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Restriction fragment length polymorphism diversity in soybean

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Summary. Fifty-eight soybean accessions from the genus *Glycine*, subgenus *Soja*, were surveyed with 17 restriction fragment length polymorphism (RFLP) genetic markers to assess the level of molecular diversity and to evaluate the usefulness of previously identified RFLP markers. In general, only low levels of molecular diversity were observed: 2 of the 17 markers exhibited three alleles per locus, whereas all others had only two alleles. Thirty-five percent of the markers had rare alleles present in only 1 or 2 of the 58 accessions. Molecular diversity was least among cultivated soybeans and greatest between accessions of different soybean species such as *Glycine max* (L.) Merr. and *G. soja* Sieb. and Zucc. Principal component analysis was useful in reducing the multidimensional genotype data set and identifying genetic relationships.

Key words: Restriction fragment length polymorphism *Glycine max* – *Glycine soja*

Introduction

Soybean [*Glycine max* (L.) Merr] ($2n=40$ chromosomes) is a crop of major importance in the United States. In spite of intensive study, genetic knowledge of this plant is deficient. This is evidenced by the lack of detailed resolution in the soybean genetic map. Currently, by using morphological characters and isozyme analyses, only 13 linkage groups have been identified, with most consisting of only two or three markers (Palmer and Kilen 1987). Linkage studies in soybean frequently involve crosses in which only a few markers are segregating, making linkage detection inefficient. A better method for establishing linkage relationships is to analyze a single population segregating for a large number of markers. Molecular markers in the form of isozymes and restriction fragment length polymorphisms (RFLPs) are potentially available in large numbers. The soybean genetic map will grow rapidly as these markers are developed and analyzed.

The genus *Glycine* subgenus *Soja* consists of two species, the cultivated soybean, *G. max*, and the wild annual, *G. soja* Sieb. and Zucc. Several groups have reported that soybean accessions in the subgenus *Soja* lack diversity at the DNA sequence level. Doyle and Beachy (1985) found no length heterogeneity and no restriction site polymorphisms for six enzymes in the 18S and 25S rDNA tandem repeat. Doyle (1988) surveyed 33 *G. max* and *G. soja* accessions for variation in the 5S RNA genes and found only a single variant. Quemada (1986) was able to demonstrate 5S gene heterogeneity between two *G. max* plant introductions only by DNA sequencing; no heterogeneity was associated with restriction sites. Apuya et al. (1988) has reported the development of RFLP markers differentiating two *G. max* plant introductions, 'Minsoy' (PI 27890) and 'Noir I' (PI 290136). Again, a lack of diversity was observed, inasmuch as only one single-copy probe in five was polymorphic when screened against DNA digestions with five different restriction enzymes. This low frequency of DNA sequence diversity makes the construction of a genetic map tedious and expensive. Identification of a more diverse population would facilitate the construction of a molecular genetic map in soybean.

Modern U.S. soybean cultivars were derived by hybridization of Plant Introductions. Several authors have noted that relatively few accessions have made large ge-

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netic contributions to the pedigrees of elite cultivars (Delanney et al. 1983; Specht and Williams 1984). They noted that ten accessions contributed 88% of Northern germplasm and that seven lines contributed 70% of the Southern germplasm. Pedigree analysis of elite lines suggests that the DNA sequence diversity among them will be minimal. Identifying cultivars with maximum diversity will allow effective use of RFLP markers in mapping efforts and in breeding programs. We have screened 58 soybean accessions and cultivars, including 8 *Glycine soja*, 2 *"G. gracilis"* Skvortz., and 48 *G. max* plant introductions and cultivars with RFLP markers. (While *"Glycine gracilis"* has been taxonomically joined with *G. max* by Hermann (1962), it is used here to identify morphologically different accessions.) The purpose of this study was to identify RFLP marker diversity such that diverse parents could be used in constructing populations for genetic mapping. In addition, little is known about the allelic structure of soybean RFLP markers. By screening a large number of accessions, the number of alleles and their frequency could be characterized.

Materials and methods

Fifty-eight soybean lines were screened for RFLP diversity (Table 1). Germplasm accessions were chosen on the basis of several criteria, including suitability for future quantitative genetic studies, morphological and isozyme diversity, and their importance in soybean breeding programs. The majority of the accessions were obtained from Dr. R. Bernard, USDA, Urbana/IL. Commercial cultivars were obtained from Dr. W. Fehr, Iowa State University. Two breeding lines, A81-356022 and A80-244036, developed at Iowa State University, were included in this study.

