Validation of microsatellite markers for assisted selection of soybean resistance to cyst nematode races 3 and 14

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Abstract – The objective of this work was to validate microsatellite markers associated with resistance to soybean cyst nematode (*Heterodera glycines* Ichinohe) races 3 and 14, in soybean (*Glycine max* L.) genotypes, for use in marker-assisted selection (MAS) programs. Microsatellites of soybean linkage groups A2, D2 and G were tested in two populations, and their selection efficiencies were determined. The populations were 65 $F_{2:3}$ families from Msoy8001 (resistant) x Conquista (susceptible) cross, and 66 $F_{2:3}$ families of S5995 (resistant) x Renascença (susceptible) cross, evaluated for resistance to races 3 and 14, respectively. Families with female index up to 30% were considered moderately resistant. Markers of A2 and G linkage groups were associated with resistance to race 3. Markers Satt309 and GMENOD2B explained the greatest proportion of phenotypic variance in the different groups. The combinations Satt309+GMENOD2B and Satt309+Satt187 presented 100% selection efficiency. Resistance to race 14 was associated with markers of G linkage group, and selection efficiency in the Satt309+Satt356 combination was 100%. The selection differential obtained by phenotypic and marker assisted selection showed that both can result in similar gains.

Index terms: *Glycine max*, *Heterodera glycines*, soybean breeding, SCN, SSR, MAS.

Validação de marcadores microssatélites para a seleção assistida de resistência de soja ao nematóide-de-cisto raças 3 e 14

Resumo – O objetivo deste trabalho foi validar marcadores microssatélites associados à resistência às raças 3 e 14 do nematóide-de-cisto (*Heterodera glycines* Ichinohe) da soja (*Glycine max* L.), para serem utilizados em programas de seleção assistida por marcadores moleculares (SAM). Microssatélites dos grupos de ligação A2, D2 e G da soja foram testados em duas populações, e suas eficiências de seleção foram determinadas. As populações foram 65 famílias $F_{2:3}$, do cruzamento Msoy8001 (resistente) x Conquista (suscetível), e 66 famílias $F_{2:3}$, do cruzamento S5995 (resistente) x Renascença (suscetível), avaliadas para a resistência às raças 3 e 14, respectivamente. Famílias com índice de fêmeas de até 30% foram consideradas moderadamente resistentes. Marcadores dos grupos de ligação A2 e G apresentaram associação com a resistência à raça 3. Os marcadores Satt309 e GMENOD2B explicaram a maior proporção da variância fenotípica nos diferentes grupos. As combinações Satt309+GMENOD2B e Satt309+Satt187 apresentaram eficiência de seleção de 100%. A resistência à raça 14 foi associada com marcadores do grupo de ligação G, e a eficiência de seleção da combinação Satt309+Satt356 foi de 100%. Os diferenciais de seleção fenotípica e de seleção assistida mostraram que os dois tipos de seleção podem proporcionar ganhos similares.

Termos para indexação: *Glycine max*, *Heterodera glycines*, melhoramento de soja, NCS, SSR, SAM.

Introduction

One of the great limitations in developing soybean cultivars resistant to soybean cyst nematode (SCN) is phenotypic evaluation, because it is time consuming, expensive and influenced by the environment, restricting the number of plants to be assessed. Marker-assisted selection (MAS) for this characteristic has become an important tool in breeding, because it allows selection of lines based on the alleles of genetic markers linked to resistance, reducing the number of lines to be assessed in a

greenhouse (Young & Mudge, 2002; Concibido et al., 2004).

Several quantitative trait loci (QTL) associated to SCN resistance have already been mapped in soybean, on several resistance sources and for different SCN races (Concibido et al., 2004; Guo et al., 2006). Two regions on the soybean genome are very important for wide resistance to SCN, the regions of the *rhg1* gene (LG G) and the *Rhg4* gene (LG A2). These two loci account for almost all the variability in soybean for resistance to SCN race 3 (Weisemann et al., 1992; Webb et al., 1995) and a great part of the variability for the other races (Concibido et al., 1997, 2004; Wang et al., 2004; Guo et al., 2005, 2006). Schuster et al. (2001) detected another region with large effect on LG D2 conferring resistance to race 14. It is suggested that the minor genes reported in other LG may be involved in race specificity, but they need to be validated.

