SINGLE-SEED DESCENT, SINGLE-POD DESCENT, AND BULK METHODS IN SOYBEAN

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Funada, Mizuki, M.S., Department of Plant Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, July 2011. Single-seed Descent, Single-pod Descent, and Bulk Methods in Soybean. Major Professor: Dr. Ted Helms.

Soybean [*Glycine max* (L.) Merr.] breeders need to use the most efficient and inexpensive method to advance populations during the inbreeding process. Most breeders in the soybean industry prefer the bulk method because it requires less hand labor during harvest. This study used molecular markers to compare the single seed descent (SSD), the single-pod descent (SPD) and the bulk methods. The objective was to identify the most efficient method of inbreeding by determining which method had the greatest number of unique lines and the fewest pairs of redundant lines. The number of pairs of redundant lines was determined by scoring each of 100 $F_{4:5}$ lines, developed from each of the three inbreeding methods, using 21 polymorphic SSR markers. A similarity coefficient (S_{xy}) was used to determine the level of similarity between each possible pair of lines within each inbreeding method. The SSD method was used as a control to identify the number of lines that were identical by descent for the SPD and bulk methods. Unique lines were lines that were not paired with any other line within that inbreeding method for the specified level of genetic similarity. At the $S_{xy} \geq 0.875$ level of genetic similarity, 49% of the lines were unique for the SSD, 44% of the lines were unique for the SPD, and 39% of the lines were unique for the bulk method, but the difference in the number of unique lines among methods was not significant. For the SSD method, zero lines were identical by descent with another line. For the SPD method, at the $S_{xy} \geq 0.875$, there were 16 pairs of lines that were identical by descent. For the bulk method, at the $S_{xy} \geq 0.875$, there were 42 pairs of lines that were identical by descent. This result was evidence that due to

genetic sampling, there were more redundant lines for the bulk method and SPD than for the SSD method. The number of unique lines developed by each of the three inbreeding methods is the most important measure of the relative efficiency of each method. However, there was no significant difference in the number of unique lines among these methods. Therefore, the most efficient method should be the method that requires the least time and labor during harvest. The SSD method requires too much time during harvest to be practical for a commercial soybean breeder. The decision as to whether to use the SPD or bulk method will depend on which of those two methods is the most practical for that specific breeding program.

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INTRODUCTION

Soybean [*Glycine max* (L.)] is the most important grain legume crop in the world. Soybean has an important nutritional role and has a high protein and oil content. With those components, soybean has become one of the major sources of protein and edible oil for human consumption, animal feed, and industrial uses. North Dakota produced about 3.1 million metric tons (M MT) of soybean in 2008, which was 3.9% of the soybean produced in the United States. Also, North Dakota soybean acreage has been increasing yearly (USDA-NASS 2008).

Soybean was domesticated from a wild plant species. Artificial selection has significantly changed domesticated plant species into beneficial cultivars with higher yield, faster growth, larger seeds, or better taste compared to their wild relatives (Hymowitz, 1970). Hence, breeding is essential for the improvement of soybean varieties. Each breeder decides which methods are the most rapid, efficient, and inexpensive to enable them to produce improved crop cultivars with the maximum utility for farmers and consumers.

Recently, plant breeders have combined traditional breeding methods with biotechnology and molecular genetics to develop improved varieties. The basic principles of breeding have not changed, but molecular techniques have enhanced the process. The development of molecular markers is one example of an enhanced breeding technique. Molecular markers are applied to genotype each progeny. Conventional plant breeding programs relied on phenotypic data for selection. However in the 1980s, plant breeding was improved by DNA-based molecular markers. Since then, plant breeders have begun to decipher and comprehend the genetic variation essential for key traits, and

use polymorphic molecular markers to aid in the selection of new cultivars with desired quantitative and qualitative traits (Bernardo, 2008). Scientists have discovered and mapped new resistance genes for disease and pest resistance, and qualitative trait loci (QTLs) controlling agronomic traits. However, breeders still need to develop segregating plant populations to maintain genetic variation down through generations of inbreeding.

In the steps of breeding for a self-pollinated crop, plant breeders select two parental genotypes, and cross them to develop F_1 seeds. The F_1 seeds are planted, and F_2 seeds are harvested from the F_1 plants. The breeder determines which breeding procedure is the best choice for that crop, based on the breeding goals. Three of the standard inbreeding methods of a self-pollinated crop are single seed descent (SSD), single pod descent (SPD), and bulk methods.

Most breeders in the soybean industry choose the bulk method because it is an easy method to advance populations during inbreeding. However, there is a need to compare the bulk method to other methods to determine the most efficient method. In this study, SSD, SPD, and bulk methods, were compared to determine the most efficient method. The bulk method would be expected to reduce the number of unique F_2 derived lines in the population from generation to generation compared with the SSD and the SPD methods. The reason for this is that the bulk method would be expected to result in many redundant lines and the extinction of lines.

The objective of this research was to determine the number of lines which are redundant and the number of unique lines in a sample of 100 $F_{4:5}$ lines for the SSD, SPD and bulk methods, for different levels of genetic similarity. This research was designed to identify the most efficient method of inbreeding.

LITERATURE REVIEW

Definitions of Three Principal Inbreeding Methods

The SSD method is theoretically the most efficient method to develop inbred lines. One seed is randomly picked from each plant. This method maximizes the genetic variance for quantitative traits and results in homozygous genotypes though inbreeding, while minimizing time, space, and note taking. It is possible to select for characters of high heritability and to grow several generations in a year (Brim, 1966).

The SPD method involves collecting one pod from each plant. There are several seeds in each pod, which results in the development of duplicate lines due to the process of genetic sampling. All the pods from a population are threshed together and a sample of threshed seeds is planted for the next generation. Therefore, two or three seeds are harvested from each plant, and it is likely that some F_2 plants will be represented two or three times in the F_3 generation. It is also likely that some F_2 plants will not be represented in the F_3 generation. Therefore, the SPD method reduces the genetic variation and changes allele frequency (Empig and Fehr, 1971).

The bulk method also provides a procedure to inbreed a segregating population until the desired homozygosity is achieved, and the above genetic sampling problem also exists for the bulk method. Survival in a plot is dependent on the number of seeds each individual produces and the proportion of seeds from each plant that reach maturity and produce offspring.

The bulk procedure often takes into account an intangible measure of superiority that might normally be overlooked by a breeder. Traits like plant height, maturity, and adaptation rapidly change because natural selection increases the frequency of desired

genotypes. This is an easy way to maintain populations during inbreeding (Empig and Fehr, 1971). Natural selection affects seed size, plant height, and lodging. Natural selection favors tall, late-maturing plants, with a large number of small seeds. Natural selection can alter the allele frequency of plants that have been inbred using the SSD, SPD, and the bulk method (Sleper and Poehlman, 2006).

Empirical Evaluations of Inbreeding Methods

According to Gill et al. (1995), the honeycomb method was highly effective for producing superior lines with increased yield per plant and pods per plant compared with other methods. The honeycomb method is based on single plant selection. A single superior plant is selected from a grid, based on phenotype. The second best method was the bulk method, followed by the SSD and SPD methods. Their research found that the SSD, the SPD, and the bulk methods were equally effective in deriving superior lines. However, the differences between these methods were not clear.

Tee and Qualset (1974) suggested that the SSD and the bulk methods influenced the population for characters like height, yield, and maturity. They developed two different segregating populations in their greenhouse, each from different bi-parental crosses. For one population, which was inbred using the bulk method, the plant height and yield increased from the F_4 to F_6 generations, due to natural selection. However, in the second population, there were no significant difference between the SSD and the bulk method. The genetic variances of the two methods were similar. Therefore, they could not determine which method was appropriate for an accelerated-generation program. They were unable to determine the number of lines derived from the different F_2 or F_3

plants because they used morphological markers, but did not use any molecular markers to identify relationship among pairs of lines.

Another experiment, using molecular markers, reported that there were no redundant lines at the F_5 level of inbreeding when the SSD method was used (Keim et al., 1994). They assumed that any redundant lines did not exist in the SSD because this method was a control. However, 18% of lines were redundant when the SPD method was used. The SPD method is more cost effective and is more practical than the SSD method because generation advance using the SSD method requires more time and labor. The molecular marker experiment reported by Keim et al. (1994) did not compare the bulk method to the other methods. However, the bulk method is the method used by most private company soybean breeding programs.

Computer Simulations of Inbreeding Methods

Muehlbauer et al. (1981), used computer simulation to compare the SSD and the bulk methods. Results of the simulation showed 81.5% of initial F_2 plants were represented at the F_6 level of inbreeding for the SSD method. Also, 24.6% of the original F_2 plants were represented at the F_6 level of inbreeding using the bulk method. The bulk method resulted in more redundant genotypes than the SSD method because of genetic sampling problems.

Wells and Weiser (1989) used a computer simulation to compare the SSD and the SPD methods. They assumed that three seeds per pod were sampled with two different levels of probability of survival. If the probability of survival was relatively high, the genetic variance of 100 individuals produced by the SSD method, and the expected

amount of genetic variance of 200 individuals derived by the SPD method, were equal. The SSD method was more efficient than the SPD.

Genetic sampling, using the bulk method, eliminated more unique lines than the SSD and the SPD methods. A greater number of seeds sampled per plant resulted in a greater number of redundant lines. For example, the extinction rates were 66.7% at the F_4 level of inbreeding when ten seeds were sampled from each plant using the bulk method and 55.0% at the F_4 level of inbreeding when two seeds were sampled from each plant using the SPD method (Kervella and Fouilloux, 1992).