The recombinant DNA clones used as probes in this study were generously provided by Dr. K. G. Lark (University of Utah), with the exception of pK-3, which was isolated from a PstI genomic library (Keim and Shoemaker 1988). Seven of the 17 RFLPs used in this study had been first identified by screening two Plant Introductions, PI 27890 and PI 290136 (Apuya et al. 1988). All other markers were previously unidentified. RFLP markers can be either dominant (presence or absence of a fragment) or codominant (two or more distinct restriction

Table 1. Germplasm accessions surveyed with RFLP markers

Wild and semi-wild accessions

Species	PI No.	Origin	PCA No.
soja	424004A	Korea	1
soja	468904	China	2
soja	468905	China	3
soja	468906	China	4
soja	468918	China	5
soja	342618B	USSR	6
soja	326581	USSR	7
soja	468916	China	8
gracilis	153292	Belgium	9
gracilis	79593	Manchuria	10

Table 1. (continued)

Glycine max accessions

Name	PI. No.	Origin	Type	PCA No.
–	408272B	Korea	PI	11
–	398303	Korea	PI	12
–	407900	Korea	PI	13
Minsoy	27890	France	PI	14
Noir I	290136	Hungary	PI	15
T248	83945-4	Japan	PI	16
–	437477B	USSR	PI	17
–	91732	China	PI	18
Medium Green	(Guelph)	Japan	PI	19
Wilson	19183	Unknown	PI	32
A81-356022	–	Hybrid	C	20
Pride B216	–	Hybrid	C	20
Puckered-1	–	Hybrid	C	20
Stine 3200	–	Hybrid	C	20
Trivalley Charger	–	Hybrid	C	20
Williams 82	–	Hybrid	C	20
Midwest Oil Seed 3010	–	Hybrid	C	20
A80-244036	–	Hybrid	C	21
Altona	–	Hybrid	C	22
Asgrow X25AF	–	Hybrid	C	23
BSR101	–	Hybrid	C	24
Corsoy	–	Hybrid	C	25
Hark	–	Hybrid	C	26
Harper	–	Hybrid	C	27
Hodgson 78	–	Hybrid	C	28
Harosoy	–	Hybrid	C	29
Jilin 3	427099	Hybrid	C	30
Maple Arrow	–	Hybrid	C	31
Aoda	81043	Japan	A	33
Mandarin	36653	China	A	34
Mandarin Ott.	36653	China	A	34
Peking	17852B	China	A	35
Illini	FC30761	China	A	36
Richland	70502-2	China	A	37
–	54610	China	A	38
–	240664	Phil.	A	31
CNS	71569	China	A	49
Palmetto	71587	China	A	40
Dunfield	36846	China	A	41
Seneca	FC03654A	Man.	A	42
Tokyo	8424	Japan	A	43
A.K. Harrow	FC30761	China	A	44
Polysoy	22308	Unknown	A	45
Biloxi	23211	China	A	46
S100	FC30761	China	A	47
Manchu	30593	China	A	48
Mukden	50523Q	China	A	49
Roanoke	71597	China	A	50

PCA No. – Principal Component Analysis No., PI – Plant Introduction, C – Cultivar, A – Ancestral lines to modern cultivars, FC – Forage Crop number, Man. – Manchuria, Phil. – Philippines