However, care should be taken when presuming whether the QTL linkage marker will remain in different gene pools or in different experiments. QTL detection is influenced by several factors such as the QTL magnitude, the existence of other linked QTL, the mapping population size, phenotypic assessment accuracy, genotyping errors, lost data and environmental effects (Collard et al., 2005; Francia et al., 2005). Thus, for application in breeding, the already published QTL should be validated in independent populations (Fasoula et al., 2004), that is, the efficacy of these markers should be tested in determining the target phenotypic type in independent populations and in different gene pools (Fasoula et al., 2004; Francia et al., 2005). In the case of SCN, the selection efficiency of the marker should be determined, considering the resistance sources and the SCN race for which it is intended to introduce the resistance.

Although many QTL have been reported in soybean for resistance to SCN, few have been confirmed in additional populations from the same or different gene pools. One of the few studies of QTL validation for resistance to SCN was carried out by Glover et al. (2004), who confirmed the presence of one QTL conferring resistance to SCN race 14 in the J linkage group in PI 88788. Therefore, QTL validation is a critical step before marker-assisted selection is used in breeding programs.

The objective of this study was to validate microsatellite markers, previously identified as being associated to QTL that conferred genetic resistance to SCN races 3 and 14, in the A2, D2 and G linkage groups, using plant populations obtained from resistance sources different from those where the QTL were mapped, and, in case they were present, to verify their selection efficiency.

Material and Methods

Two populations of $F_{2:3}$ families were used, one obtained from Msoy8001 (resistant) x Conquista (susceptible) cross, and the other from S5995 (resistant) x Renascença (susceptible) cross. Sixty-five families from the first cross were phenotypicly evaluated for resistance to SCN race 3, and in the second, 66 families were evaluated for resistance to race 14.

These experiments were carried out in a greenhouse at Embrapa Soybean Center, Londrina, Paraná, Brazil. A completely randomized design was used, and four to six plants were assessed per $F_{2:3}$ family, under temperature conditions ranging from 25 to 30°C, and 16 hours of light. The cross Msoy8001 x Conquista was assessed from February to March 2003 for resistance to race 3, and the cross S5995 x Renascença was assessed from July to August 2004 for resistance to race 14.

The inocula for races 3 and 14 were kept in a susceptible cultivar in a greenhouse. The $F_{2:3}$ populations, the parental lines, the soybean differential cultivars (Peking, Pickett, PI 90763 and PI 88788) and the susceptible control (Lee) were included in the experiments. The seeds were placed to germinate in sand at 25°C. Every two to three-day old seedling was transplanted into a 0.5 L clay pot containing a mixture of soil and sand at the 1:2 ratio. Four thousand eggs per pot were inoculated simultaneously at transplant.

Thirty days after inoculation, the plants were removed from the pots and their roots washed under a strong jet of water, in a 20 mesh sieve, attached to another 60 mesh sieve, and cysts were counted under a stereoscopic microscope.

The data obtained were analyzed statistically using the model for a completely randomized design with an unequal replication treatment (each family was considered a treatment). The genetic parameters were estimated using the Genes program (Cruz, 2006).

The mean of each $F_{2:3}$ family was transformed in female index (FI), estimated as follows: FI = 100(cyst average numbers in one given family/cyst average numbers found in the susceptible parent).

The data for the susceptible parents from each population were used in the denominator of the expression above to

calculate the FI for each family, and assess the genetic differences among parents of each cross. To confirm the SCN race, the FI was calculated substituting the denominator of the expression above by the number of cysts and females in Lee (susceptible standard cultivar), as proposed by Riggs et al. (1988).

For phenotypic selection, each of the $F_{2:3}$ families was classified using the resistance criterion proposed by Schmitt & Shannon (1992), by which plants with FI<30% are considered moderately resistant and plants with FI<10% are considered resistant.