Alike in State and Identical by Descent

When two individuals have the same form of an allele or an individual is homozygous at a locus, there are two different possibilities and two different genetic concepts to consider. Falconer (1960) defines two alleles that are alike in state when the "two genes are regarded as being identical if they are not recognizably different in their phenotypic effects, or by any other functional criterion; in other words, if they have the same alleleomorphic state." An individual that is a homozygous at a locus has two alleles at that locus that are alike in state. Two alleles that are alike in state may have been inherited from the same F_2 or F_3 plant, which would be a common ancestor. If two alleles are the result of DNA replication that occurred in a common ancestor based on biological inheritance, the two alleles would be both identical by descent and alike in state. It is also possible that two alleles that are alike in state are not the result of DNA replication from a common ancestor. Two alleles that are identical by descent must be alike in state. Two alleles that are alike in state are not necessarily identical by descent. Keim et al. (1994) compared the SSD to the SPD methods using an alike in state criterion.

Muehlbauer et al. (1981) and Kervella and Fouilloux (1992) used identical by descent as their criterion.

MATERIAL AND METHODS

Population Development

The parents were 'OAC Atwood' and 'RG600RR'. OAC Atwood has purple flower color and tawny pubescence. RG600RR has purple flower color and gray pubescence. The cross between OAC Atwood and RG600RR was made at Casselton, ND in the summer of 2005. The F_1 seeds were planted in the 2005-2006 Chile, S.A. nursery. The F_2 seeds were planted at Fargo, ND in the summer of 2006. The F_2 populations were identified as segregating, based on segregation of pubescence colors. Approximately 200 F_2 plants were sampled for each generation for each method. For the SSD method, one seed was sampled from each of 200 F_2 plants and advanced to the F_3 generation. For the SPD method, one two or three-seeded pod was sampled from each of 200 F_2 plants. For the SPD method, all 200 pods were placed in the same sack and later threshed in bulk. For the bulk method, every seed of all 200 F_2 plants were harvested with a plot combine and bulked.

The F_3 populations were sent to Chile, S.A. in the 2006-2007 season. For the SSD method, the 200 F_3 seeds were planted in the Chile winter nursery. For the SPD and bulk methods, random samples of 200 F_3 seeds were planted in the Chile winter nursery. These procedures were repeated in Chile when F_4 seeds were harvested from F_3 plants. At Casselton, ND in the fall of 2007, approximately 150 F_4 plants for the SSD, SPD, and bulk methods were individually threshed, and seeds from each F_4 plant were put in a separate envelope to form $F_{4:5}$ lines.

In 2008, 100 $F_{4:5}$ lines from each of the three inbreeding methods were chosen at random. Ten seeds of each $F_{4:5}$ line were planted in pots and grown in the greenhouse at North Dakota State University to collect leaf samples for DNA extraction.

Laboratory Experiments

DNA extraction from soybean tissue

Two parents, OAC Atwood and RG600RR, and the 300 lines of soybean were sampled for leaf tissue. Two trifolioate leaves from each of 10 plants per line were put into envelopes. After the leaf tissues were collected, these were immediately placed in liquid nitrogen. Approximately 2 g of leaf tissue was frozen with liquid nitrogen for each experimental line and later were ground, using a chilled mortar and placed into a 50 ml plastic, capped centrifuge tube. Then 10 ml of preheated CTAB isolation buffer at 60°C was added. These tubes were incubated for 30 minutes in water at 60°C. After incubating, 10 ml of chloroform: isoamyl alcohol (24:1) was added to each tube and shaken vigorously. These tubes were centrifuged for 15 minutes at 3500 rpm. A small piece of miracloth from EMD Chemicals in a clean 50 ml centrifuge tube was used to remove the aqueous phase (top layer) with a pipette. The aqueous layer was transferred to the clean tube allowing it to strain through the miracloth which eliminated large particles. Then, 10 ml of cold isopropyl alcohol was added to precipitate the DNA. The tubes were gently mixed until the white strands of DNA became visible. At this point, the samples were placed in a refrigerator for several hours or overnight. After that, 10 ml of DNA wash solution was added and left for at least 20 minutes in the cooler. When adding the wash solution, the pellet was dislodged to remove traces of chloroform. All tubes were centrifuged a second time for 15 minutes at 3000 rpm. DNA washing

was repeated one additional time. The supernatant was removed and the sample was allowed to dry by placing it upside down. After the DNA was dry, the DNA was transferred into a 500 µl tube and 500 µl of H_2O and 0.5 µl of RNase A were added.

Concentrated DNA quantification

Dye mixing consisted of 45 ml of H_2O, 5 ml of 10×TNE, and 50 µl of Hoerst 33258 dye prepared in a 50 ml tube. Two ml of dye solution was added to a fluorometer cuvette to make a control with DNA concentration of zero in a fluorometer. Then, 2 µl of Calf Thymus DNA was added into the cuvette and the scale set to 1000. This concentration of DNA indicates 1000 µg/ml of sample. The cuvette was emptied and rinsed with ddH_2O. The concentrated DNA quantification was measured using the solution of 2 ml of dye solution and 2 µl of DNA sample. This process was continued until all samples were analyzed.

Selection of SSR primers and PCR reactions

Twenty-one SSR markers which were identified as polymorphic between the two parents, OAC Atwood and RG600RR, were selected from a random set of 230 SSR markers. The 21 markers were also selected to represent numerous different chromosomes (Table 1). Markers that were located closer than 100 cM were considered linked. For example, the loci of the two primers, Satt070 and Satt126, were both located on chromosome 14. The start position of Satt070 was 73 cM, and the other start position of Satt126 was 28 cM. The genetic distance between those loci was 45 cM. The distance was less than 100 cM. Therefore, they were considered to be linked to each other. To verify whether the observed molecular marker genotypic scoring process was accurate,

the allelic relative frequencies of each SSR marker for each method were calculated (Table 1).

Table 1. Chromosome number and chromosome region of each SSR marker.

Sequence name	Chromosome number[†]	Start position	Relative Frequencies[‡]
		-------cM-------	
Sat_033	3	58	0.66
Sat_372	18	108	0.50
Satt070	14	73	0.54
Satt126	14	28	0.39
Satt135	17	27	0.37
Satt173	10	58	0.51
Satt198	1	69	0.17
Satt254	1	56	0.70
Satt309	18	5	0.50
Satt353	12	8	0.61
Satt386	17	125	0.44
Satt424	8	61	0.36
Satt474	14	75	0.53
Satt565	4	0	0.46
Satt589	8	34	0.39
Satt597	11	74	0.49
Satt614	20	32	0.63
Satt634	2	65	0.72
Satt643	6	95	0.45
Satt687	14	114	0.59
Satt703	2	99	0.48

[†]Information including of chromosome numbers and start positions were cited from SoyBase Genetic Map Feature Retrieval Page: http://soybase.org/MarkerDB/index.php

[‡]The allelic relative frequencies of each SSR marker.

The polymerase chain reaction (PCR) master mix of each sample was prepared by combining 13.2 µl of H_2O, 2 µl of 10× PCR Buffer, 1 µl of 2mM dNTPs, 2 µl of 5 µM Primer R + F, and 0.6 µl of 5 units/µl Taq polymerase enzyme. DNA samples were added, then 20 µl of master mix were filled on a PCR plate. The PCR plate was placed in a thermal cycler to control temperature and time requirements as per the PCR profile for

soybean SSR primers. The thermal cycling was programmed at 95°C for two minutes as the first cycle. A cycle involved denaturizing at 92°C for 1 minute, annealing at 47°C for 1 minute, and extension at 68°C for 1 minute. The cycle was repeated 33 times. The last cycle was at 68°C for five minutes as the final extension. After the PCR reaction was completed, the plate was kept at or below 4°C for a few minutes.

Electrophoresis of PCR reactions

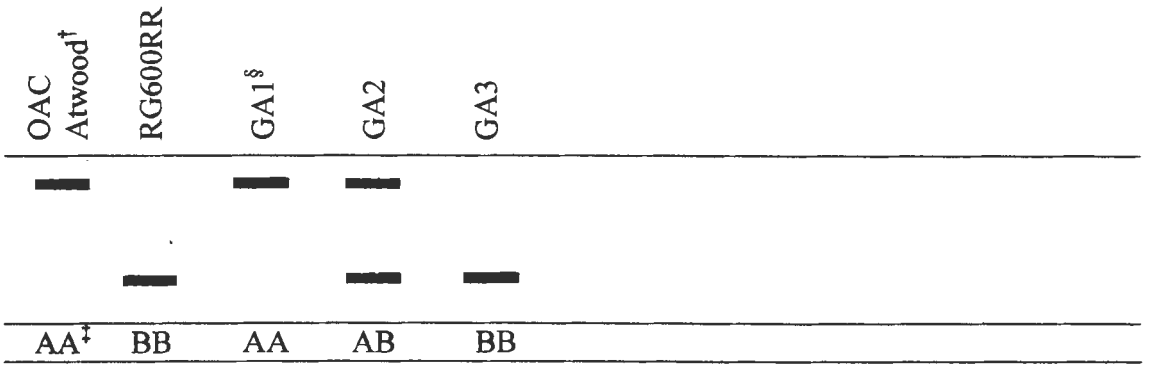
Four µl of ethidium bromide was added to a 2% agarose gel, prepared, and placed in a buffer-filled electrophoresis apparatus, then electrical current was applied via the power supply. One µl of 10× dye was added to each PCR reaction. Both the PCR reactions with dye and the standard DNA ladder sample were loaded into a well of the gel. A standard DNA ladder with several different sized fragments of DNA was used to compare it to the DNA from the PCR reaction. The ladder was used for comparison to an unknown fragment size. The power source was plugged into an outlet and the voltage was set to about 90 V. The gel was left to run until the dye migrated about 1-2 cm from the wells, which required 60-90 minutes. After the electrophoresis was complete, the power supply was turned off and removed from the electrophoresis apparatus. A photograph was taken of the gel. Finally, all samples were analyzed to determine the genotype. If the SSR amplified bands were difficult to analyze in the 2% agarose gel, a high resolution (4% SFR) agarose gel was used to classify banding patterns.

