P < 0.0001$ for Satt038 and the combination of Satt038 and Satt130, and $P < 0.0002$ for Satt130. The skewing towards the Evans genotype and toward the susceptible phenotype were taken into consideration when calculating the chi-squared values. In this analysis, lines with SCN indices less than 30% were classified as resistant, while those with indices greater than 30% were classified as susceptible. Heterozygotes were not included in the analysis.

Satt038 alone was 95% accurate in predicting SCN phenotype (71/75). Satt130 is farther from the resistance locus, so it is not surprising that it was only 74% accurate (49/66). However, because Satt130 is on the opposite side of the SCN resistance locus, the use of both Satt038 and Satt130 allowed 98% prediction accuracy of phenotype when both loci carried alleles from the same parent (40/41). In this comparison, any lines that were recombinant between the markers were excluded from the analysis. The fact that these two markers flank the SCN resistance locus allows the monitoring of alleles at this locus except in the case of rare double-crossovers between the resistance locus and the flanking microsatellites. However, the use of both markers may increase the linkage drag accompanying the SCN resistance allele.

Satt130 may be useful in selecting lines that have crossovers near the SCN resistance locus in order to minimize linkage drag. This could be important because yield reduction associated with SCN resistance (Shannon, 1989) appears to be due to a yield depression gene in the same region as the resistance locus on MLG G (Mudge et al., 1996).

Table 2. Contingency table analysis comparing SCN phenotype (F_{45}) with genotypes at either or both of Satt038 and Satt130 (F_{45}).

	Satt038 Genotype‡		Satt130 Genotype‡		Satt038 and Satt130 Genotype‡	
Phenotype†	Evans	PI 209332	Evans	PI 209332	Evans	PI 209332
Susceptible	44 (59%)	2 (3%)	33 (50%)	11 (17%)	28 (68%)	1 (2%)
Resistant	2 (3%)	27 (36%)	6 (9%)	16 (24%)	0 (0%)	12 (29%)
		$\chi = 59.08$		$\chi = 13.82$		$\chi = 36.54$
		$P < 0.0001$		$P < 0.0002$		$P < 0.0001$

† Based on mean number of cysts recovered from 10 F_{45} plants; if this was less than 30% of the number recovered from the susceptible check, Evans, plants were scored as resistant; otherwise, they were scored as susceptible.

‡ Leaves from an F_{45} line were bulked and genotyped for Satt038 and Satt130 to determine if they carried the Evans or PI 209332 alleles. Heterozygous lines were not included; when the genotype at both Satt038 and Satt130 was considered, lines with a crossover between the two loci were excluded.

