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Restriction fragment length polymorphisms as genetic markers in soybean, *Glycine max* (L.) merrill

N. R. Apuya, B. L. Frazier, P. Keim, E. Jill Roth and K. G. Lark
University of Utah, Department of Biology, Salt Lake City, UT 84112, USA

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Summary. Restriction Fragment Length Polymorphisms (RFLP) have been identified between widely distant cultivars ('Minsoy' and 'Noir 1') of soybean *Glycine max* (L.) Merrill. Using as probes randomly chosen clones of DNA, one in five probes revealed a polymorphism. More than half of these polymorphisms appear to result from rearrangements of the genomic DNA. Twenty seven markers were analyzed for linkage in F_2 plants. Eleven of these markers were contained in four linkage groups. Five cultivars were compared in a search for new alleles. When RFLP markers corresponding to low copy DNA were used to analyze three other cultivars – 'Sooty', 'Forrest' and 'Mandarin (Ottawa)' – few new alleles were found. Using these probes, five different markers could be used to differentiate the five cultivars. Complex probes, which correspond to repeated DNA, revealed different polymorphisms in different cultivars and a single such probe could be used to distinguish the five cultivars from each other.

Key words: Soybean – Restriction fragment length polymorphism – Genetics – Allele – Variation

Introduction

Soybean (*Glycine max* (L.) Merrill) is a self-fertilizing, inbreeding plant. Because of this, it may be expected that cultivars which are able to grow in diverse climates and habitats also would have diverged genetically. It is not surprising that a broad range of climate and photoperiod adaptations (maturity groups) exist able to grow in tropical or in northern temperate locations. Plants have been collected from China (where soybean has been under cultivation for more than 3,000 years) as well as Korea, Japan and a number of other countries (Hymowitz and Kaizuma 1981) and more than 3,000 cultivars currently are available in the germplasm collection of the USDA.

Despite breeding programs using this extensive germplasm, relatively little genetic information is available for this important crop plant. *G. max* has 40 chromosomes ($n=20$) on which only 17 linkage groups have been located (Palmer and Kilen 1987,. R.G. Palmer, personal communication). Clearly, using conventional technology the process of constructing a genetic map has been slow. Although evolutionary studies of *Glycine max* are available (Hymowitz 1970, 1984; Hymowitz et al. 1977; Hymowitz and Newell 1980, 1981) the limited amount of genetic information has made it difficult to compare the evolutionary history of individual plant introductions.

Crosses between soybean plants are difficult to carry out because the flower opens after pollination. (Each cross involves dissection of the flowers participating in the cross.) Plants of different maturity groups must be scheduled to mature in such a way that cross pollination is possible. Although techniques are available for carrying out genetic studies of soybean cells in tissue culture (Roth et al. 1985), few genetic markers are available which can be used in both intact plants and in cell culture (Roth and Lark 1984).

We have investigated the use of Restriction Fragment Length Polymorphisms (RFLP) as markers for use in soybean genetics. RFLP markers represent changes in the length of the fragments of genomic DNA produced by digestion with specific restriction enzymes. Restriction fragment length polymorphisms can furnish a large set of genetic markers which can be arranged into a genetic map using a single cross (F_1) and its segregating progeny (F_2). Thus, these molecular markers furnish an ideal approach to study a plant in which crosses are difficult to carry out. These markers are co-dominant, have full penetrance (since they analyze the DNA direct-

ly) and are, in principle, unlimited in number. They have been used successfully to map human chromosomes (Feder et al. 1985) and more recently to construct genetic maps of plants such as maize, tomato and lettuce (Helentjaris et al. 1985, 1986a, b; Bernatzky and Tanksley 1986a, b, c; Vallejos et al. 1986; Landry et al. 1987). In animal cells, chromosome loss in tissue culture (e.g., from human-mouse cell hybrids) has provided a rapid means of assigning markers to specific chromosomes (Weiss and Green 1967; Kao et al. 1982; Neve et al. 1983). In a similar manner, RFLP markers can be used in conjunction with plant cell culture technology (Roth and Lark 1984; Roth et al. 1985) to rapidly assign loci to specific chromosomes.

In this paper we describe RFLP markers for *Glycine max* and show that they will be useful in the construction of a genetic map and as markers in breeding programs. Our results suggest a conservation of the soybean genome and that a large part of the polymorphisms which exist may be due to genomic rearrangement.