Table 2. Frequency of RFLP alleles in soybean accessions

Probe/enzyme		Polymorphic fragment	Cultivars	Ancestors	All *G. max*	*G. gracilis*	*G. soja*	All accessions
NE-10	EcoRI*	10 kb (d)	0.78	0.75	0.80	0.5	1.0	0.81
NE-10	EcoRI*	5 kb (d)	0.11	0.05	0.10	1.0	0.5	0.19
pK-3	HinDIII	7 kb	1.0	1.0	0.96	1.0	0.88	0.95
		6 kb	0	0	0.04	0	0.12	0.05
M224	EcoRI	3.5 kb (d)	1.0	1.0	0.96	0.5	1.0	0.97
M224	EcoRI	1.3 kb (d)	1.0	1.0	0.96	1.0	1.0	0.97
M121	BclI*	6.7 kb	0.17	0.2	0.18	0	0.25	0.19
		4.7 kb	0.83	0.8	0.82	1.0	0.75	0.81
M109	BclI*	5.5 kb	0.83	0.45	0.63	0	0.62	0.60
		2.3 kb	0	0.1	0.06	1.0	0.38	0.14
		0.9 kb	0.17	0.45	0.31	0	0	0.26
pG-15	EcoRI*	8.4 kb	0.89	0.95	0.90	1.0	0	0.77
		4.2 kb	0.11	0	0.08	0	1.0	0.21
		3.5 kb	0	0.05	0.02	0	0	0.02
pG17-3	EcoRI*	9.6 kb	0.22	0.60	0.39	0.5	0.88	0.46
		6.6 kb	0.78	0.40	0.61	0.5	0.12	0.54
pG17-3	EcoRI	4.4 kb	0	0.05	0.04	0	0	0.03
		3.8 kb	1.0	0.95	0.96	1.0	1.0	0.97
pG17-3	HinDIII	3 kb (d)	1.0	0.95	0.96	1.0	1.0	0.97
M69	DraI*	4.3 kb (d)	0.28	0.45	0.43	1.0	0.62	0.47
M69	DraI*	2.5 kb (d)	0.72	0.80	0.73	0	0.62	0.69
M69	DraI*	2.0 kb	0.89	0.60	0.71	1.0	0.50	0.69
		1.9 kb	0.11	0.40	0.29	0	0.50	0.31
M373	EcoRI*	4.2 kb	0.11	0.10	0.12	0.5	0.88	0.24
		3.8 kb	0.89	0.90	0.88	0.5	0.12	0.76
pGly-3	HinDIII*	4.4 kb	0.11	0.45	0.24	0	0	0.21
		3.9 kb	0.89	0.55	0.76	1.0	1.0	0.79
pG21-3	DraI	1.5 kb	0	0	0.02	0	0	0.02
		8.8 kb	1.0	1.0	0.98	0	1.0	0.98

* Indicates the markers used in principal component analysis
(d) designates a dominant RFLP marker (presence or absence of a fragment). Fragments are arranged with their alleles

fragments differing in molecular weight; Apuya et al. 1988). In a survey where all the lines are homozygous, a dominant marker will yield as much information as a codominant marker with two alleles. Both types of markers have been used in this study, and the type is indicated in Table 2.

DNA was isolated from the leaves of greenhouse-grown seedlings (Keim et al. 1988) and digested with the appropriate restriction endonuclease (Table 2). The DNA was then separated by agarose gel electrophoresis (Maniatis et al. 1982) and transferred to nylon membrane (Biotrace RP, Gelman, Ann Arbor/MI) by using a Vacublot apparatus (American Bionetics, Hayward/CA) and 0.4 *N* NaOH as the transfer medium. DNA clones were radioactively labelled with dCTP (alpha P-32) using random primers (Boehringer-Mannheim, Kit No. 1004-760) and by primer extension (Apuya et al. 1988). Radioactive probes were hybridized as described previously (Apuya et al. 1988).

A form of Euclidean genetic distance was calculated between pairs of accessions. In each instance, the number of RFLP loci containing different alleles was divided by the total number of RFLP loci scored to give the frequency of RFLP differences. The average diversity within and between soybean groups represents the average of these pair-wise comparisons. Principal component analysis was done by using PC-SAS (Statistical Analysis System, Cary/NC). Markers with little variation generate only small amounts of information. Hence, the 11 most informative RFLP loci (those in which the predominant allele has a frequency of less than 0.9; Table 2) were used to establish an RFLP genotype for each accession. These genotypes were compared to generate a correlation matrix from which the principal components were derived.

Results

Multiple bands were observed with many probes. These probes can be useful for detecting variation at different