Statistical Analysis

After the electrophoresis was completed for each of the 21 SSR markers, the SSR amplified band was determined for each $F_{4:5}$ line from photographs of each gel. OAC Atwood was considered to be AA, and the RG600RR genotype was considered to be BB

(Fig. 1). For example, if the GA1 genotype had the same band as OAC Atwood, this genotype was recorded as AA. If the GA3 line had the same SSR band as RG600RR, this genotype was recorded as BB. If GA2 was heterozygous at an SSR locus, the genotype was recorded as AB. Based on this rule, the genetic composition of each line was determined. The data matrix for each genotype and SSR marker was filled in with the genotypic score.

Fig. 1. Explanation of homozygous and heterozygous marker genotype scoring from the two parents, OAC Atwood and RG600RR.



†Indicates cultivar is a parent. Both OAC Atwood and RG600RR were parents.
‡Indicates a genotype such as homozygous or heterozygous.
§GA indicates lines derived from cross of OAC Atwood and RG600RR.

In addition, the pair-wise coefficient of similarity was used to identify which lines were redundant for each of the three methods of inbreeding. The SSR marker scores were used to provide the genetic composition of each line. All pair-wise coefficients of similarity values were determined for each method using the following formula:

$$S_{XY} = \frac{2 \times \Sigma N_{XY}}{N_X + N_Y}$$

Where S_{XY} is the coefficient of similarity (Keim et al., 1994), N_{XY} is the number of SSR alleles in common between a pair of genotypes for one marker locus; N_X is two

times the number of SSR loci scored for genotype X; and N_Y is two times the number of SSR loci scored for genotype Y. For example, GA1 (line X) was compared with GA2 (line Y). Then, GA1 was compared with all the other 99 lines within an inbreeding method, which is from GA3 to GA100. Likewise, the coefficient of similarity was computed for GA3 with all the other lines within the same inbreeding method.

The score of N_{XY} was determined, using the following the rule: if both lines were completely homozygous (AA and AA, or BB and BB) the N_{XY} for that marker was scored as a two for that locus because both alleles were the same between both lines; if two genotypes were different at a locus (AA and BB), then the N_{XY} of that marker was scored as a zero for that locus, because both alleles were different; if line X was homozygous and line Y was heterozygous (AA and AB, or BB and AB), the N_{XY} value of that marker was scored as a one because one allele was the same between both lines for that locus.

Table 2 provides an example of how to calculated S_{XY} for hypothetical genotypes. A comparison between GA1 and GA2, based on the data of Table 2 for each of the 21 SSR loci resulted in N_{XY} scored as a two for each of the 21 SSR loci because both alleles were exactly same between GA1 and GA2. Therefore, the coefficient of similarity was equal to unity.

$$SXY = \frac{2\times\Sigma N_{XY}}{N_X+N_Y}$$

$$= \frac{2\times 42}{42+42} = 1$$

A comparison between GA1 and GA3, with half of the loci in common between these two genotypes (AA and AB), resulted in an N_{XY} value of one for each of the 21

SSR loci, because these two genotypes had one SSR allele in common for each locus. Therefore, the coefficient of similarity was equal to one-half.

$$SXY = \frac{2\times1\times21}{42+42} = 0.5$$

When genotypes GA1 and GA4 were compared, these lines have no alleles in common (AA and BB), so N_{XY} was scored as a zero because both alleles were different. Therefore, the coefficient of similarity was equal to zero.

$$SXY = \frac{2\times0\times21}{42+42} = 0$$

Table 2. Hypothetical data to show how to calculate the similarity coefficients.

Sequence name	Genotype			
	GA1[†]	GA2	GA3	GA4
SSR 1	AA[‡]	AA	AB	BB
SSR 2	AA	AA	AB	BB
SSR 3	AA	AA	AB	BB
SSR 4	AA	AA	AB	BB
SSR 5	AA	AA	AB	BB
SSR 6	AA	AA	AB	BB
SSR 7	AA	AA	AB	BB
SSR 8	AA	AA	AB	BB
SSR 9	AA	AA	AB	BB
SSR 10	AA	AA	AB	BB
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
SSR 21	AA	AA	AB	BB

[†]GA indicates lines derived from cross of OAC Atwood and RG600RR.
[‡]Indicates a genotype such as homozygous or heterozygous.

The number of paired lines, which have similarity coefficient (S_{xy}) values greater than or equal to 0.75, and 0.875 similarity levels was computed for each of the three inbreeding methods. Lines were only paired with other lines within the same inbreeding

method. The number of pairs of lines was computed for each of the three methods. Two different criteria were used to compare the three methods. One criterion was to compare the number of pairs of lines at a given level of genetic similarity. A second criterion was to compare the number of lines that were not paired with any other line at a given level of similarity. Lines that were not paired with any other line were defined as unique lines.

The number of paired comparisons for the SSD method was determined as a base number at each level of similarity. The SSD method eliminates any possibility that two $F_{4:5}$ lines could be derived from a common F_2 or F_3 plant. Therefore, any lines paired at each S_{xy} level were paired due to alike-in-state relationship and were not paired because of an identical-by-descent relationship.

The number of paired comparisons of the SPD and bulk methods was affected by genetic sampling. The number of additional paired comparisons, which were due to genetic sampling was determined for the SPD by subtracting the number of paired comparisons for the SSD from the number of paired comparisons for the SPD, calculated at the same level of S_{xy}. Genetic sampling also occurred in the bulk method, so the additional number of paired comparisons which were due to genetic sampling were determined by subtracting the number of paired comparisons of the SSD from the number of paired comparisons for the bulk method at the same level of S_{xy}. The number of paired comparisons at a specified level of S_{xy} that was greater than the number of paired comparisons for the SSD method, was a measure of the identical-by-descent relationship between lines for the SPD or bulk methods.

The Z-test was used to determine whether the coefficient of similarity was significantly different between methods at given level of S_{XY} (Freund and Walpole, 1980). The Z-test was based on the binomial distribution and was calculated using the formula:

$$Z = \frac{\widehat{\theta_1} - \widehat{\theta_2}}{\sqrt{\frac{\theta_1 (1-\theta_1)}{n_1} + \frac{\theta_2 (1-\theta_2)}{n_2}}}$$

Where θ_1 was the relative frequency of paired comparisons in one method; and θ_2 was the relative frequency of paired comparisons for a different method.

$$\widehat{\theta_1} = \frac{(\text{Number of paired comparisons in SSD})}{4950}$$

$$\widehat{\theta_2} = \frac{(\text{Number of paired comparisons in SPD})}{4950}$$

The number of paired comparisons that were equal or greater than the specified S_{xy} level, was determined for each method. The Z-test was used to determine whether the coefficient of similarity was significantly different between methods at each level of S_{xy}. For example, in the SSD method there were 133 paired comparisons with an $S_{xy} \geq 0.75$. The total number of paired comparisons was 4950. Then θ_1 was estimated to be $\frac{133}{4950} =$ 0.03. At an $S_{xy} \geq 0.75$ level there were 210 paired comparisons out of a total of 4950 for the SPD method and θ_2 was estimated to be 0.04. The critical Z-value was 1.96 at the P = 0.05 level of Type I error.

For both the SSD and SPD methods, the number of paired comparisons used to calculate S_{xy} values was $n = \frac{100(99)}{2} = 4950$. Due to a small amount of missing data, the number of paired comparisons for the bulk method was n = 4753. No SSR marker data

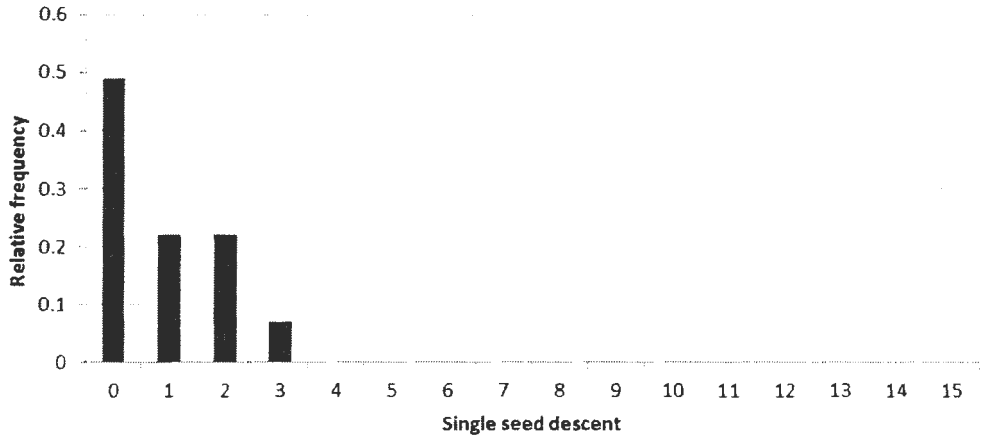
was available for two lines that had been inbred by the bulk method, this reduced the number of pair-wise comparisons to n=4753 for that method.