Materials and methods

Plant material

The soybean cultivars, 'Minsoy' (PI 27,890), 'Noir 1' (PI 290,136), 'Sooty', 'Forrest', and 'Mandarin (Ottawa)' were used in these studies. Seed of soybean cultivars 'Minsoy', 'Noir 1', and an F_1 sexual hybrid between 'Minsoy' and 'Noir 1' were obtained from Dr. Reid Palmer, Iowa State University, Ames, Iowa. Seed of soybean cultivars 'Sooty' and 'Forrest' were obtained from Dr. Lila Vodkin, University of Illinois, Urbana, Illinois. Seed of the soybean cultivar 'Mandarin' was obtained from the Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada. Plants were grown in a greenhouse in a standard potting soil mixture at temperatures of 21°–30 °C during the day and 10°–15 °C at night. Daylengths were 14 h of light, 10 h of darkness. F_1 hybrids were allowed to set seed and the resulting F_2 seed was germinated. F_2 plants were grown in hydroponics in a modified Hoagland nutrient solution (Leggett and Frere 1971).

Tissue culture cell lines from 'Minsoy', 'Noir 1' and the F_1 hybrid were produced from the stems and leaves of sterile seedlings. Soybean seeds were sterilized 2 h in chlorine gas in a glass desiccator. The seeds were germinated in sterile glass jars on filter papers saturated with water. Two weeks after germination, leaves and stems were cut into sections and placed in petri dishes on filter papers saturated with B5C medium (Gamborg et al. 1968) containing 0.2% casein hydrolysate (ICN Pharmaceuticals Inc., Cleveland, Ohio), 0.4 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), 3.0 mg/l napthaleneacetic acid (NAA), and 0,2 mg/l kinetin. Callus formed after two weeks. The callus was then put into suspension in B5C medium containing 1 mg/l 2,4-D. Cell lines were maintained in this medium.

Enzymes and chemicals

Restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersberg, Maryland) and New England Biolabs (Beverly, Massachusetts). Radioisotopes and *E. coli* DNA polymerase I used in nick translations were purchased from New England Nuclear (Boston, Massachusetts). DNase I, pancreatic RNase, agarose, 1,10 phenanthroline, EDTA, salmon sperm DNA, dextran sulfate, IPTG and sodium dodecyl sulfate were purchased from SIGMA Chemical Co. (St. Louis, Missouir). Proteinase K was purchased from Boehringer Mannheim (Indianapolis, Indiana). Formamide, large subunit of DNA polymerase I (Klenow fragment), M13 15bp sequencing primer, and X-gal were purchased from Bethesda Research Laboratories.

DNA isolation

DNA from leaves, roots, stems, and tissue culture lines was isolated by a modification of the procedure of Dellaporta et al. (1983). The material was ground with a mortar and pestle to a fine powder in liquid nitrogen. The frozen powder was quickly added to extraction buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 150 mM NaCl, 1% SDS, 5 mM 1,10 phenanthroline, and 50 µg/ml proteinase K) at 65 °C. Approximately 10 ml of extraction buffer was used per gram of tissue. The mixture was maintained at 65 °C for 30–60 min. One-fifth volume of 5 *M* potassium acetate was added and the mixture was placed on ice for 30 min. The mixture was centrifuged at 3,000 × g for 20 min. DNA was precipitated from the supernatant with 0.54 volumes of isopropanol. The DNA was purified by phenol extractions and equilibrium centrifugation in cesium chloride.

Preparation of M13 probes

DNA from the leaves of an F_1 hybrid plant was digested to completion with *Sau*3AI and ligated into the RF form of M13 (MP9, Bethesda Research Laboratories) which had been digested with *Bam*HI. The ligation mixture was transformed into DH5α competent cells (Hanahan 1985) and then plated onto the indicator strain UT580 (K12 F+ F' traD36 proAB lacIqZ m15/hsp r-m+k (prolac) ara Tn10zji) on plates containing IPTG and X-gal. Colorless plaques (those containing inserts) were picked into wells of microtiter dishes containing 200 µl L-broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract). These were incubated overnight at 37 °C. To differentiate single copy clones from high copy clones, the clones were probed with radioactive total soybean cell DNA (see lambda clones below). Following washing (see below) the membranes were placed on XAR-1 film (Kodak) with intensifying screens (Dupont Cronex Lightning-Plus) for two weeks. Clones were picked that gave very little or no signal. The clones were further characterized for insert size by running 20 µl of M13 phage from each microtiter well on an 0.8% agarose gel along with M13 of known size. M13 clones containing inserts of greater than 300 bp were selected.

