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Identification of quantitative trait loci controlling nodulation and shoot mass in progenies from two Brazilian soybean cultivars

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Abstract

Nitrogen (N) demand of soybean [*Glycine max* (L.) Merrill] can be supplied via biological nitrogen fixation (BNF), however, higher yielding cultivars increase plant demand for N. Phenotypes differing for traits associated with biological nitrogen fixation result from the expression of the multiple genes of both the host plant and the microsymbiont, but limited studies have been done on the genetics of the soybean BNF. Integrated maps of soybean with simple sequence repeat (SSR) markers [Cregan, P.B., Jarvik, T., Bush, A.L., Shoemaker, R.C., Lark, K.G., Kahler, A.L., Kaya, N., Van Toai, T.T., Lohnes, D.G., Chung, J., Specht, J.E., 1999. An integrated genetic linkage map of the soybean genome. Crop Sci. 39, 1464–1491.] offer an excellent opportunity for the identification of traits related to BNF. This study aimed at the identification of quantitative trait loci (QTLs) controlling BNF and nodulation in an F_2 population of 160 plants derived from an intraspecific cross between two Brazilian cultivars, Embrapa 20 × BRS 133, previously identified as having good potential for mapping of QTLs [Nicolás, M.F., Arias, C.A.A., Hungria, M., 2002. Genetics of nodulation and nitrogen fixation in Brazilian soybean cultivars. Biol. Fertil. Soils 36, 109–117.]. From 252 SSR markers tested in the parental genotypes 45 were polymorphic with high heterozygotes resolution. Mapping was performed with those 45 SSR markers for nodulation [nodule number (NN) and nodule dry weight (NDW)] and plant growth [shoot dry weight (SDW)] phenotypes in $F_{2:3}$ lines. A total of 21 SSR loci were mapped with a likehood of odds (LOD) value of 3.0 and a maximum Haldane distance of 50 cM, and were distributed in nine linkage groups with coverage of 251.2 cM. The interval mapping analysis with Mapmaker/QTL revealed two genomic regions associated with NN and NDW, with a contribution of putative QTLs of 7.1 and 10%, respectively. The regression analysis identified 13 significant associations between the marker loci and QTLs due to additive effects, with some of them being significantly associated with more than one phenotypic trait. Effects were observed in all variables studied, ranging from 2 to 9%. A one-way analysis of variance (ANOVA) also detected 13 significant associations, related to dominance effects. A two-way-ANOVA showed six epistatic interactions among non-linked QTLs for SDW, NN and NDW, explaining up to 15% of the trait variation and increasing the phenotypic

Abbreviations: ANOVA, analysis of variance; cM, centimorgan; QTL, quantitative trait loci; SSR, simple sequence repeat

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expression from 8 to 28%. The data obtained in this work establish the initial stage for additional studies of the QTLs controlling BNF and indicate that effective marker-assisted selection using SSR markers may be feasible for enhancing BFN traits in soybean breeding programs.

Keywords: Biological nitrogen fixation; *Bradyrhizobium*; Quantitative trait loci (QTL); *Glycine max*; Simple sequence repeats (SSR); Nodulation

1. Introduction

Nitrogen (N) demand of soybean [*Glycine max* (L.) Merrill] can be supplied via biological nitrogen fixation (BNF) through the inoculation with selected *Bradyrhizobium japonicum*/*B. elkanii* strains, eliminating the need for N fertilizers and resulting in an economy estimated in US$ 3 billion per crop season. However, as the mean yield in Brazil has increased from 1166 kg ha^{-1} in 1968/1969 to 2737 kg ha^{-1} in 2003/2004, plant demand for N has also increased (CONAB, 2004; Hungria et al., in press).

The importance of the plant genotype to the success of symbiosis is well known. Although pioneer studies have demonstrated differences among Brazilian genotypes in relation to BNF, in the last two decades breeders have often not evaluated the symbiotic performance, while paying more attention to disease resistance and yield (Hungria et al., in press). Consequently, differences among cultivars were observed when 152 genotypes recommended for different regions of Brazil were evaluated for BNF capacity, with some of them accumulating up to 4 times more NDW and 2.5 times more N in tissues (Bohrer and Hungria, 1998; Hungria and Bohrer, 2000).

The symbiosis between soybean and bradyrhizobia result from a complex process involving many genes of both partners that leads to the formation of N_2-fixing nodules in roots (Verma et al., 1992; Provorov and Vorob'ev, 2000). In relation to the microsymbiont, several laboratories have reported research progresses and relevant information has been obtained with the sequencing of the genome of *B. japonicum* strain USDA 110 (Kaneko et al., 2002). Nevertheless, the plant genes determining nodulation and BNF are still not well known, mainly, due to the large size of the plant genome. There are a few studies on nodulation characteristics described for ''nitrate-tolerant-symbiotic'' (nts) soybean mutants (Carroll et al., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992). Advances were also achieved by the detection and isolation of several nodulin genes and plant proteins that are nodule enhanced or nodule specific, with functions in oxygen transport, cell wall architecture, sugar and N metabolism, among others (Verma et al., 1992). More recently, an integrated genetic linkage map of the soybean genome was published including some loci related to nodulation (*Rj1*, *Rj2* and *Nts* loci) (Cregan et al., 1999). Further progresses in the genetics have been obtained from studies with single nucleotide polymorphisms (SNPs) and bacterial artificial chromosome (BAC) libraries (Shoemaker et al., 2003) and will certainly come from the recent consortium established to sequence the genome of the soybean (Anonymous, 2004).