Selection efficiency (SE) of the markers linked to the resistance locus was based on the comparison between the phenotypic (IF) and the genotypic (markers) evaluations. Only nonsegregating families were considered for SE estimation, which was calculated as follows: SE = 100[(MFMF + mfmf)/(MM + mm)], in which: MFMF is the number of families selected correctly as resistant, based on the markers and on phenotypic evaluation; mfmf is the number of families selected correctly as susceptible, based on the marker and on phenotypic evaluation; MM + mm is the total number of families selected, based on the markers only, resistant and susceptible.

The selection differential (SD) estimation was calculated according to Cruz (2006), as follows:
$SD = \bar{X}_S - \bar{X}_0$, in which: $\bar{X}_S$ is the selected families mean; $\bar{X}_0$ is the population mean.

The DNA was extracted from the soybean leaves by the CTAB method (Keim et al., 1988), and then quantified in a spectrophotometer and stored at 4°C until use.

Thirty microsatellite markers were tested in the parents of Msoy8001 x Conquista cross, previously identified as being associated to resistance to race 3, in the A2, D2 and G linkage groups. For S5995 x Renascença cross, 25 microsatellite markers were tested (from the D2 and G linkage groups). Markers that generated polymorphisms among the parents were amplified in DNA bulks of each family, that is, in a solution containing equal amount of DNA from all the plants of the same family. Amplification reactions were carried out in a total volume of 15 µL, containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.4 mM $MgCl_2$; 100 µM of each desoxinucleotide; 0.3 µM of each primer, a unit of *Taq* polymerase and 30 ng genomic DNA.

The amplification reactions were carried out in a Perkin Elmer thermocycler, model 9600, programmed for thirty cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; at the end of the 30 cycles, a stage of 7 min at 72°C was performed.

Amplification products were separated by electrophoresis in 3% agarose gel, or in 10% native vertical polyacrylamide gels, using a TAE1X buffer (0.09 M Tris-acetate and 0.002 M EDTA). After electrophoresis, gels were stained with ethidium bromide (10 mg mL^{-1}) and photographed.

To identify markers linked to the resistance QTL, the co-segregation among the amplified markers in the populations and the phenotypic type of each $F_{2:3}$ family (FI per family) were analyzed statistically, by single marker analysis, the ANOVA method. The individual segregation of the molecular markers was tested using the chi-square test.

The genetic distances were established for the markers of the same linkage groups with minimum LOD of three, and 30% maximum recombination percentage. Localization and characterization of the possible QTL effect were carried out through QTL mapping by single interval (Lander & Botstein, 1989) and composed interval methods (Zheng, 1994). The estimates of the additive and dominance values, the QTL determination coefficient (corresponding to the greatest statistical significance peak of the QTL) and the position of the possible QTL were declared, when the likelihood ratio (LR) values were greater than the critical cutting ones ($\alpha = 0,05$), in each linkage group. The critical and LR values were determined by 1,000 permutations (Churchill & Deorge, 1994). All the analyses were carried out by the GQMOL program (Cruz & Schuster, 2005).

Results and Discussion

The genetic variability in Msoy8001 x Conquista population, for resistance to race 3, was shown by the high variation in the mean number of cysts detected between parents (from 1.9 to 165.67, respectively), and by the finding of transgressive segregation in the mean number of cysts in the families in the population (means ranged from 0.5 to 179).

In S5995 x Renascença population (assessed for resistance to race 14), the mean number of cysts in the parents ranged from 76.37 to 284.87. In the population this variation ranged from 35.63 to 224. The high value of the mean number of cysts, found in the resistant S5995 parent, showed that genes were segregating in this population, which conferred moderate resistance to SCN race 14. Transgressive segregation was also observed.

Though a continuous distribution of the mean FI values in the $F_{2:3}$ populations were observed, only data obtained from the evaluation for resistance to race 3 presented

normal distribution at 1% probability by Lilliefors test (calculated D = 0.117); this fact may indicate a greater number of genes with smaller effects, segregating for resistance to race 3 compared to race 14.

However, it is known that a few genes with greater effect confer wide resistance to several races of this pathogen (Cervigni et al., 2004; Guo et al., 2005). In this study, one evidence of a few resistance genes segregating in the populations is the high heritability in the broad sense for resistance to the two races (heritability of 64.27 and 78.68 for races 3 and 14, respectively). The heritability results obtained in this study are in agreement with those of the literature (Webb et al., 1995; Yue et al., 2000).