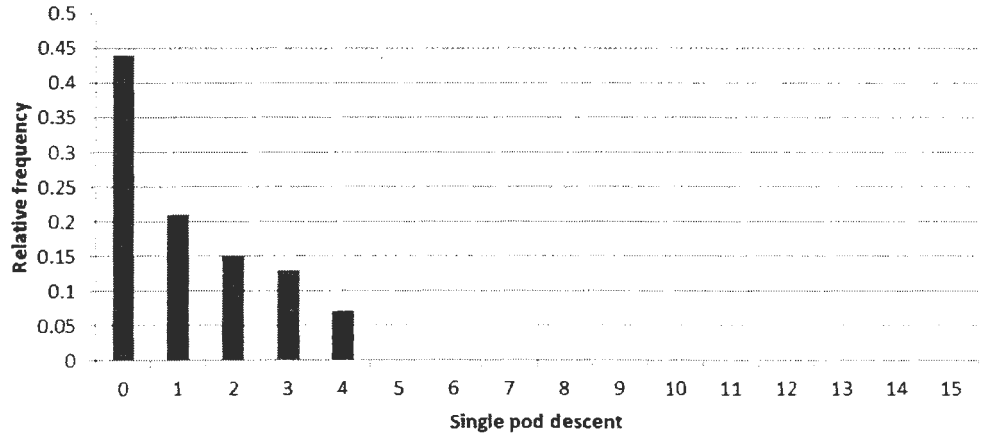
RESULTS AND DISCUSSION

The allelic relative frequencies of each SSR marker for SSD were shown in Table 1. The relative frequencies of the SPD and bulk methods were very similar to those of the SSD for each of the 21 SSR markers (data not shown). The overall mean relative frequency of the SSD, averaged across all 21 markers, was 0.497 which was very close to the expected value of 0.5. This result shows that genotypes were properly scored for SSR bands for all three inbreeding methods. Z-test was used comparing with two proportions to determine whether these are significantly different when the sample size is 30 or large (Freund and Walpole, 1980).

Three bar graphs at the $S_{xy} \geq 0.875$ level of similarity are shown in Fig. 2. The relative frequencies of lines that were not paired with any other line were 49% for the SSD, 44% for the SPD and 39% for the bulk method. Lines that were not paired with any other line were considered to be unique lines. For the SSD method, no line was paired with three other lines (Fig. 2). For the SPD method, some lines were paired with four other lines. For the SSD and SPD methods, no line was paired with five or more other lines. However, for the bulk method, some lines were paired with five or more lines. This result shows that due to genetic sampling, there are more redundant lines for the bulk method than either the SSD or SPD methods.

Table 3 shows the number of paired comparisons at the $S_{xy} \geq 0.75$, and $S_{xy} \geq 0.875$ levels for each of the three breeding method, based on the 21 SSR markers evaluated per line. If the number of lines paired with another line is compared for the same inbreeding method for $S_{xy} \geq 0.75$ versus when $S_{xy} \geq 0.875$, there are more paired comparisons when $S_{xy} \geq 0.75$. For example, for the SSD method, 133 lines were paired





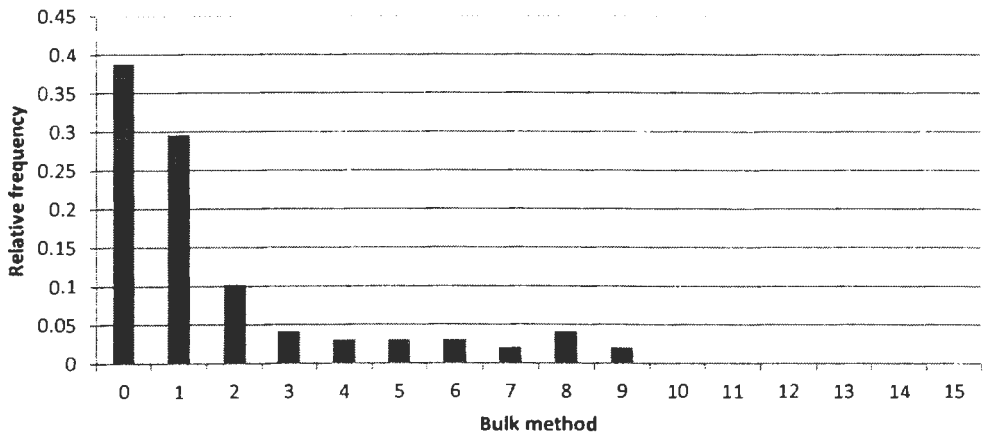


Fig. 2. The relative frequency of the number of paired comparisons per line at the $S_{xy} \geq 0.875$ level of genetic similarity coefficient for three methods of inbreeding.

with another line when $S_{xy} \geq 0.75$, but only 43 lines were paired with another line for that same inbreeding method when $S_{xy} \geq 0.875$ (Table 3). This result shows that more lines had at least 75% or more of their alleles alike in state with another line and fewer lines had at least 87.5% of their alleles that were alike in state with another line within the same inbreeding method. As the similarity coefficient is increased, we would expect fewer lines to be alike in state with any other line. Chance segregation of alleles during meiosis causes lines to be different. For example, if the coefficient of similarity was 99% or greater, then due to chance segregation we would expect very few pairs of lines to have this high a level of similarity.

Table 3 shows that for the SSD method at the $S_{xy} \geq 0.75$ level, zero lines are paired with another line in the identical by descent column. However, for the SSD method there are 133 paired comparisons at the $S_{xy} \geq 0.75$ level in the alike in state column. Redundant lines cannot be descended from a common F_2 or F_3 plant when the SSD method is used, which explains why there are zero paired comparisons under the IBD column for the SSD method. There are 77 pared comparisons for the SPD method at the $S_{xy} \geq 0.75$ level under the IBD column because 210 minus 133, which is equal to 77. The 77 paired comparisons are due to lines descended from the same F_2 plant, which is explained by an identical by descent relationship when either two or three seeds were sampled per pod from an individual F_2 plant. Likewise, the 124 paired comparisons for the bulk method when $S_{xy} \geq 0.75$ are due to identical by descent relationships.

The number of paired comparisons at the $S_{xy} \geq 0.75$ level was greater for the SPD when compared to the SSD (Table 3). The number of paired comparisons at the at 75% similarity level ($P = 0.05$) was greater for the bulk method than the SPD. This result

would be expected because no two lines would be descended from the same F_2 plant for the SSD method. However, more than one line could be descended from the same F_2 plant for the SPD and bulk methods. Only two or three lines can be descended from the same F_2 plant when the SPD method is used because each pod has only two or three seeds. Because each plant has more than three seeds, more than three lines can be descended from the same F_2 plant when the bulk method is used. When we look at the identical by descent column of Table 3, these relationships are evident. A method that results in harvesting more seeds from each F_2 plant produces more paired comparisons, due to an increase in the number of lines that are identical by descent.

At the $S_{xy} \geq 0.875$ level, there was no difference in the number of paired comparisons between the SSD and SPD (Table 3). This result is evident by looking at the identical by

Table 3. The number of pair-wise comparisons at the 75%, and 87.5% similarity levels for each method, using 21 SSR markers per line.

	$S_{xy} \geq 0.75$[#]		$S_{xy} \geq 0.875$[¶¶]	
Methods[†]	IBD[‡]	AIS[††]	IBD	AIS
SSD	0[§]	133	0[‡‡]	43
SPD	77[¶]	210	16[§§]	59
Bulk	124	257	42	85

[†]SSD is the single seed descent method, and SPD is the single pod descent method. 4950 paired-comparisons in the SSD and SPD method, and 4753 paired-comparisons in the bulk method

[‡]IBD is identical by descent.

[§]The number of paired comparisons for the SSD versus the SPD methods was significantly different ($P = 0.01$) when $S_{xy} \geq 0.75$.

[¶]The number of paired comparisons for the SPD versus the bulk method was significantly different ($P = 0.01$) when $S_{xy} \geq 0.75$.

[#]The similarity coefficient is greater than or equal to 0.75.

[††]AIS is alike in state.

[‡‡]The number of paired comparisons for the SSD versus the SPD was not significantly different ($P = 0.05$) when $S_{xy} \geq 0.875$.

[§§]The number of paired comparisons for the SPD versus the bulk method was significantly different ($P = 0.01$) when $S_{xy} \geq 0.875$.

[¶¶]The similarity coefficient is greater than or equal to 0.875.

descent column of Table 3. However, at the $S_{xy} \geq 0.875$ level, there were more paired comparisons for the bulk method than for the SPD. This result shows that there were more redundant lines for the bulk method than for the SPD when $S_{xy} \geq 0.875$, which would be expected due to greater genetic sampling problems for the bulk method.

The number of unique lines was the same for all three methods at both the $S_{xy} < 0.75$ and the $S_{xy} < 0.8\ 75$ levels (Table 4). If the number of unique lines is the criterion to determine which method is the best choice, then all three methods were equally efficient. It is reasonable to expect that the more lines that are unique, the greater would be the chance of identifying a superior line. $S_{xy} < 0.75$ and $S_{xy} < 0.8\ 75$ were values of the expected genetic similarity between different lines for repetitive sampling during inbreeding of multiple seed descent soybean population. If S_{xy} was less than 75%, $F_{4:5}$ derived lines were the result of repetitive sampling from the F_2 generation. If S_{xy} was less than 87.5%, $F_{4:5}$ derived lines were the result of repetitive sampling from the F_3 generation (Keim et al., 1994). In this research, the $S_{xy} < 0.8\ 75$ level was the most appropriate value to compare between different lines because experimental lines were advanced to the $F_{4:5}$ generation.

When two lines have more than 87.5% their alleles alike in state, the breeder would be testing two lines that are very similar. Since the total number of lines that can be tested is a fixed number, an increased proportion of unique lines would be expected to increase the chance that a superior line will be identified. The number of unique lines was the same for all methods, which suggests that all three inbreeding methods would be equally likely to identify a superior line.

Table 4. The number of unique lines for three inbreeding methods, at the 75%, and 87.5% level of genetic similarity.