The information already available in genetic maps or the construction of new ones may lead to marker-assisted selection programs targeting desirable agronomic traits (Dudley, 1993). Important advances have been achieved by the characterization and mapping of QTLs in soybean and another crops.

In relation to the mapping of QTL controlling BNF, Nodari et al. (1993) detected four QTLs affecting nodule number in common bean (*Phaseolus vulgaris* L.), with some of them showing relation with effects of the pathogen *Xanthomonas axonopodis* pv. *phaseoli* (Boscariol et al., 1998; Tsai et al., 2000), while others show relation with mineral N (Souza et al., 2000). In our laboratory, we first reported a study of quantitative trait inheritance of BNF in Brazilian soybean cultivars with different capacities of BNF: J-200 and Bossier (high), Embrapa 20 (medium) and BRS 133 (low). Two crosses, Bossier × Embrapa 20 and Embrapa 20 × BRS 133 have shown adequacy to use in studies of QTLs controlling BNF and nodulation (Nicolás et al., 2002). The objective of this work was to use

simple sequence repeat (SSR) markers in the F_2 population of Embrapa 20 and BRS 133 to identify QTLs controlling nodulation and shoot development.

2. Materials and methods

2.1. Genetic materials

To obtain segregant generations, the singles crosses, including reciprocals, were made between the two selected cultivars with different capacities for BNF: Embrapa 20 (medium) and BRS 133 (low). In summer of 1998 (January) two seeds of each parent were planted per plot of 5 kg capacity and hybridizations were performed. The F_2 and F_3 generations were obtained by March of 1999, after self-pollination (single-seed-descent methods, SSD). At harvest, seeds were taken manually from each pool, and three to five seeds were randomly sample to produce the next generation. The remaining seeds were kept at the Embrapa Soja soybean germoplasm bank. In the summer (January) of 1999, seeds of the parents, F_1 and F_2 generations, were planted simultaneously with the production of the segregant generation F_3, to produce seeds of the same age, thereby minimizing intrinsic and environmental effects. In May of 1999, an $F_{2:3}$-mapping population consisting of 160 individual plants obtained by such method were used to evaluate the correlation between the molecular markers and phenotypic evaluations [nodule number (NN); nodule dry weight (NDW); NDW/NN ratio] and plant growth [shoot dry weight (SDW)], as described before (Nicolás et al., 2002).

2.2. Biological nitrogen fixation analysis

For the phenotypic evaluations, 160 $F_{2:3}$ lines of the cross Embrapa 20 × BRS 133 and both parental genotypes were grown under greenhouse conditions. Plants were grown in 5 kg capacity pots containing 4 kg of non-sterile soil and sand, with one plant growing per pot. The experiment was arranged in a complete randomized design with eight replications of each of the $F_{2:3}$ lines and parental genotype. The plants were inoculated at V_2 stage (completely unrolled leaf at the first node above the unifoliate node, Fehr and Caviness, 1977), by adding 1 mL of the mixed inoculum (*B. japonicum*/*B. elkanii*, SEMIA 587:SEMIA 566, 1:1, v/v).

At five weeks after emergence, plants were harvested for the evaluation of the parameters of NN, NDW, NDW/NN and SDW. The data related to nodulation and shoot mass were used in the statistical analysis. There was no need of analyzing total N in tissues, as with soybean growing under similar conditions to this experiment, Hungria and Bohrer (2000) have shown that there is a high correlation between the parameters of SDW and total N accumulated by the plants (TNP) ($r = 0.915^{**}$). Furthermore, in that same paper, authors have shown similar correlations between SDW and NDW ($r = 0.665^{**}$) and TNP and NDW ($r = 0.654^{**}$) (Hungria and Bohrer, 2000).

2.3. SSR assay

For the SSR assay, two leaves (first trifoliolate leaves) of each of the 160 F_2 and parental plants were harvested and kept at −80 °C. DNA extraction procedures used were as described by Keim et al. (1988), with 0.7 mL of buffer per extraction. A group of 252 SSR markers was chosen at approximately 25 cM intervals to achieve a high marker density in 19 out of the 20 consensus soybean linkage groups as described by Cregan et al. (1999). The primers pairs were purchased from Research Genetics, Inc. (http://www.resgen.com). Reaction mixtures contained 20 ng of soybean genomic DNA, 2.5 mM $MgCl_2$, 0.7 μM of forward and reverse primers, 125 μM of each dNTP, 1× PCR buffer containing 62.5 mM KCl, 2.5 mM Tris–HCl pH 8.3, 1 unit of Taq DNA polymerase (Life Technologies, Rockville, MD, USA), in a total volume of 15 μL. Cycling consisted of: 2 min at 95 °C; 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C; a final step of 7 min at 72 °C completed the cycles performed on an MJ Research model PTC-200 thermocycler (MJ Research, Watertown, MA). The PCR products were separated by electrophoresis on a 1.5% (w/v) agarose + 1.5% (w/v) Synergel (Diversified Biotech, Boston, MA) gel, stained with ethidium bromide and visualized by UV fluorimetry followed by exposure to a Kodak DC 120 digital picture (Eastman Kodak).