Preparation of M13 template DNA

Single stranded M13 DNA was prepared by the method described in the Bethesda Research Laboratories M13 cloning/dideoxy sequencing instruction manual (see also Sanger et al. 1980). The M13 was grown on the bacterial strain UT580.

Radioactive labeling of M13 templates

M13 templates were labeled with α-^{32}P dCTP using a M13 15 bp sequencing primer (Messing 1983). Five µl of the 50 µl template preparation was used for each labeling reaction. The unincorporated radioactive triphosphate was separated from the reaction mixture using Sephadex G-50 spin columns (Maniatis et al. 1982). Yields were greater than 10^7 DPM per reaction (specific activity 10^8 to 10^9 DPM per µg of insert). The labeled templates were denatured in 50% formamide at 80 °C for 15 min.

Preparation of lambda probes

A lambda library of the soybean cultivar 'Forrest' was provided by Dr. Robert Goldberg. The average insert size of this library was 15–20 kb. To differentiate clones of single copy DNA from clones of repeated DNA, the clones were probed with radioactive soybean genomic DNA. To do this, phage particles were transferred to nitrocellulose membranes by the technique of Benton and Davis (1977), these nitrocellulose membranes were then hybridized with nick translated total soybean DNA. Following washing (see below) the membranes were placed on Kodak SB5 film for 2–3 weeks. A range of intensities of signals was observed, indicative of the relative copy number of the clones in the soybean DNA. Clones were picked that gave very little or no signal. Lambda clones G-8, G-17, and G-21 were digested with *Eco*RI and subcloned into the *Eco*RI site of pUC9 (Frazier 1987).

Other lambda clones

Lambda clone DA28-30 was a gift of Dr. Robert Goldberg, University of California, Los Angeles, California. This clone contains the coding region for glycinin genes G1 and G2 on a 15.4 kb *Eco*RI fragment in lambda phage Charon 4A (Fisher and Goldberg 1982). Subclone pGly 3 is a 5.3 kb *Eco*RI fragment from this lambda clone, cloned into pUC9 (Frazier 1987). Plasmid clone pJCX-1, a 3.0 kb *Xba*I fragment containing the coding region for the lectin gene 1 in the vector pUC12 was a gift of Dr. Lila Vodkin (Goldberg et al. 1983). The plasmid clone pS-5H1.3 (TGM) was also obtained from Dr. Lila Vodkin (Rhoades and Vodkin 1985).

Radioactive labeling of soybean, lambda and plasmid DNA

Soybean DNA and plasmid DNA were labeled with α-^{32}P using the nick translation procedure of Rigby et al. (1977). The unincorporated radioactive triphosphate was removed by washing the DNA three times with 80% ethanol. The labeled DNA was denatured in 50% formamide at 80 °C for 15 min.

DNA restriction analysis and Southern transfers

Plant DNA was digested with restriction enzymes according to the manufacturer's specifications. Digestion was carried out overnight (12–16 h) using 25 units of enzyme per 5 µg of DNA. DNA fragments were resolved by gel electrophoresis through 1% gels, 5 µg of DNA per lane. TPE (80 mM Trisphosphate pH 7.8, 8 mM EDTA) was used as the running buffer. The DNA fragments were either transferred to nylon membranes or nitrocellulose.