Method[†]	$S_{xy} < 0.75$[‡]	$S_{xy} < 0.875$[¶]
	Number of unique lines[§]	Number of unique lines[#]
SSD	9	49
SPD	6	44
Bulk	9	38

[†]SSD is the single seed descent method, and SPD is the single pod descent method. 4950 paired-comparisons in the SSD and SPD method, and 4753 paired-comparisons in the bulk method.
[‡]The coefficient of similarity was less than 75% ($S_{xy} < 0.75$) between pairs of inbred lines.
[§]The number of lines that were not paired with another line at the $S_{xy} < 0.75$ level. This was a measure of how many lines were not alike-in-state with any of the other 100 lines, at this level of genetic similarity. The number of unique lines was not different among the three methods when $S_{xy} < 0.75$.
[¶]The coefficient of genetic similarity between pairs of inbred soybean lines was less than 87.5% ($S_{xy} < 0.875$) between pairs of inbred lines.
[#]The number of lines that were not paired with another line at the $S_{xy} < 0.875$ level. This was a measure of how many lines were not alike-in-state with any of the other 100 lines, at this level of genetic similarity. The number of unique lines was the same between the three methods when $S_{xy} < 0.875$.

The experiment reported in this manuscript is different from the results of Keim et al. (1994) because they sampled 260 lines, but only 100 lines were sampled in this experiment. Keim et al (1994) advanced five seeds per plant in the last two stages of the inbreeding process, while in the experiment reported herein only one pod (two or three seeds) per plant were advanced. Despite the slightly different population size and number of seeds advanced per generation, the results reported by Keim et al. (1994) showed an 18% increase in the number of pairs of redundant lines for the SPD above the SSD, while the experiment reported herein showed a 16% increase in the number of pairs of redundant lines at $Sxy \geq 0.875$ level. The results of Keim et al. (1994) were remarkably similar to the results of the experiment reported herein. Keim et al. (1994)

suggested that the SPD would be more practical than the SSD, due to the savings of time and labor during the hand-harvesting process.

Kervella and Fouilloux (1992) found that the extinction rates of inbred lines derived from the F_3 plants were 55.0% at the F_4 level of inbreeding in the SPD method, but 66.7% of the F_4 lines were extinct using the bulk method. Thus, they suggested that the bulk method could eliminate more unique lines than the SPD method. Using a computer simulation approach, they were able to trace each line and determine the number of unique lines that were represented by each method. Muehlbauer et al. (1981) compared the SSD and the bulk method, using a computer simulation. According to their results, 81.5% of initial F_2 plants were represented at the F_6 level of inbreeding for the SSD method. Also, 24.6% of the original F_2 lines were represented at the F_6 level of inbreeding using the bulk method. Because the results reported herein were from an empirical experiment, it is not possible to determine how many F_2 plants are not represented as inbred lines. For this reason it is not possible to compare the results of Kervella and Fouilloux (1992) or the results of Muehlbauer et al. (1981) to the results of this empirical experiment.

There are several different evaluation approaches for the comparison of the SSD, SPD, and bulk methods. Keim et al. (1994) used the number of paired-comparisons at each similarity level, which was an alike in state comparison. The difficulty with using the number of paired lines, at a specified coefficient of similarity level, is that a line can be paired with more than one other line. This makes it difficult to compare methods and interpret the results.

Use of the number of unique lines as criteria is different from the criteria of Keim et al. (1994), Muehlbauer et al. (1981), and Kervella and Fouilloux (1992). Although the research reported herein reported the number of paired comparisons, a second criterion was to compare the number of lines that were not paired with any other line, these were defined as unique lines. None of the other studies in the scientific literature compared the SSD, SPD or bulk methods by comparing the number of unique lines developed, using these methods. Determination of the number of unique lines is an alike in state criterion.

From a practical standpoint, a soybean breeder has a goal of development of improved cultivars. The number of lines that are not alike in state with any other line at the specified level of genetic similarity is the most important factor to evaluate the efficiency of each method. The greater the number of unique lines developed by an inbreeding method, the more likely that an improved line will be identified through phenotypic evaluation. Therefore, the more unique lines there are, the more likely that important genetic difference would exist among those lines. However, in this experiment there was no significant difference in the number of unique lines produced using either the SSD, SPD and bulk methods at the 87.5% or less level of genetic similarity. This suggests that the most efficient inbreeding breeding method would be based on the savings of time and labor when advancing F_2 and F_3 populations to develop the F_4-derived inbred lines.

The bulk method does not require as much time to advance the F_2 or F_3 populations, compared to either the SSD or SPD methods. However, advancing populations using the bulk method requires the use of a small-plot combine. Also, the bulk method eliminates the opportunity of the breeder to select for desirable phenotypes

among F_2 or F_3 plants. The SPD method does not require use of the plot combine and so it does not compete for use of the harvester. The choice of the best method for advancing populations will depend on the specific goals and resources available to each breeder.

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Table 1A. Genetic data for 1-48 lines with 21 SSR markers for the SSD method.

Obs		GA1	GA2	GA3	GA4	GA5	GA6	GA7	GA8	GA9	GA10	GA11	GA12	GA13	GA14	GA15	GA16	GA17	GA18	GA19	GA20	GA21	GA22	GA23	GA24	GA25	GA26	GA27	GA28	GA29	GA30	GA31	GA32	GA33	GA34	GA35	GA36	GA37	GA38	GA39	GA40	GA41	GA42	GA43	GA44	GA45	GA46	GA47	GA48
1	Sat_033	3	3	3	3	3	3	3	3	1	3	1	3	3	1	1	3	3	1	1	1	1	3	2	3	1	1	3	3	1	.	3	3	3	1	1	3	1	2	2	3	3	1	3	3	1	1	1	3
2	Sat_372	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	2	1	3	3	3	1	3	1	1	2	3	3	3	1	1	3	3	1	3	1	1	3	3	2	3	3	1	3	1	3	1	3	3
3	Satt070	2	2	3	3	3	3	2	3	2	3	1	1	3	1	1	3	3	1	1	1	1	3	3	3	1	2	3	3	3	3	3	3	3	1	1	3	1	3	2	1	3	1	3	1	2	2	1	3
4	Satt126	3	3	1	1	1	1	3	3	1	1	1	3	1	1	1	2	3	3	3	3	1	1	1	1	3	3	3	3	3	.	1	1	1	3	3	2	2	1	2	1	1	3	1	1	3	2	3	1
5	Satt135	2	2	2	1	1	1	2	2	1	2	1	1	2	3	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	Satt173	1	1	3	2	2	2	1	3	3	3	1	3	3	3	3	3	1	3	3	3	3	1	2	3	3	1	3	3	3	2	1	1	3	1	2	1	2	2	2	3	3	2	3	3	2	2	2	1
7	Satt198	1	1	1	3	2	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	3	3	1	1	1	1	2	3	3	1	2	3	1	1	3	1	1	3	3	3	3	2	3	1	1
8	Satt254	3	2	3	3	3	3	2	3	2	1	1	3	3	3	3	3	1	1	1	1	3	3	3	2	1	1	1	1	3	3	3	3	3	2	3	1	2	3	2	1	3	3	3	3	3	3	2	3
9	Satt309	1	1	2	2	2	2	1	1	1	1	3	3	2	3	3	2	3	3	3	3	3	3	1	2	3	3	3	1	1	1	1	1	1	1	3	3	3	3	1	3	3	1	3	1	3	1	1	3	3
10	Satt353	3	3	3	3	3	3	3	3	3	3	1	3	3	1	1	3	2	1	1	1	1	3	3	3	3	1	3	3	3	3	1	1	3	2	2	3	3	1	3	3	1	2	1	3	1	1	3	2
11	Satt386	3	3	1	3	3	3	3	1	1	1	3	1	1	2	2	3	1	1	1	1	1	1	1	3	1	1	2	2	3	2	1	1	3	3	1	1	1	1	1	1	3	1	3	2	1	1	3	3
12	Satt424	3	3	1	3	3	3	3	1	3	1	1	1	1	3	3	1	1	1	1	1	1	1	2	1	1	1	3	2	2	1	1	1	1	2	2	1	1	1	2	2	3	3	1	3	1	2	1	3
13	Satt474	2	2	3	3	3	3	2	3	2	3	1	1	3	1	1	3	3	1	1	1	1	2	3	3	1	2	3	3	3	1	3	3	3	1	1	1	1	3	3	1	3	1	3	1	3	2	1	3
14	Satt565	1	1	3	2	2	2	1	2	3	3	3	1	3	3	3	1	3	1	1	1	3	3	3	2	1	1	3	3	1	2	1	1	3	3	1	1	3	1	3	2	3	1	3	1	1	3	3	3
15	Satt589	3	3	1	3	3	3	3	3	1	1	2	1	1	1	1	3	3	2	2	2	3	2	1	1	2	3	2	2	1	1	1	1	3	3	1	1	3	1	2	2	1	1	1	1	1	1	3	1
16	Satt597	3	3	3	1	1	1	3	3	3	3	1	2	3	3	3	1	3	3	3	3	3	3	1	1	2	1	2	3	1	1	2	1	1	1	2	3	3	1	2	2	3	2	3	2	3	3	1	2	1
17	Satt614	2	2	3	1	2	1	2	3	1	3	1	3	3	3	3	3	3	1	1	1	3	1	2	3	1	1	3	3	3	3	1	1	3	3	3	3	3	2	3	2	1	3	1	1	3	1	1	1
18	Satt634	3	3	3	3	3	3	3	3	1	3	2	3	3	3	3	3	2	2	2	2	1	3	2	2	3	2	3	3	3	2	3	3	1	3	1	3	3	3	3	3	3	1	3	2	3	3	3	3
19	Satt643	2	2	1	3	3	3	2	3	3	2	3	3	1	3	3	1	3	1	1	1	3	3	3	3	3	1	1	1	1	2	1	1	2	3	3	3	1	1	2	3	1	3	1	1	1	3	1	3
20	Satt687	3	3	1	3	3	3	3	3	3	2	1	1	1	3	3	3	3	3	3	3	1	2	2	1	1	3	2	2	1	1	3	3	3	1	2	3	3	3	3	3	3	2	3	3	3	3	3	2
21	Satt703	3	3	1	1	1	1	3	1	1	1	1	1	1	3	3	3	1	3	3	3	1	1	1	3	3	3	1	1	1	1	2	2	3	3	1	1	3	2	2	3	1	1	1	1	3	3	3	1

Table 2A. Genetic data for 49-100 lines with 21 SSR markers for the SSD method.