2.4. Data analysis and linkage mapping

Trait means, regression analysis and ANOVA were performed using the Statistical Analysis System (SAS, 1989). Segregation ratios for each marker in the F_2 mapping population were tested by χ^2 test to a 1:2:1 genotypic ratio ($P \leq 0.05$). Most probable map order and linkage distances were determined by multi-point linkage analysis using the computer program Mapmaker/Exp at LOD = 3.0 with a maximum Haldane distance of 50 cM (Haldane and Waddington, 1931; Lincoln et al., 1993).

Each pairwise combination between a quantitative trait and an SSR loci was subjected to regression analysis to establish the presence of linked QTL and to estimate the phenotypic variation of a trait explained by additive effects of the detected QTL. Linkage between a marker locus and a quantitative trait was assumed when there was a significant difference ($P \leq 0.05$) between the comparisons of the genotypic means of the marker classes. The proportion of the phenotypic variance explained by each detected QTL was estimated by the coefficient of determination (R^2) (Edwards et al., 1987).

One-way-ANOVA was also conducted for all pairwise combinations between marker loci and quantitative traits using the general linear model proposed by Edwards et al. (1987). This analysis was used to indicate the deviations of the marker locus from the linear effects and to evaluate dominance effects (Dudley, 1993).

In addition to the main effects, two-loci interactions of unlinked QTLs were examined using the two-way-ANOVA. The ''interaction'' factor calculated in the two-way-ANOVA was an estimate of the interaction between the two QTLs in determining the phenotype. A significant interaction factor suggests that the effect rendered by the two QTLs together is not simply the sum of their independent effects (Tanksley, 1993).

The QTLs were also analyzed by the interval mapping approach with Mapmaker/QTL. LOD score

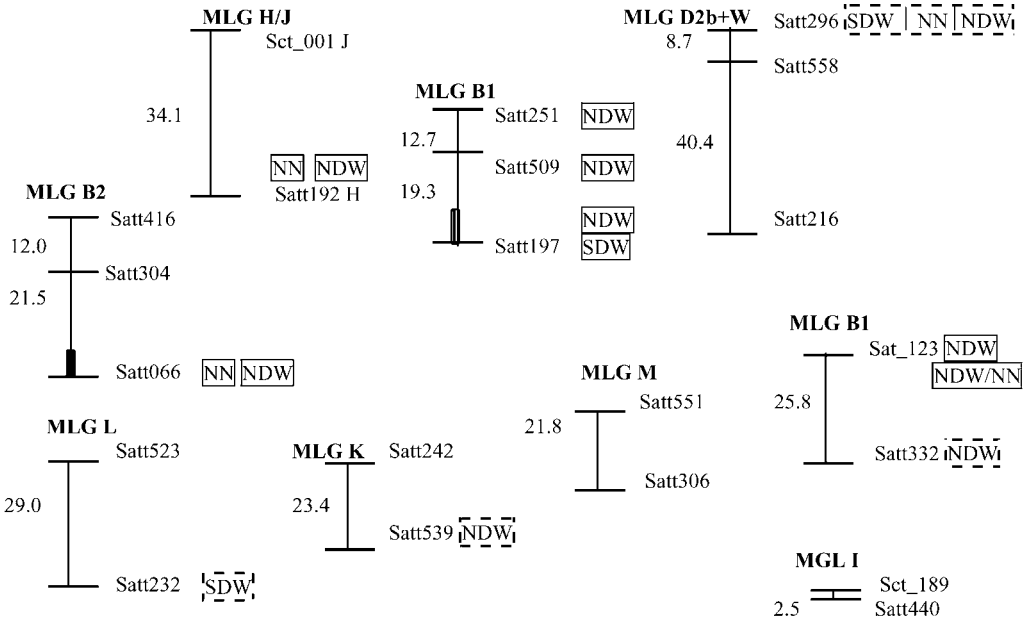

Fig. 1. Partial linkage map of the soybean based on genotypic information from 21 SSR loci of an F_2 mapping population from Embrapa 20 × BRS 133 cross. Marker loci are shown on the right side of each linkage group (MLG) and genetic distances (cM) are indicated on the left as determined by Mapmaker/Exp for LOD 3.0 and Haldane distance of 50 cM. SSR markers and linkage groups as described by Cregan et al. (1999). Shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW) and NDW/NN ratio denote markers significantly associated with traits. Boxes represent: additive (complete boxes) and dominant (dashed boxes) effect, respectively. Boxes on the linkage group represent a QTL for NDW MLG B1: LOD 2.8 and a QTL for NN MLG B2: LOD 2.3, as determined by Mapmaker/QTL.