The result of the chi-square test provided further evidence of few genes with great segregating effect for the different races in the two populations. By this test, the presence of two recessive genes confers resistance to race 3 in Msoy8001 x Conquista population. In this population, seven families were classified as resistant (FI<10%), and 58 families as susceptible (S), fitting the segregation of 1R:15S by the chi-square test (χ^2 calculated = 2.26; 12.87% probability). Regarding resistance to race 14, in S5995 x Renascença population, two recessive and independent genes were detected conferring moderate resistance. The families of this population were classified as 32 moderately resistant and 34 susceptible, fitting a 7R:9S segregation (χ^2 calculated = 0.612; 43.81% probability).

In the population evaluated for resistance to race 3, 23 polymorphic markers between parents were used. Seven of these presented distortion of the expected Mendelian segregation. Markers of LG A2 and LG G presented association with resistance by the single marker analysis, and those of G linkage group were more significant (Table 1). One QTL was mapped by the

Table 1. Evaluation of microsatellites, associated with resistance to soybean cyst nematode races 3 and 14, using the single marker method.

Race	Marker	LG	Anova	Mean (FI)[1]		
			Prob(F)	Homoz(S)	Heter	Homoz(R)
Race 3	Satt187	A2	0.014*	84.68	72.23	41.91
	Gmenod2B	A2	0.00**	101.23	71.49	43.83
	BLT65	A2	0.014*	81.02		54.92[2]
	Satt177[3]	A2	ns	70.50	72.90	52.81
	Satt424	A2	ns	59.54	75.48	74.78
	Satt163	G	0.004**	92.19	70.34	47.48
	Satt309	G	0.0**	102.21	71.59	36.78
	Satt610	G	0.0**	96.05	72.87	32.33
	Sat_168	G	0.001**	101.94	71.06	29.36
	Satt130	G	0.005**	89.64	70.52	40.16
	Satt570[3]	G	0.00**	80.44	89.61	37.34
	Satt275[3]	G	0.24*	85.65	74.07	48.16
	Satt356	G	0.014*	91.73	80.21	50.97
	Satt082[3]	D2	ns	52.85	77.60	71.03
	Satt226	D2	ns	78.01	76.96	86.90
	Satt301	D2	ns	67.19	74.19	63.11
	Satt311	D2	ns	66.95	65.77	70.86
	Satt514	D2	ns	73.19	73.42	68.03
	Satt574	D2	ns	66.82	70.12	71.88
	Satt341[3]	D2	ns	89.51	63.37	61.36
	Sat_114[3]	D2	ns	78.01	76.96	86.90
	Satt543	D2	ns	74.02	64.31	71.19
Race 14	Satt177	A2	ns	103.07	100.26	97.01
	Satt389	D2	ns	93.43	98.43	110.11
	Satt309	G	0.00**	151.75	93.04	61.42
	Sat_168	G	0.00**	132.56	92.69	61.74
	Sat_141	G	0.00**	142.70	96.96	64.01
	Satt570	G	0.00**	121.44	101.57	66.44
	Satt356[3]	G	0.00**	139.54	109.51	71.35
	Satt217	G	0.003**	124.86	100.48	73.22

[1]Homoz(S) = susceptible homozygote; Heter = heterozygote; Homoz(R) = resistant homozygote. [2]Mean of the resistant heterozygote and homozygote plants, because the BLT 65 marker is dominant. [3]Markers that present segregation distortion. [ns]Nonsignificant. * and **Significant at 5 and 1% probability, respectively.

composed interval method, explaining 29.5% of the phenotypic variation in Satt187 and GEMENOD2B (LG A2) interval, and another two were mapped on LG G, explaining 33.8% and 41.2% in Satt570 – Satt163 and Satt309 – Sat_168 intervals, respectively (Table 2). Figure 1 shows the positions of the respective QTL. The QTL mapped on LG A2 was in the same region studied by Weisemann et al. (1992), characterized as a region of *Rhg4* gene, of great effect in resistance to race 3. The QTL found in the upper part of LG G were in the region where QTL were detected of great effect in several sources of resistance, conferring partial resistance to SCN races 1, 2, 3, 5, 6, 9 and 14, summarized by Concibido et al (2004). Cregan et al. (1999) identified the gene close to Satt309 microsatellite as being *rhg1*. These authors also verified that Satt309 and Sat_168 were highly efficient in identifying the alleles of resistant and susceptible lines, and that Sat_168 can be used alternatively to Satt309, in cases where the sources of resistance and susceptibility to SCN have identical alleles.