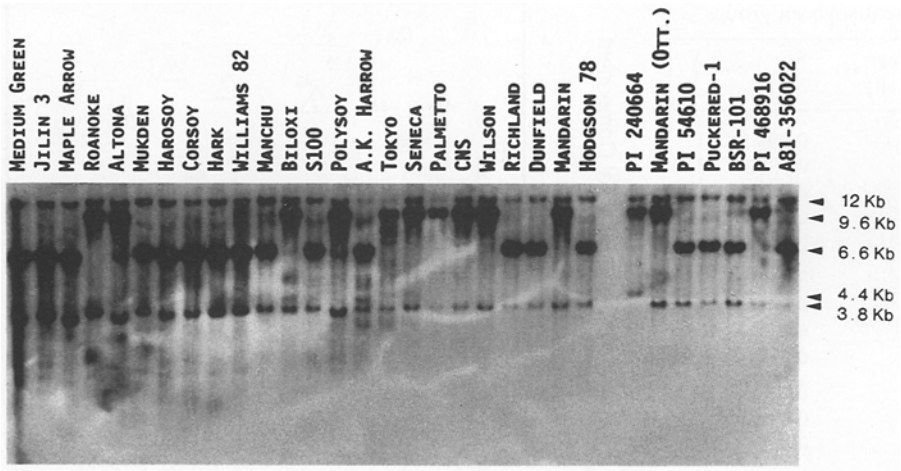

Fig. 1. Autoradiogram of EcoRI-digested DNA probed with pG17-3. DNA samples from 31 different soybean cultivars or accessions were digested with the restriction endonuclease EcoRI and analyzed by the procedure of Southern (Maniatis et al. 1982) with radioactive DNA from the recombinant plasmid pG17-3. DNA fragments of five different molecular weights are observed

loci in the genome (Apuya et al. 1988). Figure 1, e.g., is an autoradiograph where 5 EcoR1 fragments are detected with probe pG17-3. The 6.6- and 9.9-kb fragments were previously described as polymorphic between 'Minsoy' and 'Noir I', whereas the 4.4- and 3.8-kb fragments were not (Apuya et al. 1988). A 12.0-kb fragment was also observed with this probe but was monomorphic in all accessions tested.

The allelic relationship between polymorphic fragments can be inferred from their distribution in homozygous soybean lines. The 9.6- and 6.6-kb fragments in Fig. 1 were never observed together in a single accession, but every accession had one or the other. This type of exclusive relationship implies that the 9.6-kb fragment is allelic with the 6.6-kb fragment. This has been confirmed by genetic segregation analysis (Apuya et al. 1988). Using this criteria of mutual exclusivity, we have assigned tentative allelic relationships between different polymorphic fragments. In 15 of 17 loci, only two alleles were observed in these accessions (Table 2). Two probes, M109 and pG8-15, had patterns consistent with three alleles at one locus. The 3.5-kb allele of pG8-15 was very rare and was only observed in one accession ('Tokyo'), whereas the M109 2.3-kb allele was present in 14% of the accessions (Table 2).

The rarity of multiple alleles is consistent with low levels of genetic diversity. Additional evidence of this is the presence of a predominant allele at some loci. Six of the RFLP loci surveyed had a particular allele present in 95% or more of the accessions (Table 2: pK-3, M224 EcoRI 3.5 kb, M224 EcoRI 1.3 kb, pG17-3 EcoRI 3.8 kb, pG17-3 HinDIII 3 kb, and pG21-3 DraI 0.8 kb). Such RFLP loci generate very little information for distinguishing between accessions. In future studies, these markers would be useful in only a few populations. However, other RFLPs proved more informative. The most informative locus in this survey was the M109 RFLP in which there were three alleles with a frequency of 0.60, 0.26, and 0.14 (Table 2). Such a probe would be useful in many populations and, therefore, valuable for future studies.

For some RFLP loci, only slight variation in allele frequency was seen between different groups of soybean. For example, the M121 alleles have a ratio of 19:81 for all accessions, and except for the two *"G. gracilis"* accessions, all groups were within 6% of this ratio (Table 2). In contrast, the frequencies of the EcoRI 9.6 and 6.6-kb fragments detected by pG17-3 differed by nearly 40% between ancestral accessions and cultivated lines (Table 2). The 9.6-kb allele (or genes linked to it) could have been selected against (or for the 6.6-kb allele) as cultivars have been derived from the original introductions. Alternatively, the frequencies in allele frequencies were observed when *G. max* accessions were compared with *G. soja* accessions. In four cases, there were major shifts in allele frequencies (M109, pG8-15, pG17-3 EcoRI, M373) (Table 2). Most dramatic was the pG8-15 marker in which the 8.4-kb allele was present in 90% of the *G. max* accessions but absent from all *G. soja* accessions tested. Changes in allele frequency should accompany species differentiation, and in other genera, the differences are even greater (Figdore et al. 1988).