3.0 was accepted as the threshold for detecting QTL location. The position of the LOD peak in each significant interval suggested the location of a QTL (Lander and Botstein, 1989; Lincoln et al., 1993).

3. Results and discussion

3.1. Polymorphism of SSR markers in the parental genotypes and linkage mapping

A total of 252 SSR markers covering 19 out of the 20 consensus soybean linkage groups described by Cregan et al. (1999) were screened in Embrapa 20 and BRS 133 and 45 were polymorphic between the parents, showing better separation of bands on gel and thus higher heterozygote genotype resolution. Although a genome cover provided by the 45 SSR is low, the data obtained in this work establish the initial stage for additional studies of the QTLs controlling BNF. Deviation from the expected 1:2:1 ratio was not detected for the 45-screened SSR loci. Out of these 45 primers selected only 21 were scored and mapped in the F_2 population of this study with a maximum Haldane distance of 50 cM. Nine linkage groups were detected, representing eight linkage groups described by Cregan et al. (1999) (Fig. 1). The total map coverage was of 251.2 cM with an average of 38.6 cM between adjacent loci and the order between linked markers loci is comparable to the integrated linkage map described by Cregan et al. (1999). One exception was recorded for Sct_001 locus (MLG J) that was linked to Satt192 and included on the same linkage group, and one reason for that might be differences in the DNA sequences of the Brazilian genotypes (more than one locus) in relation to the

Table 1
Linkage distribution of markers showing significant associations with phenotypic differences for shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW) and the NDW/NN ratio per plant as determined by the regression analysis

Linkage group[a]	Marker	Significance level of F test	R^2	SDW A_1A_1[b]	mg pl^{-1} A_1A_2[b]	A_2A_2[b]	Significance level of F test	R^2	NN A_1A_1	Nodule pl^{-1} A_1A_2	A_2A_2
MLG B2	Satt066	–	–	–	–	–	0.0003	0.09	52.94b	57.82b	64.36a
MLG H/J	Satt192	–	–	–	–	–	0.05	0.02	55.64a	59.57a	60.90a
MLG B1	Satt197	≤0.02	0.04	2.56b[§]	2.66ab	2.73a	–				
MLG B1	Satt251	–	–	–	–	–	–	–	–	–	–
MLG B1	Satt509	–	–	–	–	–	–	–	–	–	–
MLG B1	Sat_123	–	–	–	–	–	–	–	–	–	–
n.a.	Satt307	–	–	–	–	–	0.05	0.03	55.15b	59.06ab	61.14a
n.a.	Satt554	–	–	–	–	–	0.004	0.05	62.22a	58.77ab	53.81b

Linkage group[a]	Marker	Significance level of F test	R^2	NDW A_1A_1	mg pl^{-1} A_1A_2	A_2A_2	Significance level of F test	R^2	NDW/NN A_1A_1	mg $nodule^{-1}$ A_1A_2	A_2A_2
MLG B2	Satt066	0.02	0.04	86.33b	91.83ab	97.47a	–	–	–	–	–
MLG H/J	Satt192	0.02	0.03	86.64b	93.61ab	95.78a	–	–	–	–	–
MLG B1	Satt197	0.003	0.06	85.85b	92.31ab	101.0a	–	–	–	–	–
MLG B1	Satt251	≤0.02	0.04	86.58b	91.71ab	97.48a	–	–	–	–	–
MLG B1	Satt509	≤0.005	0.05	84.94b	91.90ab	97.99a	–	–	–	–	–
MLG B1	Sat_123	0.03	0.03	86.25b	92.64ab	96.79a	0.02	0.04	1.55b	1.72a	1.75a
n.a.	Satt307	–	–	–	–	–	–	–	–	–	–
n.a.	Satt554	≤0.02	0.04	97.67a	91.03ab	86.82b	–	–	–	–	–

[a] Linkage groups as shown in Fig. 1; n.a., not assigned.
[b] A_1 and A_2 marker loci alleles in association with QTL loci alleles from Embrapa 20 and BRS 133 parents, respectively.
[§] For each trait, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

primer utilized, resulting in an assignment on another linkage group.