Obs	GA49	GA50	GA51	GA52	GA53	GA54	GA55	GA56	GA57	GA58	GA59	GA60	GA61	GA62	GA63	GA64	GA65	GA66	GA67	GA68	GA69	GA70	GA71	GA72	GA73	GA74	GA75	GA76	GA77	GA78	GA79	GA80	GA81	GA82	GA83	GA84	GA85	GA86	GA87	GA88	GA89	GA90	GA91	GA92	GA93	GA94	GA95	GA96	GA97	GA98	GA99	GA100
1	3	1	3	.	3	.	.	.	3	1	3	1	1	3	3	3	3	1	.	3	1	1	.	3	.	3	1	1	1	3	3	3	3	3	3	3	3	3	3	3	.	3	.	3	3	3	3	.	3	3	3	2
2	1	3	1	3	1	3	3	3	1	1	3	1	3	3	3	3	2	1	1	3	1	1	1	1	3	3	3	2	3	2	3	2	1	1	1	2	2	2	2	2	2	3	3	1	3	2	2	2	3	3	2	2
3	1	1	3	3	1	1	1	1	3	1	3	1	3	2	3	3	3	1	1	2	2	1	1	1	3	3	3	1	1	3	3	3	3	1	1	3	3	2	3	3	2	1	1	3	3	2	1	2	2	1	2	3
4	1	1	3	3	3	1	1	1	1	3	1	1	2	.	2	2	2	1	3	1	1	3	3	1	.	2	3	3	.	1	3	1	1	1	1	1	2	.	1	2	1	1	1	2	1	2	2	1	1	1	1	3
5	1	1	1	1	.	3	3	3	1	1	3	3	2	3	3	2	2	1	1	1	1	1	1	3	3	1	1	3	3	3	3	3	3	1	1	3	3	3	3	3	2	2	3	3	3	3	3	2	2	3	3	3
6	1	2	3	1	2	2	2	3	1	2	1	3	3	2	1	2	3	1	1	1	3	1	1	1	3	1	3	3	2	1	1	1	1	1	1	1	1	3	1	2	1	3	2	2	1	1	3	1	3	3	3	3
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	1	2	3	1	3	3	3	3	3	1	1	1	1	.	3	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3	3	3	1	3	3	3	3	2	3	3	1	3	3	3	1	3	2	2	3	1	3	3
9	3	3	3	3	3	2	2	2	1	1	3	1	2	2	3	1	1	3	1	2	1	1	1	1	3	3	3	1	2	1	3	1	1	2	2	1	2	2	1	1	1	3	3	2	3	2	2	1	1	3	2	1
10	1	3	3	3	3	2	2	2	1	3	3	1	3	2	2	1	1	3	3	2	3	3	3	3	3	2	3	3	2	3	3	3	1	3	3	3	3	1	3	1	1	1	3	1	1	2	1	1	3	1	1	1
11	1	1	1	1	1	3	2	3	3	1	3	3	3	3	3	2	2	3	1	2	1	1	1	1	3	3	3	1	2	3	1	3	1	3	3	3	1	1	3	1	1	3	3	2	1	2	1	1	2	2	1	1
12	1	1	3	3	3	1	1	1	1	1	3	1	2	3	2	3	3	1	1	1	1	1	1	1	1	1	3	1	2	1	2	1	1	3	3	1	2	1	1	3	3	1	1	3	3	1	1	2	1	1	1	3
13	1	1	1	3	1	1	1	1	3	1	3	1	3	2	3	3	3	1	1	2	2	1	1	1	3	3	3	1	1	3	3	3	3	1	1	3	3	2	3	3	2	1	1	3	3	2	2	2	2	1	2	3
14	1	3	3	2	3	1	1	1	1	1	1	1	1	1	1	1	1	3	1	2	3	2	2	3	1	1	1	1	1	3	3	3	1	1	1	3	3	1	3	2	1	3	3	3	1	2	1	1	3	1	1	3
15	3	3	.	.	2	3	3	3	2	3	1	3	2	3	1	3	3	.	.	.	.	1	1	1	1	1	1	2	3	1	2	1	1	1	1	1	1	1	1	2	1	3	3	1	3	1	1	.	2	1	1	1
16	1	1	1	3	1	2	2	2	2	1	3	3	3	1	1	2	2	1	2	2	1	2	2	3	3	1	3	2	2	3	1	3	1	1	1	3	2	2	3	1	1	1	3	1	1	3	2	1	1	3	2	1
17	3	3	3	3	3	3	3	3	3	1	2	1	2	3	3	3	3	2	3	3	1	3	3	1	1	3	2	1	3	3	3	3	2	3	3	3	3	2	3	2	2	3	3	1	1	2	2	2	1	1	2	3
18	3	3	3	3	3	3	3	3	3	3	2	3	1	1	3	2	2	1	1	3	3	1	1	1	2	3	2	1	3	1	2	1	3	3	3	1	2	2	1	3	3	3	3	3	3	1	2	3	3	3	2	2
19	3	1	1	1	1	3	3	3	3	1	1	2	1	2	2	2	2	2	1	2	1	1	1	3	2	2	1	1	3	1	2	1	1	3	3	1	2	2	1	1	1	1	1	3	1	2	2	1	2	1	2	3
20	1	3	1	3	1	2	1	2	3	1	1	1	1	1	1	1	1	1	3	2	1	3	3	2	1	1	3	3	2	3	3	3	3	1	1	3	3	2	3	2	2	1	1	1	1	3	2	2	3	3	2	3
21	3	3	1	3	1	1	3	1	1	1	1	3	3	2	3	3	3	3	1	3	1	1	1	3	1	3	3	1	3	1	3	1	1	3	3	1	3	3	1	3	1	3	3	3	1	1	3	1	1	3	2	3

Table 3A. Genetic data for 1-48 lines with 21 SSR markers for the SPD method.

obs		GBS1	GBS2	GBS3	GBS4	GBS5	GBS6	GBS7	GBS8	GBS9	GBS10	GBS11	GBS12	GBS13	GBS14	GBS15	GBS16	GBS17	GBS18	GBS19	GBS20	GBS21	GBS22	GBS23	GBS24	GBS25	GBS26	GBS27	GBS28	GBS29	GBS30	GBS31	GBS32	GBS33	GBS34	GBS35	GBS36	GBS37	GBS38	GBS39	GBS40	GBS41	GBS42	GBS43	GBS44	GBS45	GBS46	GBS47	GBS48
1	Sat_033	3	3	3	3	3	3	3	1	3	1	3	3	3	1	3	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	2	.	1	1	2	1	3	1
2	Sat_372	3	2	2	2	1	1	1	1	1	3	3	2	1	3	3	1	3	1	1	2	3	3	2	2	2	1	1	1	1	1	2	3	3	1	3	2	2	3	3	1	1	2	3	3	1	3	2	3
3	Satt070	1	3	3	3	3	3	3	3	2	2	1	3	3	3	1	3	1	3	3	3	1	2	3	3	2	3	1	1	2	2	3	1	1	3	3	2	3	3	3	3	1	3	3	3	3	3	3	3
4	Satt126	3	3	3	1	3	3	3	1	3	2	3	1	3	3	3	3	3	1	3	1	3	3	3	2	3	2	3	3	3	3	3	1	3	2	2	2	2	1	1	1	1	3	2	1	2	1	2	1
5	Satt135	2	1	1	1	1	1	1	1	.	3	1	2	1	3	2	2	3	2	1	3	1	1	1	.	3	1	1	1	1	1	1	2	1	2	2	1	3	3	1	2	3	1	1	1	3	1
6	Satt173	3	3	3	3	3	3	3	3	3	1	3	3	3	2	2	3	3	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	2	3	1	3	1	1	.	1	1	2	1	3	1
7	Satt198	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
8	Satt254	3	1	1	3	3	2	2	2	3	3	2	3	1	3	2	1	3	3	1	3	3	1	3	2	3	2	2	3	3	3	3	3	3	3	1	2	2	1	1	3	2	.	2	3	2	1	1	1
9	Satt309	3	3	3	2	2	3	3	3	1	3	1	2	1	3	1	1	3	1	1	2	2	1	2	2	2	1	1	1	1	1	2	3	3	1	2	1	2	3	3	3	3	2	3	2	3	1	3	2
10	Satt353	3	1	1	1	1	1	1	2	1	3	3	1	3	2	3	3	1	3	3	1	3	1	1	1	.	3	1	1	1	1	1	3	3	2	3	3	1	3	1	1	2	1	3	3	1	1	1	3
11	Satt386	1	2	2	2	2	1	1	3	1	2	2	3	1	2	3	1	1	3	1	1	2	3	2	2	1	1	1	1	1	1	3	3	1	3	1	3	3	1	3	3	1	2	3	1	3	1	3	1
12	Satt424	3	2	2	1	1	1	1	3	1	2	2	1	3	1	2	3	3	3	3	1	2	1	1	3	1	3	1	1	1	1	1	3	1	1	1	1	1	1	3	1	1	1	1	1	2	1	3	1
13	Satt474	1	3	3	3	3	3	3	3	3	2	1	.	3	.	1	3	.	3	3	3	1	2	3	3	.	3	1	1	2	2	3	1	1	3	3	3	3	3	3	3	1	.	3	3	3	3	3	3
14	Satt565	2	2	3	3	1	3	1	1	1	3	1	1	1	3	3	3	3	2	3	3	2	3	1	3	3	1	3	3	3	3	2	3	3	3	3	2	2	3	2	3	2	2	1	3	1	3	1	3
15	Satt589	3	3	3	3	3	1	1	1	1	1	3	3	1	1	3	1	3	1	1	3	3	1	3	3	2	1	1	1	1	1	3	1	1	3	1	2	2	1	1	1	3	1	1	1	3	3	1	1
16	Satt597	2	3	3	3	3	3	3	3	3	3	3	3	1	3	3	1	2	1	1	3	3	2	3	3	3	1	3	3	3	3	3	3	3	1	3	2	2	3	1	1	1	3	3	3	1	3	1	3
17	Satt614	2	1	1	3	3	3	3	1	3	3	1	3	3	1	1	3	2	3	3	1	1	1	3	1	2	3	3	3	3	3	3	2	3	3	3	2	2	3	3	3	3	3	3	3	3	1	3	3
18	Satt634	3	3	3	3	3	3	3	3	3	3	2	3	3	3	2	3	3	3	3	3	3	1	3	3	3	2	3	3	3	3	3	3	2	2	3	2	2	3	2	3	3	3	1	3	3	3	1	3
19	Satt643	1	1	1	3	1	2	2	1	3	1	1	3	3	1	1	3	3	1	3	1	2	1	3	1	3	3	3	3	3	3	3	3	1	2	3	1	2	1	1	1	1	3	3	2	1	1	3	1	1
20	Satt687	1	3	3	3	3	1	1	3	1	1	3	3	1	2	3	1	1	3	1	.	2	3	3	3	1	1	1	1	1	1	3	2	3	3	1	2	2	1	1	3	3	3	3	1	3	3	1	1
21	Satt703	3	2	2	1	1	1	1	2	1	1	1	1	3	1	1	3	3	1	3	1	2	1	1	2	2	3	1	1	1	1	1	1	1	1	2	2	3	3	3	3	1	1	3	3	1	3	3