In this study, 58 accessions were screened and genetic distances were calculated (see Materials and methods) for all possible combinations (1653) of the accessions. The average of these comparisons between and within soybean groups suggests where genetic diversity exists in soybeans. As expected, the greatest average RFLP diversity was found between species (Table 3). *Glycine soja* and *"G. gracilis"* were also diverse (35%). RFLP diversity within groups was consistently less than between groups. The lowest within-group diversity was for the cultivated varieties of *G. max* (16%). Seven of these lines (A81-356022, Pride B216, Trivalley Charger, Stine 3200,

Table 3. RFLP diversity between different soybean groups

Comparison		Average diversity	SE	*N*
Cultivars	× Cultivars	0.16	0.008	171
	× Ancestors	0.23	0.005	360
	× *G. max* P.I.	0.30	0.008	162
	× *G. gracilis*	0.34	0.014	36
	× *G. soja*	0.33	0.008	144
Ancestors	× Ancestors	0.26	0.007	190
	× *G. max* P.I.	0.32	0.020	200
	× *G. gracilis*	0.39	0.016	40
	× *G. soja*	0.36	0.007	160
G. max P.I.	× *G. max* P.I.	0.37	0.020	45
	× *G. gracilis*	0.36	0.020	20
	× *G. soja*	0.36	0.010	80
G. soja	× *G. soja*	0.22	0.017	28
	× *G. gracilis*	0.35	0.090	16
G. gracilis	× *G. gracilis*	0.24	–	1
All comparisons		0.28	0.007	1,653

SE – Standard Error – Standard Deviation/$\sqrt{N}$
N – The number of comparisons between individuals of different groups

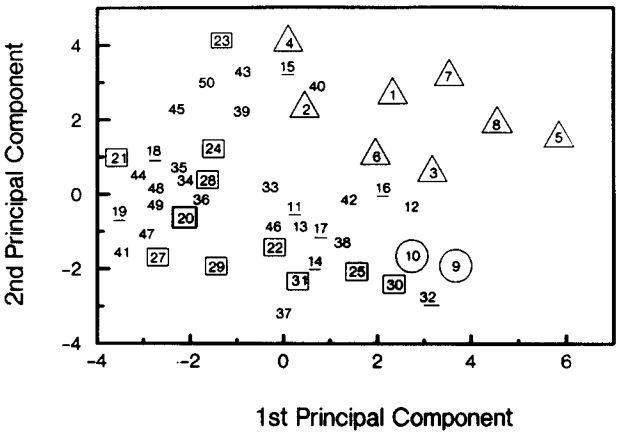

Fig. 2. Principal component analysis of survey genotype data. The first two principal components were calculated (see text) and plotted. The number plotted represent individual accessions and correspond to the numbers listed in Table 1. Different groups of accessions can be identified by the symbols associated with the numbers: *G. soja* accessions are marked with *triangles*, "*G. gracilis*" accessions are *circled*, *G. max* cultivars are *boxed*, and *G. max* Plant Introductions are *underlined*. Ancestral lines to the cultivars have no additional markings. The first and second principal components accounted for 20% and 16% of the variance, respectively

Midwest Oil Seed 3010, Puckered-1, and Williams 82) were identical at all 17 RFLP loci. The greatest diversity within a group was for *G. max* Plant Introductions (37%).

A genetic distance matrix is a multidimensional data set, which can be so overpowering in size (1653 data points in this case), that it makes important relationships among accessions difficult to identify. Principal component analysis was used to identify multidimensional relationships that account for large portions of the genetic variance in a data set. Figure 2 represents the first two principal components of the genotype data that account for 36% (20% and 16%) of the total variance. Note that all *G. soja* accessions have high principal component scores and dominate the upper right quadrant of the graph. The two *"G. gracilis"* accessions are closely associated and have high values on the first principal component. Separation of the *G. soja* accessions from the *"G. gracilis"* accessions can be made on the basis of the second principal component score, although these two species are similar on the first principal component axis. Cultivars ranged from very high to very low values on both principal components, but none simultaneously had high values in both, which clearly separated them from *G. soja*. Most cultivated lines had values of less than 1 in both components, placing them in the lower left quadrant of the graph. The seven identical cultivars (number 20, Fig. 2) were in this area of the graph. Many of the important ancestral lines of U.S. cultivars are also found in this portion of Fig. 2. An affinity between cultivars and their ancestors would be expected. One cultivar ('Jilin 3') was separated from the others by high values on the first principal component. This cultivar is the result of a breeding program in China and was developed independently of U.S. cultivars. All other cultivars in this survey are somewhat related by pedigree. 'Mandarin' and 'Mandarin (Ott.)' did not have identical RFLP genotypes, but were similar enough to receive the same principal component scores (number 34, Fig. 2). Asgrow X25AF had the highest value on the second principal component, which separated it from the other cultivars. Its pedigree was not unusual for U.S. cultivars, but it does have the unique feature of containing a putative transposable element (Groose et al. 1988). Outlying accessions such as the PI 468916, Asgrow AX25AF, and PI 153292 tended to have high RFLP diversity scores compared with other accessions (dat not presented).