3.2. Markers associated to BNF and nodulation

Results of the regression analysis with marker loci as the independent variable and SDW, NN, NDW and NDW/NN as the dependent variables showed 13 significant associations ($P \leq 0.05$) between marker loci and QTL loci due to additive effects, although some marker loci were significantly associated with more than one trait (Table 1). Six marker loci were located on three molecular linkage groups (MLG): MLG B2 (Satt066), MLG H/J (Satt192), MLG B1 (Satt197, Satt251 and Satt509) and another one including Sat_123 (MLG B1); considering the Haldane distance of 50 cM, it was not possible to link the two linkage groups belonging to MLG B1 (Table 1 and Fig. 1). Two other marker loci showed significant association with QTLs (Satt307 and Satt554), but it was not possible to assign them on the genetic linkage map (Table 1). SDW and NDW were significantly associated at Satt197, while NDW and NDW/NN were associated at Sat_123 marker loci. Four and seven significant associations were detected for NN and NDW, respectively, and Satt066 and Satt192 marker loci and Satt554 unassigned marker locus were significantly associated for both parameters. The unassigned Satt307 was also significantly associated to NN, while Satt251 and Satt509 were significantly associated to NDW. The phenotypic variation (R^2) explained by each marker locus-QTL locus was of 4% for SDW and NDW/NN, 2–9% for NN and 3–6% for NDW (Table 1). Interesting, Meksem et al. (2001) detected a major QTL for soybean seed glycitein content at Satt197 and Satt251 marker loci. Since the glycitein is an isoflavone glycoside in soybean, which is a specific inducer of the *nodD*$_1$ gene in *B. japonicum* (Smit et al., 1991), this genomic region could be a candidate for positional cloning and sequencing, in order to comparison of EST sequences from Soybean Public EST Project.

The low level of variation of these QTLs is consistent with the quantitative nature of BNF and nodulation inheritance in soybean. QTLs of minor effects associated with nodulation traits (NN) in legumes were also detected when 70 individual plants from an F_2-mapping population of common bean was analyzed, with 12 QTLs ($P \leq 0.05$) contributing with 9–18% of the phenotypic variation (Nodari et al., 1993). More recently, Souza et al. (2000) also identified QTLs ($P \leq 0.01$) explaining 4–11% of NN variation in an $F_{8:9}$ (recombinant inbred lines, RILs) population of common bean.

The interval mapping analysis revealed only two genomic regions similar to those found using the regression analysis. One interval was included between Satt066 and Satt304 markers on linkage group MLG B2, which had a significant effect ($P \leq 0.01$) on NN phenotypic expression. The other interval was found between Satt197 and Satt509 on linkage group LG B1 and was significantly associated with NDW (Fig. 1). The LOD score of the region marked by Satt066 and Satt304 was above the threshold score of 2.3 and the LOD score of the region marked by Satt197 and Satt509 was 2.7. The contribution to NN and NDW of putative QTLs was of 7.1 and 10%, respectively, quite similar to the values obtained by the regression analysis, despite the differences in number of regions detected for the QTLs (Table 2). The discrepancy in the number of QTLs might be related to the different significance levels adopted for regression and interval mapping analysis ($P \leq 0.05$ and ≤ 0.01, respectively). Another explanation could be the accuracy of methods to detect QTLs, since the results obtained by regression analysis approximates those obtained by the interval mapping when the QTL is located at the marker locus (Lander and Botstein, 1989; Nodari et al., 1993). In our linkage mapping, as the distances between two adjacent marker loci were higher than 20 cM (mean of 38.6 cM), the map could not be sufficiently saturated to be detected by the interval mapping method. Nodari et al. (1993) have also observed similarities between regression analysis and interval mapping analysis for QTL associated with common bean resistance to *Xanthomonas campestris* pv. *phaseoli*.

Regression analysis indicated that for most marker loci—QTL loci identified, increased values of phenotypic traits were most often associated with alleles from BRS 133. Two exceptions were when alleles from Embrapa 20 were significantly associated with increased NN and NDW at unassigned marker locus Satt554. These results provide a strategy to examine the efficiency of marker-assisted selection in another soybean mapping population, using an

Table 2
Linkage distribution of markers showing additive genetic effect (*D*) and dominance genetic effect (*H*) significantly associated with phenotypic differences for shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW) and the NDW/NN ratio per plant as determined by one-way-ANOVA