Table 4A. Genetic data for 49-100 lines with 21 SSR markers for the SPD method.

	GB4	GB5	GB5	GB5	GB5	GB5	GB5	GB5	GB5	GB5	GB5	GB6	GB6	GB6	GB6	GB6	GB6	GB6	GB6	GB6	GB6	GB7	GB7	GB7	GB7	GB7	GB7	GB7	GB7	GB7	GB8	GB8	GB8	GB8	GB8	GB8	GB8	GB8	GB8	GB9	GB9	GB9	GB9	GB9	GB9	GB9	GB9	GB9	GB9	GB10
Obs	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0
1	1	3	3	.	3	3	3	.	3	.	2	3	3	1	1	.	.	.	3	3	3	.	.	3	3	.	1	1	1	3	3	3	3	3	3	.	.	.	.	.	3	3	3	3	2	3	1	1	1	3	2	1
2	1	1	3	3	3	1	2	3	3	3	2	1	1	3	3	3	3	3	1	1	1	2	1	1	1	1	3	2	1	1	1	3	3	3	3	3	1	2	3	1	3	3	1	1	2	1	3	3	3	3	1	3	3
3	3	3	3	2	3	1	2	2	3	2	1	1	3	1	1	3	1	1	3	1	1	2	3	1	3	1	3	1	1	1	2	1	1	1	3	2	3	1	1	3	1	1	1	3	3	3	1	1	1	2	1	1
4	1	1	.	.	3	.	3	1	3	1	1	3	3	1	.	3	1	1	3	3	1	.	3	1	.	.	3	2	1	1	1	3	3	.	3	1	3	1	1	1	3	1	1	1	.	1	.	.	.	1	.	.
5	2	2	1	1	1	3	2	1	1	1	2	3	3	1	1	1	1	1	3	3	3	.	1	1	.	.	1	1	1	1	1	1	1	.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	1	3	3	3	3	1	2	1	3	1	1	1	1	2	2	2	3	1	3	1	1	3	3	3	3	1	1	3	3	1	1	3	1	.	3	3	1	3	1	3	2	3	1	3	1	1	1	3	3	3	2	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	3	2	3	3	3	3	3	1	1	3	1	3	1	2	3	3	3	3	3	2	1	3	2	1	1	1	2	2
8	3	2	3	1	3	2	2	2	3	1	3	2	3	3	2	1	2	3	3	2	2	3	3	3	1	3	1	1	3	3	3	1	1	1	3	3	1	3	3	3	1	3	3	3	3	3	3	3	1	1	3	3	2
9	3	1	2	1	2	3	2	3	1	3	3	3	2	3	3	3	3	3	2	3	3	1	3	1	3	1	3	3	1	1	1	2	3	2	2	1	3	1	1	1	1	1	1	2	1	1	1	3	3	1	1	1
10	1	3	2	1	2	3	2	3	2	3	3	3	3	1	1	3	2	1	3	3	3	3	1	1	3	1	1	1	1	3	3	3	1	1	2	1	3	3	3	2	1	3	1	3	2	3	2	3	3	1	2	1
11	3	3	1	1	1	3	2	2	1	3	3	3	3	1	1	1	2	1	3	3	3	3	3	1	3	1	1	1	1	3	3	1	3	3	1	1	1	3	3	2	3	3	1	3	3	3	1	1	1	1	1	1
12	1	3	3	1	3	3	2	1	1	2	3	3	3	1	1	1	1	1	3	3	3	1	3	1	3	1	1	3	1	1	1	3	3	1	3	1	1	1	1	3	1	1	3	1	.	1	1	1	.	1	1	3
13	3	3	3	2	3	1	2	2	3	2	1	1	3	1	1	3	1	1	3	1	1	3	3	1	3	1	3	1	1	1	3	1	1	1	3	3	3	1	1	3	3	3	1	1	1	2	2	1				
14	3	3	3	1	3	3	3	3	3	3	2	1	2	1	1	2	2	2	2	2	3	1	1	1	3	1	3	1	1	3	3	1	3	1	1	1	3	3	1	3	1	1	3	3	1	1	1	3	3	2		
15	1	3	3	1	3	3	3	2	3	3	.	.	3	2	2	1	2	2	3	3	3	3	1	3	3	3	3	3	3	3	3	3	3	.	3	3	1	1	3	2	3	3	3	2	3	3	1	3	3	3	.	3
16	1	1	3	1	3	2	3	1	3	1	1	3	1	1	1	2	1	1	1	3	2	1	1	2	1	2	3	3	2	1	1	2	2	3	3	1	3	1	1	3	3	1	1	3	3	3	3	2	2	3	3	3
17	2	1	3	3	3	1	3	3	3	2	2	3	1	3	3	3	.	3	1	3	1	1	1	3	3	3	3	1	3	3	3	1	3	2	3	3	3	3	3	3	2	3	3	3	3	3	1	1	1	3	2	1
18	3	3	1	3	1	1	1	3	1	3	3	1	2	3	3	3	3	3	1	1	1	1	3	3	1	3	3	1	3	3	3	1	3	1	1	3	3	3	3	2	1	3	3	3	1	3	3	1	1	1	3	3
19	1	3	1	1	2	3	2	2	2	1	2	3	2	3	3	1	.	3	1	3	3	2	1	2	3	3	3	3	3	3	3	3	3	3	3	1	1	1	1	.	1	3	3	1	1	3	1	3	2	2	2	3	3
20	3	2	1	1	1	3	2	3	1	3	3	3	3	1	1	3	2	1	3	3	3	3	1	3	3	1	2	1	3	1	1	1	1	2	1	3	2	1	1	1	1	3	1	3	1	3	1	1	1	3	3
21	3	1	3	3	3	3	3	3	3	3	2	3	1	3	1	3	3	1	1	3	3	1	3	1	1	1	3	2	1	3	3	3	3	1	3	3	1	1	3	3	1	3	3	3	3	2	1	2	2	2	2	1

Table 5A. Genetic data for 1-48 lines with 21 SSR markers for the bulk method.