Discussion

We have found in a survey of 58 genotypes that most soybean RFLP loci have only two alleles per locus and that for some loci, one allele was very rare. This is consistent with a previous study (Griffin 1986) involving ca. 1400 accessions and 16 isozyme loci in which multiple alleles were rare. Forty-five of the accessions evaluated in this study were included in Griffin's study, making RFLP/isozyme comparisons possible. Isozyme and RFLP allele frequencies are comparable (data not pre-

sented), inasmuch as only 2 of 16 isozyme loci had three alleles (*Aco*4 and *Ap*) in these 45 accessions. In both cases, the third allele was rare, having a frequency of less than 5%. Molecular diversity, as detected by both these types of markers, is present only at low levels in soybean.

The reasons that soybean contain low molecular diversity may be two-fold. First, as a self-pollinated crop, individuals are highly homozygous. Consequently, all deleterious mutations that would contribute to molecular diversity are eliminated by selection. In contrast, these same types of changes can be more easily tolerated in open-pollinated plants. Second, many of the cultivated soybeans in the United States are derived from a common set of parents (Delanney et al. 1983; Specht and Williams 1984). Common ancestry decreases diversity in cultivars, which is illustrated by the seven cultivars exhibiting identical genotypes for 17 RFLP loci.

RFLP markers were grouped into two types: informative and uninformative. Informative markers have alleles with near equal distributions and become more informative with increasing numbers of alleles (e.g., M109). Uninformative markers have only two alleles, one of which is very rare (e.g., pG21-3). The RFLP markers identified in a previous study (Apuya et al. 1988) proved to be of both types in this survey. The informative markers identified in this study will be increasingly useful for future studies in new populations.,

Roth et al. (1988) have suggested that some molecular diversity in soybeans results from *specific* and frequently employed DNA recombination events. Identical alleles would be created independently in different lineages. Presumably, certain genomic locations would be more susceptible to this type of molecular variation and, thus, vary more frequently, creating informative markers. A second hypothesis revolves around the evolutionary history of soybeans. If the common ancestor of the subgenus *Soja* was in the recent evolutionary past, soybean accessions in this study may have been derived from a recent common population. In such a case, informative markers would have been variant in that ancestral population, whereas the rare variants (uninformative loci) would have arisen more recently in individual lines. The low level of soybean outcrossing would prevent the spread of variation among different populations or lines.

Soybean RFLP mapping has lagged behind that in other crop species largely because of a lack of molecular diversity (Apuya et al. 1988). Identification of a cross containing a greater frequency of RFLPs would enhance the construction of the first complete soybean genetic map. One genotype, potentially important in breeding programs, was selected from cultivated soybeans. The seven identical genotypes represent the most agronomically important germplasm from this study. The second parent should be as diverse as possible in both RFLP markers and agronomic traits, to facilitate the correlation of markers with quantitative trait loci. As shown in Fig. 2, the *G. soja* accessions have the most marker diversity from the "typical" cultivar (number 20). In addition, *G. soja* accessions differ greatly from cultivated *G. max* in many agronomic traits. A cross between an elite cultivar and a *G. soja* accession, therefore, offers the most RFLP diversity for genetic mapping. Such populations have been constructed and previously characterized for agronomically important quantitative traits that are very diverse (Carpenter and Fehr 1986; Cianzio and Fehr 1987; Graef 1988). We have recently screened the *G. max* breeding line A81-356022 and *G. soja* PI 468916 with more than 100 random single-copy probes and found a high level of polymorphism. Forty to fifty percent of these probes were polymorphic, which is ca. two-fold higher than in the Minsoy × Noir I population (Apuya et al. 1988). This diversity is facilitating current genetic mapping and quantitative genetic studies.

The low levels of diversity in many of the soybean accessions and cultivars could slow RFLP applications in breeding unless more informative markers are found. In the future, it will be essential to identify agronomically elite populations that contain sufficient levels of diversity before large-scale studies are undertaken. Researchers aware of this should be able to effectively use RFLP markers in their breeding programs. As Fig. 2 illustrates, some cultivated soybeans are diverse in RFLPs (e.g. Asgrow X25AF versus Jilin 3) and may, therefore, be useful. Identification of diverse cultivars with RFLP markers could encourage their use in breeding, thereby broadening the genetic base and leading to greater genetic gains.

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