Linkage group[a]	Marker	SDW				NN				NDW				NDW/NN			
		P[b]	R^2	P		P	R^2	P		P	R^2	P		P	R^2	P	
				D[c]	H[d]			D	H			D	H			D	H
MLG B2	Satt066	–	–	–	–	0.001	0.09	0.003	0.70	0.07	0.04	0.02	0.98	–	–	–	–
MLG H/J	Satt192	–	–	–	–	0.13	0.03	0.05	0.54	0.05	0.04	0.02	0.44	–	–	–	–
MLG B1	Satt197	0.05	0.04	0.02	0.72	–	–	–	–	0.01	0.06	0.003	0.73	–	–	–	–
MLG B1	Satt251	–	–	–	–	–	–	–	–	0.05	0.04	0.02	0.91	–	–	–	–
MLG B1	Satt509	–	–	–	–	–	–	–	–	0.02	0.05	0.005	0.89	–	–	–	–
MLG D1b+W	Satt296	0.03	0.04	0.38	0.01	0.02	0.05	0.97	0.007	0.09	0.03	0.96	0.03	–	–	–	–
MLG L	Satt232	0.05	0.04	0.27	0.03	–	–	–	–	–	–	–	–	–	–	–	–
MLG K	Satt539	–	–	–	–	–	–	–	–	0.07	0.04	0.55	0.02	–	–	–	–
MLG B1	Satt332					–	–	–	–	0.05	0.04	0.35	0.02	–	–	–	–
MLG B1	Sat_123	–	–	–	–	–	–	–	–	0.09	0.03	0.03	0.74	0.09	0.04	0.03	0.74
MLG A2	Satt233	–	–	–	–	0.03	0.04	0.92	0.008	–	–	–	–	–	–	–	–
n.a.	Satt303	–	–	–	–	–	–	–	–	0.02	0.05	0.65	0.005	0.02	0.05	0.48	0.007
n.a.	Satt307	–	–	–	–	0.12	0.03	0.05	0.67	–	–	–	–	–	–	–	–
n.a.	Satt339	0.07	0.03	0.25	0.04	–	–	–	–	–	–	–	–	–	–	–	–
n.a.	Satt373	–	–	–	–	–	–	–	–	0.03	0.05	0.37	0.01	–	–	–	–
n.a.	Satt414	–	–	–	–	0.07	0.03	0.42	0.03	–	–	–	–	0.02	0.05	0.18	0.02
n.a.	Satt554	–	–	–	–	0.02	0.05	0.004	0.73	0.05	0.04	0.01	0.71	–	–	–	–

[a] Linkage groups as shown in Fig. 1; n.a., not assigned to linkage group.
[b] Significance level of *F* test in one-way-ANOVA in conjunction with 2 degrees of freedom.
[c] Degrees of freedom due to additive effects (*D*).
[d] Degrees of freedom due to dominance effects (*H*). *P*: significance level of *F* test.

appropriate combination of the parental allelic pairs, e.g., using the Embrapa 20 alleles for QTL transference associated to Satt554 locus to increase NDW or BRS 133 alleles for QTL transference linked to Satt197 locus for SDW.

The one-way-ANOVA was also included as an alternative to detect dominance deviations from the additive model (Table 2). In this analysis, two degrees of freedom (d.f.) for markers were divided into one degree of freedom for dominance effects and one degree of freedom for additive effects (similar to those described for the regression analysis). In addition, 13 significant ($P \leq 0.05$) associations of marker locus-QTL locus due to dominance effects (H) were detected. For SDW, three associations at Satt296 (MLGD1b+W), Satt232 (MLG L) and Satt339 (unassigned marker locus) were detected, and also three associations were verified for NN at Satt296 and at the Satt233 and Satt414 (unassigned marker loci). For NDW, five associations at Satt296, Satt539 (MLG K) and Satt332 (MLG B1) marker loci and at Satt303 and Satt373 (unassigned marker loci) were observed. For NDW/NN, only two associations at Satt303 and Satt414 (unassigned marker loci) were identified (Table 2).

Dominance effects have been detected in several studies of QTL mapping, which were reviewed by Lynch and Walsh (1998). Damerval et al. (1994) detected 70 QTL loci using 70 RFLP markers in an F_2-mapping population of 60 individual maize plants, with 33 being additive effects and the remaining 37 having at least some dominance effect. In our study, 26 significant associations ($P \leq 0.05$) using 17 SSR markers were detected, 13 due to additive (D) and 13 to dominance (H) effects. Those results were expected, since we have observed dominance genetic parameters for SDW, NDW and NDW/NN ration in $F_{2:3}$ lines derived from the cross between Embrapa 20 and BRS 133 (Nicolás et al., 2002). Since the dominance effects tend to deviate F_2 means towards the progenitor bearing highest concentration of dominant genes, in this cross the selection for BNF may be more appropriate in advanced generations.

Both the regression analysis and the one-way-ANOVA revealed the occurrence of QTLs controlling more than one trait. QTL with additive effect was associated for both SDW and NDW in a common genomic region, on linkage group MLG B1 at Satt197 marker locus (Fig. 1). Also, QTL with dominance effects for SDW, NN and NDW was associated at Satt296 marker locus (MLG D1b+W) (Fig. 1). Another QTL controlling two traits, NN and NDW, was detected in two genomic regions, on MLG B2 at Satt066 and MLG H/J at Satt192, and at Satt554 (unassigned marker locus) (Table 2). The correlations observed might be due to closely linked QTL loci or due to a single QTL with pleiotropic effect. Pleiotropy has been observed for several morphologic and developmental traits in soybean and common bean (Keim et al., 1990; Mansur et al., 1993; Nodari et al., 1993; Boscariol et al., 1998; Souza et al., 2000), however those interactions are difficult to confirm due to problems is distinguishing between multiple strongly linked QTLs that affect each single trait of the single quantitative locus with the pleiotropic effect.