For S5995 x Renascença population, evaluated for race 14, nine polymorphic microsatellites in the parents were amplified in the population, but only those on G linkage group were significantly associated with resistance (Table 1). One QTL with greater effect (R^2 = 58.4%) was detected in Satt309 – Sat_168 interval (Table 2), that is, in the same region where a QTL was found for resistance to race 3 in the previous population, close to the locus of *rhg1* resistance gene (Cregan et al., 1999). The QTL associated to race 14, reported by Schuster et al. (2001), was not confirmed on D2 linkage group, but polymorphic markers were not identified in the region studied by these authors, in this population. This fact illustrates that the use of markers in breeding depends on their being polymorphic in different populations, because there are no warranty that the markers identified in one population are polymorphic in different populations. Alternatively, since S5995 is only partially resistant, this partial resistance may be due to the lack of resistance allele in this region.

The greatest assisted selection efficiency with only one marker was obtained with Satt309 microsatellite, 79% for resistance to race 3 and 94% for resistance to race 14 (Table 3). The greater selection efficiency of this marker, for resistance to race 14, was expected, because the resistance to race 3 was associated with markers in different linkage groups. Furthermore, the lower heretability of resistance for race 3 increased the chance of error both in marker-assisted and phenotypic selection.

The importance of Satt309 region for resistance to the two races was further evidence of its location close to the region of the *rhg1* gene. Further evidence of resistance being conferred by *rhg1* was that no dominance effect was detected in the intervals investigated with Satt309, in the two populations (Table 2). This is important, because resistance conditioned by the *rhg1* gene is given by a recessive allele.

The joint use of two microsatellites increased the efficiency of MAS for the two races. For race 3, in the combination of markers from different linkage groups, GMENOD2B+Satt309 or Satt309+Satt187, each presented 100% selection efficiency, that is, all families selected with the two markers were resistant by the phenotypic assessment. However, the marker combinations from the same group did not increase selection efficiency (Table 3). Cregan et al. (1999) reported that marker Satt309, that presented greater selection efficiency in this study, was 0.4 cM from the *rhg1* gene. Therefore, its use already ensures high success in assisted selection, and the joint use of another marker should be assessed regarding its location in the soybean genome to prevent reduction in the selection efficiency. For race 14, the combination of SSR

Table 2. Explained proportion of the phenotypic variance of the resistance (R^2%), additive (a) and dominant (d) components and mean degree of dominance (d/a), calculated on two $F_{2:3}$ soybean populations in relation to races 3 and 14 of soybean cyst nematode.

Resistance	LG[(1)]	Interval	Position[(2)]	LR	R^2 %	a	d	d/a
Race 3	A2	Satt187–GM2B	0.140	20.05**	29.47	-30.97	0.63	-0.02
	G	Satt570–Satt163	0.020	26.13**	33.88	-23.23	30.92	-1.33
	G	Satt309–Sat_168	0.045	32.23**	41.20	-41.07	3.93	-0.09
Race 14	G	Satt309–Sat_168	0.050	77.34**	58.40	-50.34	-22.93	0.45

[(1)]Denomination of the linkage group according to Song et al. (2004). [(2)]Position in centimorgans, GM2B = GMENOD2B. **LR exceeded the critical cut value (α = 0.01) in each linkage group; the likelihood ratio (LR) critical values were determined by 1,000 permutations.