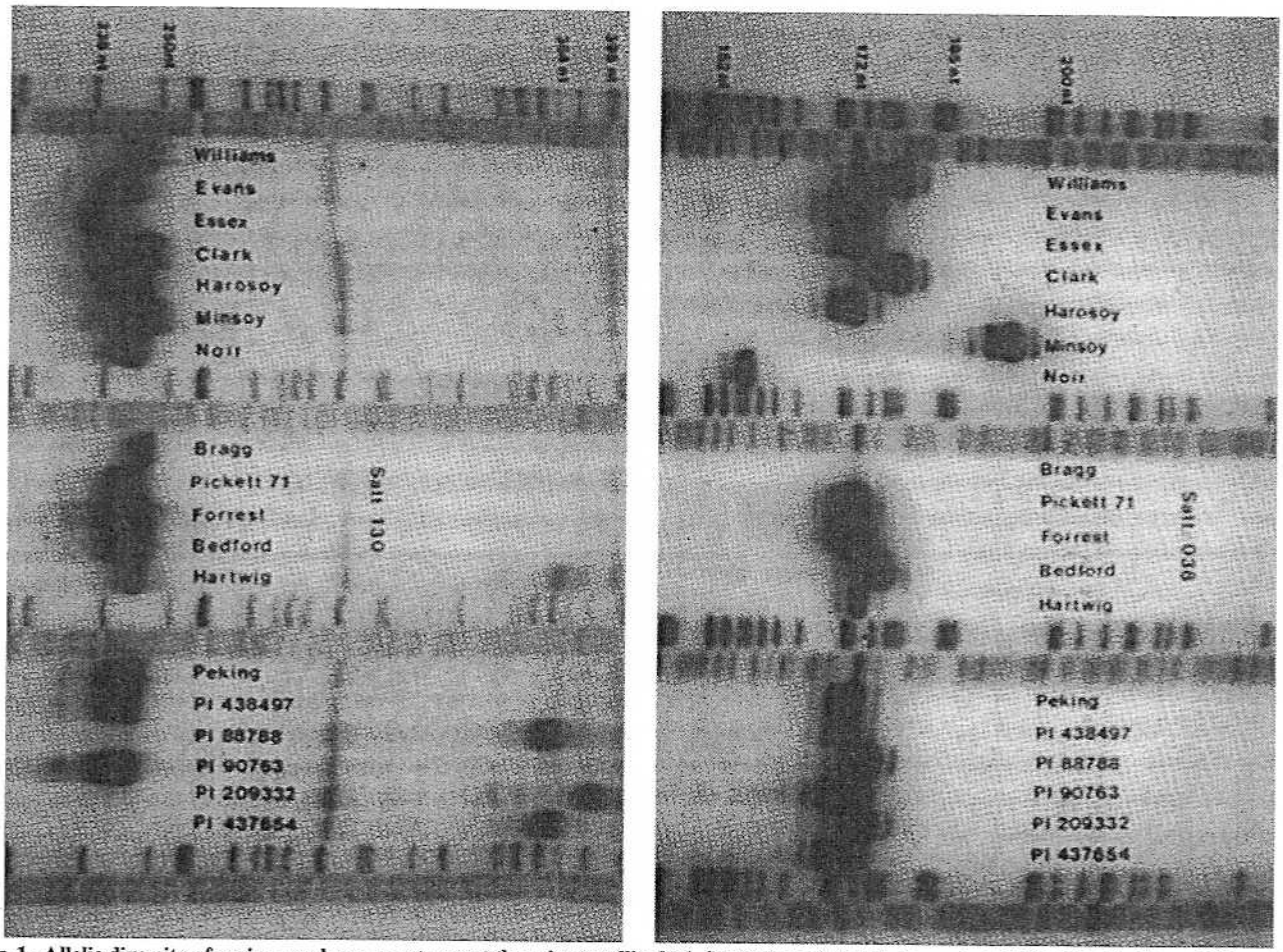

Fig. 1. Allelic diversity of various soybean genotypes at the microsatellite loci, Satt038 and Satt130. Lanes 1, 2, 10, 11, 17, 18, 25, and 26 contain size standards (sequencing reactions of M13 single-stranded DNA; fragment sizes are indicated in nucleotides at the far left). The first group of soybean genotypes (Williams–Noir I) consists of seven SCN-susceptible soybean genotypes. Bragg (SCN-susceptible) is related to the next four cultivars (Pickett 71–Hartwig), which were all bred for SCN resistance. The last group of soybean genotypes (Peking–PI 437654) are sources of SCN resistance.