3.3. Pairwise interaction between QTLs

A variance analysis with two factors (two-way-ANOVA) among all possible combinations of unlinked marker loci was performed, searching for possible interactions among specific QTLs (Tanksley, 1993). In this analysis, one marker locus significantly associated with a QTL (locus A, significantly associated at $P \leq 0.05$ from the regression analysis) and another non-linked marker locus (locus B, with QTL significantly associated at $P \leq 0.05$ or not at $P \geq 0.05$, from the regression analysis) were applied as the independent variables and the quantitative traits as the dependent variables. The ''interaction'' factor calculated in this two-way-ANOVA was utilized to estimate the interaction between the two QTLs in determining the phenotype. Six interactions between paired marker loci were found to be significant at $P \leq 0.05$ (Table 3 and Fig. 2). For SDW, two interactions were found (locus A, significant at $P \leq 0.05$ and locus B, not significant at $P \geq 0.05$), one of them between Satt197 and Satt233 marker loci and the other between Satt197 and Satt317 marker loci. The first interaction had a determination coefficient (R^2) accounting for 12% of phenotypic variation and the alleles increasing the trait were related to BRS 133 at both marker loci ($A_2A_2B_2B_2$), surpassing the phenotypic mean by 8% (Table 3). The second interaction for SDW had an R^2 value of 11%.

Table 3
Description of genotype-by-genotype interaction between two non-linked SSR marker loci associated with phenotypic differences for shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW) and NDW/NN t as determined by two-way-ANOVA

Trait	Locus A[a]		Locus B[a]		Pairs of locus	Mean	P	R^2 [b]	$A_1A_1B_1B_1$ [c]	$A_1A_1B_2B_2$	$A_2A_2B_1B_1$	$A_2A_2B_2B_2$
	P	R^2	P	R^2	Locus A × B							
SDW (mg pl^{-1})	0.02	0.04	0.68	0.005	Satt197 × Satt233	2.65	0.03	0.12	2.74	2.47	2.78	2.86
	0.02	0.04	0.07	0.03	Satt197 × Satt317	2.65	0.05	0.11	2.45	2.68	2.87	2.58
NN (no. pl^{-1})	0.05	0.02	0.17	0.02	Satt192 × Satt197	59.06	0.01	0.12	58.09	57.86	75.54	59.28
NDW (mg pl^{-1})	0.01	0.06	0.05	0.04	Satt197 × Satt192	93.06	0.02	0.15	86.04	113.18	98.93	93.91
	0.05	0.04	0.04	0.04	Satt251 × Satt554	91.81	0.008	0.15	87.56	77.42	100.47	82.35
	0.02	0.05	0.04	0.04	Satt509 × Satt554	91.81	0.05	0.14	84.91	79.04	103.64	77.54

[a] Values determined by regression analysis.
[b] Coefficient of determination as result of model with both factors plus the interaction between then: locus A + locus B + A × B.
[c] A1, B1 and A2, B2 denote alleles of marker loci in association with alleles 1 and 2 of QTL locus from Embrapa 20 and BRS 133 parents, respectively.

The combination of alleles increasing this parameter by up to 8.3% came from both parents, thus alleles from BRS 133 were associated at Satt197 marker locus and alleles from Embrapa 20 at Satt317 marker locus ($A_2A_2B_1B_1$) (Table 3). For NN, just one interaction was significant (locus A significant at $P \leq 0.05$ and locus B, not significant at $P \geq 0.05$) between Satt192 and Satt197 marker loci, and explaining 12% (R^2) of the phenotypic variation. This interaction had the highest effect among all detected in this model analysis, as the combination between the alleles of BRS 133 at Satt192 (A_2A_2) and the alleles of Embrapa 20 at Satt197 (B_1B_1) increased NN by up to 28% (Table 3). Three other interactions were detected between pairs of marker loci significantly associated to the QTL controlling NDW. Two interactions contributed with up to 15% (R^2), one between Satt197 and Satt192 markers loci and the other between Satt251 and Satt554. The best combination of parental alleles, increasing NDW mean by up to 22%, was verified between the alleles from Embrapa 20 at the Satt197 and the alleles from BRS 133 at the Satt192 ($A_1A_1B_2B_2$). The third interaction for NDW parameter was verified between Satt509 and Satt554 marker loci and account to 14% (R^2).

The comparison of the results of the genotype-by-genotype interactions (Table 3) with those of the regression analysis (Table 1) shows that the phenotypic variation was due to additive gene action and to an epistatic interaction. All interactions detected for SDW and NN, and those involving Satt197 and Satt192 marker loci for NDW resulted from epistatic interaction, since each of those beneficial alleles at a