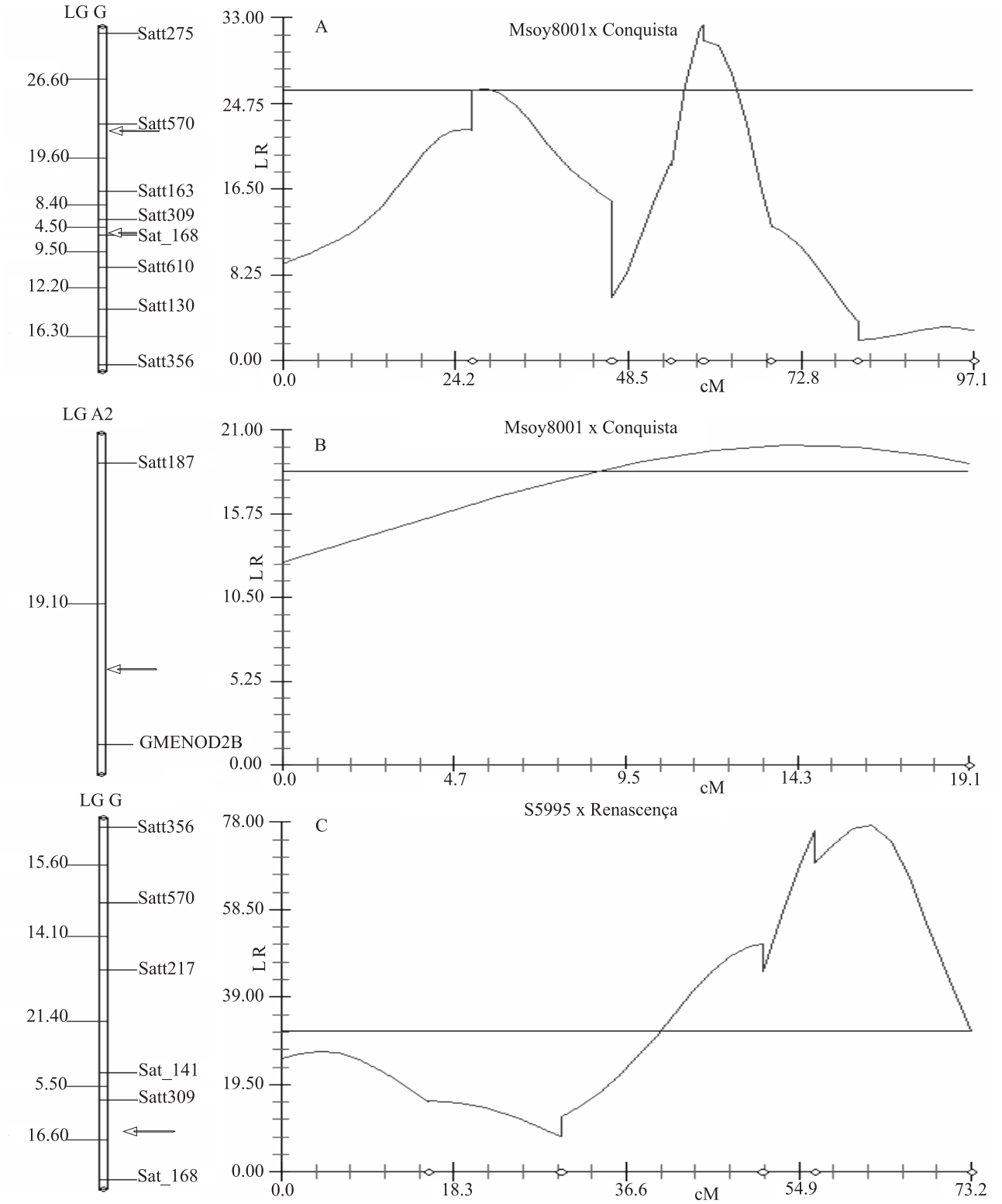

Figure 1. Identification on the linkage map (on the left) and on the maximum likelihood map (on the right) of three QTL for resistance to race 3, linkage groups G (A) and A2 (B), and one QTL for resistance to race 14, linkage group G (C) in the populations studied. The line in each graph indicates the critical value at 1% probability for the maximum likelihood (LR). On the left of the linkage map, there are the distances between markers; on the right, the pointers show the QTL positions. cM = centimorgans.

Table 3. Marker-assisted selection efficiency (SE%) for resistance to soybean cyst nematode, races 3 and 14, evaluated in two $F_{2:3}$ family populations, and comparison between marker-assisted selection (MAS) and phenotypic selection (PS), using the families mean and the selection differential (SD%).