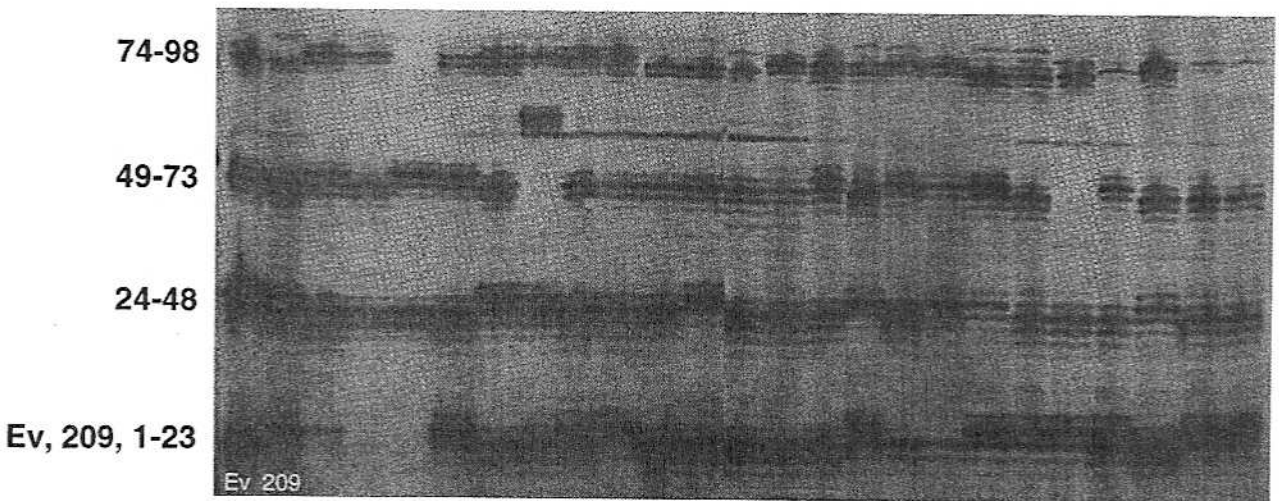

Fig. 2. Satt038 segregation pattern among 98 F_{45} progeny lines of the Evans × PI 209332 cross. Samples were loaded onto the gel in four separated groups of 25 each at 10-min intervals. For each group of 25, the first half of the samples were run into the gel to avoid leakage before the remaining samples were loaded. The lower band is the Evans allele (SCN-susceptible) and the upper band the PI 209332 allele (SCN-resistant).

tion of 98 $F_{4:5}$ lines from a cross between the SCN-susceptible cultivar, Evans, and the resistant source, PI 209332 (Anand and Brar, 1983; Anand and Gallo, 1984). This population has been described previously in detail (Concibido et al., 1996a).

SCN Phenotype

Greenhouse assays were conducted following the method described by Concibido et al. (1994) on 10 $F_{4:6}$ plants from each $F_{4:5}$ line. If the average number of cysts recovered from the roots of the plants in each line was less than 30% of the susceptible check, Evans, lines were classified as resistant. Otherwise, they were classified as susceptible. This 30% cutoff is often used by breeders during the development of SCN resistant varieties (Schmitt and Shannon, 1992).

DNA Extraction

To determine gene diversity, DNA was extracted from bulked leaf tissue of 25 to 35 plants of each genotype as described by Keim et al. (1988). For the mapping population, DNA was extracted by the method of Dellaporta et al. (1983) from bulked leaf tissue of a row of plants for each line. In some experiments, DNA samples immobilized onto disks were prepared following the method described by Lange et al., 1998. Briefly, this involved imprints of leaf samples rubbed onto a solid matrix collection card that binds DNA (Gentra Systems, Minneapolis, MN). A 3-mm disk was punched from each imprint into a well of a 96-well microplate. Disks were then washed three times for 15 min each with 100 μL Gentra DNA Purification Solution (Gentra Systems, Minneapolis, MN). The disks were rinsed with 100 μL absolute ethanol, dried at room temperature overnight or at 60°C for 30 min, and used directly in PCR.

RFLP Analysis

RFLP genotyping of the mapping population and the construction of a genetic map have been previously described (Keim et al., 1990; Concibido et al., 1996a).

Microsatellite Analysis

Primers for microsatellites in soybean were developed as described by Cregan et al. (1994). Microsatellite loci BARC-Satt038 and BARC-Satt130 (subsequently referred to as Satt038 and Satt130) were used. The forward and reverse primer sequences of each are listed in Table 1.

Microsatellites were analyzed by two methods. Both methods gave comparable results.