Obs	Src	G C 1	G C 2	G C 3	G C 4	G C 5	G C 6	G C 7	G C 8	G C 9	G C 10	G C 11	G C 12	G C 13	G C 14	G C 15	G C 16	G C 17	G C 18	G C 19	G C 20	G C 21	G C 22	G C 23	G C 24	G C 25	G C 26	G C 27	G C 28	G C 29	G C 30	G C 31	G C 32	G C 33	G C 34	G C 35	G C 36	G C 37	G C 38	G C 39	G C 40	G C 41	G C 42	G C 43	G C 44	G C 45	G C 46	G C 47	G C 48
1	Sat_033	3	3	3	3	2	3	1	1	3	1	2	1	3	3	2	3	1	2	0	3	3	3	3	1	3	3	1	3	3	1	3	2	3	3	3	.	3	1	2	3	.	1	1	3	.	0	1	3
2	Sat_372	3	3	1	2	2	3	3	3	3	3	3	3	3	2	1	2	3	1	0	2	3	1	2	3	1	3	2	3	3	1	3	1	2	3	3	3	1	3	1	3	3	1	3	3	1	0	3	3
3	Satt070	1	1	3	1	1	1	1	3	3	3	3	3	1	3	3	3	3	1	0	3	3	2	3	3	3	1	1	2	1	1	2	2	3	2	3	3	1	3	1	1	3	1	3	2	3	0	3	1
4	Satt126	3	3	3	3	3	3	1	3	2	1	3	3	3	3	1	3	3	3	0	3	3	3	3	3	1	3	2	3	3	1	2	2	3	1	1	3	1	1	2	3	3	2	2	3	2	0	1	3
5	Satt135	2	1	2	2	1	1	1	2	2	1	2	2	1	1	1	2	1	3	0	1	1	2	1	1	1	3	1	3	1	3	2	3	1	1	1	3	1	1	3	1	3	1	3	1	2	0	1	1
6	Satt173	3	3	3	2	2	3	2	1	3	1	2	1	3	3	2	2	1	2	0	3	3	3	3	3	3	3	3	1	3	3	1	3	3	3	3	3	3	3	1	3	3	1	3	3	3	3	0	3	3
7	Satt198	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
8	Satt254	3	1	1	1	2	1	1	3	3	1	3	2	1	3	1	1	2	3	0	3	3	3	3	3	1	3	3	3	1	3	3	3	3	3	1	3	3	1	3	1	3	3	3	1	3	0	1	3
9	Satt309	3	1	1	1	1	1	3	1	1	3	1	1	1	1	1	1	!	3	0	1	1	1	1	1	2	3	3	1	3	1	1	1	1	3	3	1	3	3	1	3	1	3	1	3	3	0	3	2
10	Satt353	2	3	3	3	3	3	3	3	3	3	3	3	3	3	1	3	3	2	0	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	3	1	3	3	3	3	3	3	1	1	0	3	1
11	Satt386	3	1	1	1	1	1	2	2	1	2	1	3	1	1	3	1	2	1	0	1	1	3	1	2	3	1	1	1	1	1	1	3	1	2	1	1	1	1	3	1	1	1	1	3	3	0	1	2
12	Satt424	3	3	1	3	2	3	1	3	1	3	1	3	3	1	1	3	3	1	0	1	1	1	1	3	1	3	3	3	3	1	1	1	1	1	3	3	3	3	1	3	3	.	1	1	1	0	3	1
13	Satt474	1	1	3	1	1	1	1	3	3	3	3	3	1	3	3	3	3	1	0	3	3	2	3	3	3	1	1	2	1	1	2	2	3	2	3	3	3	3	1	3	2	1	3	2	3	0	3	2
14	Satt565	1	3	3	2	2	3	1	1	2	1	2	1	1	3	1	3	1	3	0	3	3	3	3	1	2	1	3	1	2	3	2	2	3	2	1	3	1	1	1	2	1	3	2	1	3	0	1	2
15	Satt589	1	1	1	2	3	2	3	3	3	1	3	3	1	1	2	3	3	3	0	1	1	3	1	3	2	1	1	3	3	1	3	3	1	2	2	3	1	1	3	3	3	1	3	3	2	0	2	2
16	Satt597	1	3	3	2	2	3	1	3	3	3	1	3	3	3	1	3	3	3	0	3	3	3	3	3	1	1	3	3	1	1	2	3	3	3	3	3	3	3	1	3	3	3	3	1	0	3	3
17	Satt614	3	1	3	2	2	1	3	3	1	3	1	3	1	3	1	3	3	3	0	3	3	3	3	3	3	3	1	3	3	1	1	2	3	1	1	2	1	3	1	3	3	2	1	1	1	0	3	1
18	Satt634	3	3	3	1	3	2	2	2	3	3	2	1	3	3	3	2	1	2	0	3	3	2	2	2	3	2	1	3	3	3	2	1	2	2	3	3	2	3	2	2	2	1	3	3	1	0	2	3
19	Satt643	3	3	1	2	2	3	1	1	1	1	1	1	3	1	1	2	1	1	0	1	1	1	1	1	2	3	1	2	1	1	1	1	1	1	1	3	2	1	1	1	1	1	1	1	1	0	1	2
20	Satt687	3	1	2	3	2	1	3	3	3	3	3	3	3	1	1	3	3	3	3	0	1	1	2	1	3	3	3	3	3	3	3	3	1	1	3	3	3	3	3	1	3	3	3	3	3	1	0	3	1
21	Satt703	3	3	1	2	2	3	3	3	1	3	1	3	3	3	1	1	3	3	1	0	1	1	1	1	3	1	2	1	3	3	3	1	1	1	2	3	3	3	1	1	3	3	1	1	1	1	0	3	2

Table 6A. Genetic data for 49-100 lines with 21 SSR markers for the bulk method.

Obs	G C 4 9	G C 5 0	G C 5 1	G C 5 2	G C 5 3	G C 5 4	G C 5 5	G C 5 6	G C 5 7	G C 5 8	G C 5 9	G C 6 0	G C 6 1	G C 6 2	G C 6 3	G C 6 4	G C 6 5	G C 6 6	G C 6 7	G C 6 8	G C 6 9	G C 7 0	G C 7 1	G C 7 2	G C 7 3	G C 7 4	G C 7 5	G C 7 6	G C 7 7	G C 7 8	G C 7 9	G C 8 0	G C 8 1	G C 8 2	G C 8 3	G C 8 4	G C 8 5	G C 8 6	G C 8 7	G C 8 8	G C 8 9	G C 9 0	G C 9 1	G C 9 2	G C 9 3	G C 9 4	G C 9 5	G C 9 6	G C 9 7	G C 9 8	G C 9 9	G C 1 0 0
1	3	3	3	3	3	3	3	1	3	2	3	3	3	2	2	3	3	3	3	3	1	3	1	1	3	3	3	3	3	2	3	3	3	.	3	3	.	3	3	.	.	.	3	3	3	3	.	3	.	3	3
2	3	1	3	1	1	1	3	3	3	2	3	1	3	1	3	3	3	3	2	3	2	3	2	1	1	3	1	3	1	3	1	1	3	3	1	3	1	3	1	1	3	3	3	3	3	2	3	1	3	3	3	1
3	3	3	3	1	3	1	1	1	1	1	1	1	1	2	1	3	2	1	1	2	3	1	3	3	3	1	1	1	3	1	1	1	3	1	3	3	1	1	3	3	1	2	1	2	1	1	2	2	1	1	2	3
4	1	3	.	.	.	.	1	1	3	.	3	1	2	.	1	.	1	1	1	1	1	1	1	1	3	.	.	.	.	.	1	.	.	.	3	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	3	.	1	3	1	1	2	1	1	1	1	1	1	1	3	2	1	1	1	1	1	1	1	1	3	1	1	1	1	3	3	2	3	1	1	2	3	1	3	1	2	1	3	2	2	2	1	2	1	1	1	1
7	1	1	3	3	3	3	3	2	3	3	3	3	3	3	3	3	1	2	1	1	3	1	3	3	3	3	1	3	3	3	1	3	1	3	3	3	3	3	3	3	3	3	2	3	1	3	3	3	1	3	3	3	3
8	3	3	3	2	3	3	1	1	3	3	3	2	3	2	3	3	1	3	1	2	3	1	3	3	3	3	1	1	1	3	3	3	1	1	1	3	3	3	1	3	3	3	3	3	3	3	3	3	1	3	1	3	3	3
9	3	1	1	3	3	3	3	1	3	1	3	3	3	3	3	3	1	3	3	1	3	1	1	1	3	3	3	3	1	3	3	3	1	3	1	3	3	3	1	3	3	3	2	3	1	3	3	3	2	1	3	3	2	3
10	3	3	3	2	3	2	3	3	3	3	3	1	1	3	3	3	3	3	1	3	3	1	3	3	3	3	2	3	3	1	2	1	3	1	3	1	1	1	3	3	3	1	3	3	2	3	2	3	1	2	3	3
11	1	1	3	3	1	3	1	1	1	1	1	1	1	2	1	3	3	2	2	3	1	1	1	3	3	1	1	1	2	1	1	1	2	1	3	1	1	1	3	3	1	3	1	3	1	3	.	1	1	1	1	3
12	1	1	3	2	1	2	3	1	1	1	1	1	3	3	2	2	1	1	3	1	1	3	1	.	3	3	1	1	3	2	2	2	3	3	2	1	2	3	3	1	1	1	3	3	2	3	2	1	2	1	1	3
13	3	3	2	1	3	1	1	1	1	1	1	1	1	2	3	3	2	1	1	2	3	1	3	3	3	3	1	1	1	3	1	1	1	2	1	3	3	1	1	3	3	1	2	1	3	1	1	2	2	1	1	2	3
14	3	3	1	1	1	1	3	3	1	1	1	1	1	3	1	1	2	2	1	2	3	1	1	1	1	1	2	3	1	3	3	3	1	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1
15	1	1	2	3	3	3	3	3	1	1	1	3	1	3	1	1	1	1	2	3	2	3	1	3	3	3	3	3	1	3	3	3	3	2	1	3	2	3	1	3	1	1	3	1	1	1	3	1	3	1	1	1	3
16	3	3	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	1	2	3	3	3	3	3	3	3	3	3	3	1	2	1	1	1	1	3	1	2	3	1	1	1	1	3	3	3	1	3	3	3	3	3	3
17	1	3	2	3	3	3	3	3	1	3	2	3	3	3	3	3	3	2	3	3	1	3	.	3	3	2	3	3	3	3	1	3	3	3	3	1	3	3	3	.	3	3	3	3	.	3	.	3	.	3	.	2	.
18	3	3	3	3	3	1	3	1	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3	3	1	3	3	3	1	3	1	1	3	3	3	1	3	2	3	3	2	3	3	3	1	2	1	3	3	1	2
19	1	1	1	1	3	1	1	2	3	2	3	1	3	1	3	1	3	3	1	3	1	2	1	3	1	1	1	3	3	3	1	1	1	3	1	1	1	3	3	1	3	1	3	3	3	1	3	1	3	3	2	3
20	3	1	1	2	3	2	2	3	1	3	1	3	1	1	1	2	1	1	1	1	3	1	3	2	1	1	1	1	2	3	2	1	1	1	1	3	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	1	1	3	3	1	3	3	3	1	1	3	1	3	1	2	1	2	1	1	3	3	3	3	3	3	3	1	3	3	3	3	2	1	3	3	3	1	1	1	3	3	1	2	3	1	1	1	1	1	3	1	3	1	1	2	3