Race	Marker	SFN[1] for MAS	Families mean[2]		SE (%)	SD%	
			MAS	PS		MAS	PS
Race 3	Satt187	14	41.91	14.81	76	-39.68	-78.67
	GMENOD2B	20	43.83	22.31	65	-36.91	-67.88
	Satt163	21	47.48	23.35	68	-31.66	-66.40
	Satt309	18	36.78	20.00	79	-47.06	-71.21
	Satt610	15	32.32	16.09	73	-53.47	-76.83
	Sat_168	20	47.77	22.32	70	-31.24	-67.88
	Satt130	16	40.15	17.23	61	-42.21	-75.19
	Satt356	16	50.96	17.23	62	-26.65	-75.19
	Satt570	22	37.34	24.32	57	-46.25	-64.99
	GMENOD2B+Satt309	7	8.02	5.21	100	-88.44	-92.49
	GMENOD2B+Satt187	11	38.38	11.01	75	-44.75	-84.15
	GMENOD2B+Satt610	5	10.04	2.83	90	-85.55	-95.92
	Satt309 + Satt187	6	6.38	2.26	100	-84.77	-94.60
	Satt309 + Sat_168	14	40.28	14.81	78	-42.02	-78.67
	Satt309 + Satt610	14	30.66	14.81	63	-55.87	-78.67
Race 14	Satt309	17	61.41	51.82	94	-37.75	-47.48
	Satt570	19	66.44	53.19	82	-32.67	-46.09
	Satt356	26	71.35	57.83	81	-27.68	-41.39
	Sat_168	13	61.74	49.11	93	-37.42	-50.22
	Satt217	17	73.22	51.82	80	-26.40	-49.53
	Satt309 + Satt570	11	56.95	47.91	95	-42.28	-51.45
	Satt309 + Satt217	11	61.15	47.91	95	-38.03	-51.45
	Satt309 + Sat_168	12	60.67	48.56	100	-38.51	-50.78
	Satt309 + Satt356	12	56.28	48.56	100	-42.39	-50.78

[1]SFN, selected families number. [2]The two means considered the number of plants selected by MAS.

Satt309+Satt356, on the same linkage group, presented 100% selection efficiency, indicating that the QTL was probably not very close to markers, because the use of two markers flanking the QTL increased selection efficiency.

The phenotypic selection was compared with the marker-assisted selection based on the means of the families selected by the two strategies. The best marker combination for resistance to race 3 was Satt309+GMENOD2B, with a selection differential of -88.4%, while the phenotypic selection differential was -92.5%. For race 14, Satt309+Satt356 combination gave a selection, differential of -42.4%, while in the phenotypic selection, differential was -50%. The absence of a complete resistance source for race 14 may be the cause of the smaller selection differentials, compared to the resistance to race 3. Therefore, the assisted selection strategy can give similar gains to those obtained by phenotypic selection. However, phenotypic selection is influenced by the environment, and the number of plants that can be selected is limited, while assisted selection by molecular markers does not have such limitations.

In this study, QTL previously associated with resistance to SCN races 3 and 14 were confirmed in breeding populations, and were useful in marker-assisted selection, besides being highly efficient in detecting the resistant genotypes in the population. Therefore, the microsatellite markers validated in this study could be used in MAS of the QTL resistant to SCN races 3 and 14. Concibido et al. (2004) estimated that phenotypic selection can be five to six times more expensive and much slower than MAS. Therefore, the results of this study may be an important alternative to the conventional evaluation procedures in selecting genotype resistant to SCN, during the introgression of these QTL in elite cultivars, in soybean breeding programs.

Conclusions

1. A common region of soybean genome on LG G is linked with resistance to soybean cyst nematode races 3 and 14.

2. The joint use of two markers made marker-assisted selection more efficient than selection with a single marker.

3. The gains with marker-assisted selection are similar to those obtained with phenotypic selection.

Acknowledgements

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for grant and scholarship to authors; to Cooperativa Central de Pesquisa Agrícola (COODETEC), for supplying the study populations; to Embrapa Soja, for providing the facilities for carrying out the phenotypic analysis.

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Received on January 24, 2007 and accepted on July 20, 2007