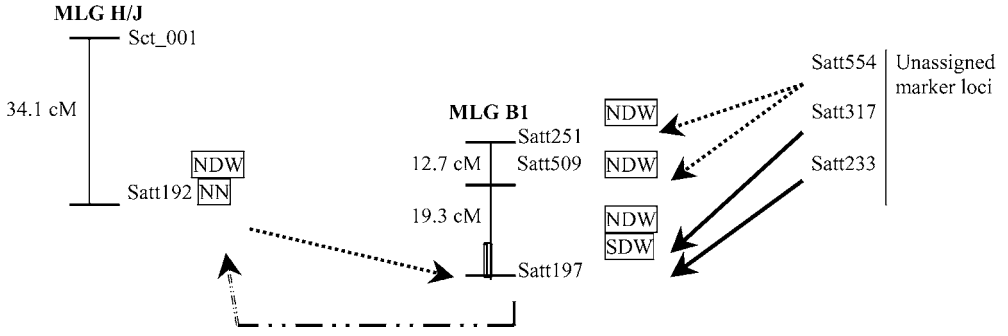

Fig. 2. Interactions between pairs of unlinked loci. Shoot dry weight (SDW), nodule number (NN) and nodule dry weight (NDW) denote markers associated with these traits. Arrows indicate interactions between QTL loci and loci that regulated them for SDW→, NN ⇢ and NDW ⇢.

marker loci were affected by opposite alleles (not beneficial to increase the trait) at the other marker loci (Table 3). In contrast, for NDW the significant interactions between Satt251 and Satt554 marker loci and the interaction between Satt509 and Satt554 marker loci had an additive gene action. Thus, the level of NDW was increased as a consequence of the enhanced accumulation of positive alleles (Table 3). Several studies have demonstrated the epistatic effects on genetic trait, e.g., Lark et al. (1995) have shown in soybean that height phenotypic variation on particular alleles at a QTL locus was conditioned by alleles at a second unlinked locus, which alone explained little or no trait variation.

Therefore, additional deductions could be derived from the pairwise interactions between QTLs, helping to explain the occurrence of QTLs controlling more than one trait as detected in the present study. A significant QTL associated to SDW ($P \leq 0.02$, Table 1) and NDW ($P \leq 0.003$, Table 1) at Satt197 marker locus on the linkage group MLG B1 was observed. Also, interactions occurred between Satt197 marker locus and Satt233 and Satt317 affecting SDW but not NDW (Table 3 and Fig. 2). The lack of an effect of Satt233 and Satt317 on NDW suggests that a separate gene might exist that only regulates SDW. Similar argument may be used for QTL linked to Satt192 marker significantly associated with NN ($P \leq 0.05$, Table 1) and NDW ($P \leq 0.02$, Table 1). However, interactions exist between Satt192 and Satt197, which are affecting NN as well as NDW (Table 3 and Fig. 2). Those interactions might indicate that a single QTL may exist controlling both traits. However, at this stage we cannot conclude if the Satt192 marker is associated to a single QTL with pleiotropic effect on NN and NDW or if Satt197 is linked to two separate and distinct QTLs controlling SDW and NDW. It will be necessary to carry out additional genetic analysis to obtain a more saturated mapping on this genomic region.

The phenotypic variation for quantitative traits accounted for by the regression analysis (4% for SDW and NDW/NN, 2–9% for NN and 3–6% for NDW, Table 1) and the two-way-ANOVA (11–12% for SDW, 12% for NN and 14–15% for NDW) could be related to the broad-sense-heretability (h_b^2) estimates as previously reported for $F_{2:3}$ lines derived from this cross (Nicolás et al., 2002). In that work, we reported that (h_b^2) estimates were 13% for NN, 26% for NDW and 20% for NDW/NN ratio and 61% for SDW. Although a multi-way analysis of variance including all the loci for each parameter was not performed in our study, it was possible to observe by the regression analysis that the markers partially explain the genetic variation for the quantitative traits in this population. Thus, the epistatic interactions detected in our study should account for an additional amount of variation in quantitative traits such as NN and NDW. Nevertheless, the variation that remains unexplained in this experiment, particularly for SDW, may be attributed to environmental variance, as well as to linkage associations between other marker loci and significant QTLs not detected in this study.

BNF plays a key role in agriculture sustainability, but very few studies have been performed trying to identify molecular markers associated with the symbiosis. The importance of BNF is even greater in developing countries, where most of the N fertilizers are imported, and estimates are that the economy resulting from the biological process with the soybean crop in Brazil are of US$ 2.87 billion per year (Hungria et al., in press).

This study represents a first step for the identification of QTLs associated with the symbiosis with Brazilian cultivars. Despite mapping only 21 SSR markers that represent about 10% of the genome, we have identified significant marker loci associated with SDW, NN and NDW, including additive and dominance effects in soybean. We have also associated molecular information to the genetic parameters ([*d*] and/or *D*, [*h*] and/or *H*) of the inheritance studies previously performed with this population (Nicolás et al., 2002). Moreover, additional studies to test the QTLs detected in an $F_{2:7}$ population of cultivars Bossier and Embrapa 20 are being carried out. At this point, seven SSR markers have been mapped and were significant associated for nodulation (Satt197, Satt192, Satt296, Satt332 and Satt509) and shoot mass (Satt232) traits, indicating that they might be effective in increasing BNF in soybean breeding programs (Santos et al., in press).

Brazil is today the second worldwide soybean grain producer and our main goal is the inclusion of those SSR markers in the Embrapa's breeding program, responsible for about 70% of the cultivars commercialized in the country.

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