Method 1. The first method followed the procedure of Akkaya et al. (1995) with a few modifications. The reaction mix contained 1.5 m*M* $MgCl_2$, 0.15 μ*M* of forward and reverse primers, 100 μ*M* of each dNTP, 0.1 μL of 3000 Ci/mmol [α-^{32}P]-dATP, 1× PCR buffer (50 m*M* KCl, 10 m*M* tris-HCl pH 9.0, 0.1% Triton X-100), 1 unit *Taq* DNA polymerase (GibcoBRL, Life Technologies, Gaithersburg, MD), and 30 ng of genomic DNA. DNA disks were not tested with this method. Samples were amplified in an MJ Research PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). The cycling protocol consisted of 32 cycles of a 25-s denaturing step at 94°C, a 25-s primer annealing step at 47°C, and a 25-s elongation step at 68°C. The samples were separated on a vertical polyacrylamide gel [6% polyacrylamide, 5.6 *M* urea, 30% formamide, and 1× TBE (0.09 *M* Tris-borate, 0.002 *M* EDTA, pH 8.3)]. Gels were dried and exposed to X-ray film.

Table 1. Forward and reverse primer sequences for Satt038 and Satt130. These two microsatellites flank the major SCN resistance locus (Concibido et al., 1996b) located on molecular linkage group (MLG) 'G' (Shoemaker and Olson, 1993).

Satt038	Forward:	5′ GGGAATCTTTTTTTCTTTCTATTAAGTT 3′
	Reverse:	5′ GGGCATTGAAATGGTTTTAGTCA 3′
Satt130	Forward:	5′ TGGTAGTGAAAGCACGAGAT 3′
	Reverse:	5′ AACACTTTGAATGGCTAAAAAC 3′

Method 2. Primers were first end-labeled with [γ-^{33}P]-dATP according to the following protocol. The reaction mixture contained 0.75 μ*M* each of forward and reverse primers, 1× kinase buffer [(70 m*M* Tris-HCl at pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* DTT (dithiothreitol)], 0.1 μL of 3000 Ci/mmol [γ-^{33}P]-dATP per sample, and 0.8 U T4 Polynucleotide kinase (New England Biolabs, Beverly, MA) per sample. The reaction volume was scaled according to the number of samples to be amplified. The reaction was incubated at 37°C for 1 h, followed by 70°C for 10 min.

The amplification reaction contained a single 3-mm DNA disk or 5 ng of DNA suspended in TE (Tris-EDTA), 1 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 2 μL of end-labeled primer mix (0.15 μ*M* of forward and reverse primers), 1× Buffer (20 m*M* Tris-HCl at pH 8.4, 50 m*M* KCl), and 1 U *Taq* DNA Polymerase in a 10-μL reaction volume. Five microliters of sterile water was added to wet the disks before the cocktail was added. Samples were amplified in an MJ Research PTC-100 thermocycler in 96-well microplates. The cycling, electrophoresis, and autoradiography protocols were the same as for Method 1.

Calculation of Gene Diversity

Gene diversity (Weir, 1990) estimates the relative amount of polymorphism seen for a marker across homozygous individuals in a self-fertilizing species. For microsatellites, gene diversity is calculated as follows:

$$\text{Gene Diversity} = 1 - \sum P_{ij}^2$$

where P_{ij} is the frequency of the *j*th allele for microsatellite *i* and is summed across all alleles (Rongwen et al., 1995). Gene diversity was calculated for Satt038 and Satt130 loci based upon allele size data obtained from the analysis of 10 soybean genotypes, Clark, Harosoy, Jackson, Williams, Amsoy, Archer, Fiskeby V, Minsoy, Noir I, and Tokyo.

Statistical Analysis

Microsatellites were mapped by Mapmaker Macintosh Version 2.0 (Lander et al., 1987). Linkage was determined by a two-point analysis in which two markers were assumed to be linked if the LOD score exceeded 3.0 with the 'Group' command (i.e., if the markers are 1000 times more likely to be linked than unlinked). Markers were ordered by a multipoint analysis to find the best order ('First Order') and were confirmed by testing all possible marker orders within each set of three markers ('Ripple').

To test the accuracy of microsatellite-based predictions of SCN phenotype, contingency table analysis was performed with Statview-II (Abacus Concepts, Berkeley, CA).

RESULTS AND DISCUSSION

Characterization of Gene Diversity in Satt038 and Satt130

Satt038 and Satt130 gave gene diversity values of 0.76 and 0.64, respectively, when analyzed on a group of 10 diverse soybean genotypes. This compares well with the