The method of Reed and Mann (1985) was used to transfer DNA to nylon membranes. The gels were treated with 0.25N HCl for 6 min and 0.4N NaOH for 5 min and then transferred to nylon membranes (Biotrace RP, Gelman Science, Inc.) using 0.4N NaOH. Membranes were rinsed in 2X SSC (Maniatis et al. 1982) and dried at room temperature. The membranes were prewashed for 1 h in 1 *M* NaCl, 0.5% SDS, 50 mM Tris pH 8.0, 1 mM EDTA and then pre-hybridized 12–16 h at 42 °C in 10–20 ml of 50% formamide, 5X SSC, 100 µg/ml denatured salmon sperm DNA, 50 mM phosphate buffer pH 6.5, 5X Denhardt's buffer, 1% SDS, 2.5% dextran sulfate. These membranes were hybridized 12–16 h at 42 °C in 5 ml of 50% formamide, 5X SSC, 100 denatured µg/ml salmon sperm DNA, 20 mM phosphate buffer pH 6.5, 1X Denhardt's buffer, 1% SDS, 5% dextran sulfate and labeled probe. After this they were washed 1 h in 2X SSC, 0.5% SDS at 42 °C, 1 h in 2X SSC, 0.5% SDS at 65 °C, 1 h 0.2X SSC, 0.2% SDS at 65 °C, 1 h in 0.2X SSC, 0.1% SDS at 65 °C, and 1 h in 0.2X SSC, 0.1% SDS. Autoradiographs were prepared by exposing the nylon membranes to Kodak SB5 X-ray film with intensifying screens (Dupont Cronex Lightning-Plus) at −70 °C. The nylon membranes were repeatedly stripped and reprobed. To strip the probe, the membranes were soaked in 0.4N NaOH for 30 min at 42 °C, and then neutralized for 15–20 min at room temperature in 0.2M Tris-HCl pH 7.5, 0.1X SSC, 0.5% SDS.

The method of Southern (1975) was used to transfer DNA to nitrocellulose. Hybridization was the same as with the nylon membranes.

Mapping of lambda clones G-17 and G-21

DNA from the lambda clones G-17 and G-21 were digested with different restriction enzymes. Single and double digests of the following restriction enzymes were used: for G-17, *Bam*HI, *Eco*RI, *Hind*III, and *Sst*I; for G-21, *Bam*HI, *Bgl*III, *Eco*RI, *Dra*I, *Hind*III, and *Pst*I. DNA fragments were separated by gel electrophoresis and fragments were transferred to nylon membranes as described. These membranes were repeatedly probed with each of several subclones; six subclones for G-17 and four subclones for G-21. Subclones were labeled by nick translation. The order of the map was determined by which subclones had fragments in common.

Linkage analysis of F_2 segregation

Linkage between RFLP markers was determined by the method of maximum likelihood, using segregation in F_2 plants and a computer program, Linkage-1 (Suiter et al. 1983). The program was the generous gift of Dr. Karl Suiter.

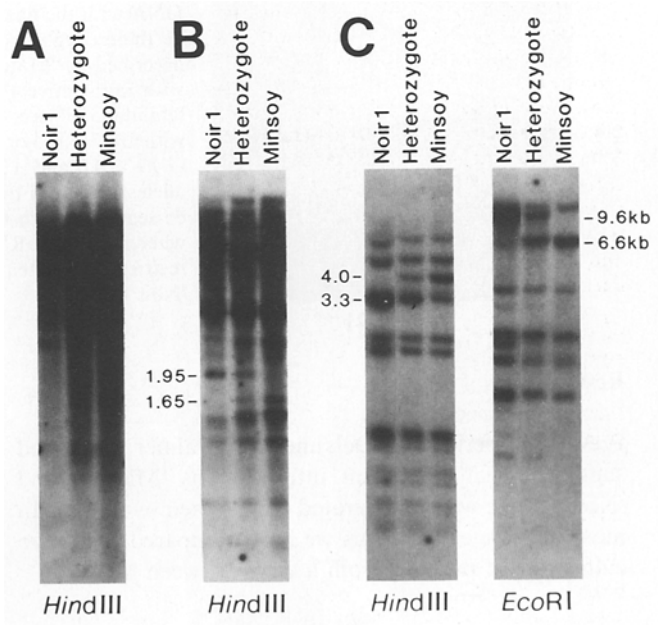

Fig. 1 A–C. Restriction fragments from digests of soybean genomic DNA, hybridized with segments of DNA cloned into phage lambda. Soybean genomic DNA was isolated from 'Minsoy', 'Noir 1' or F_1 hybrid plants and digested with the restriction enzymes shown. The fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes. These Southern transfers were hybridized with radioactive DNA: **A** λG-7; **B** λG-26; **C** λG-17. An analysis of the results is given in the text

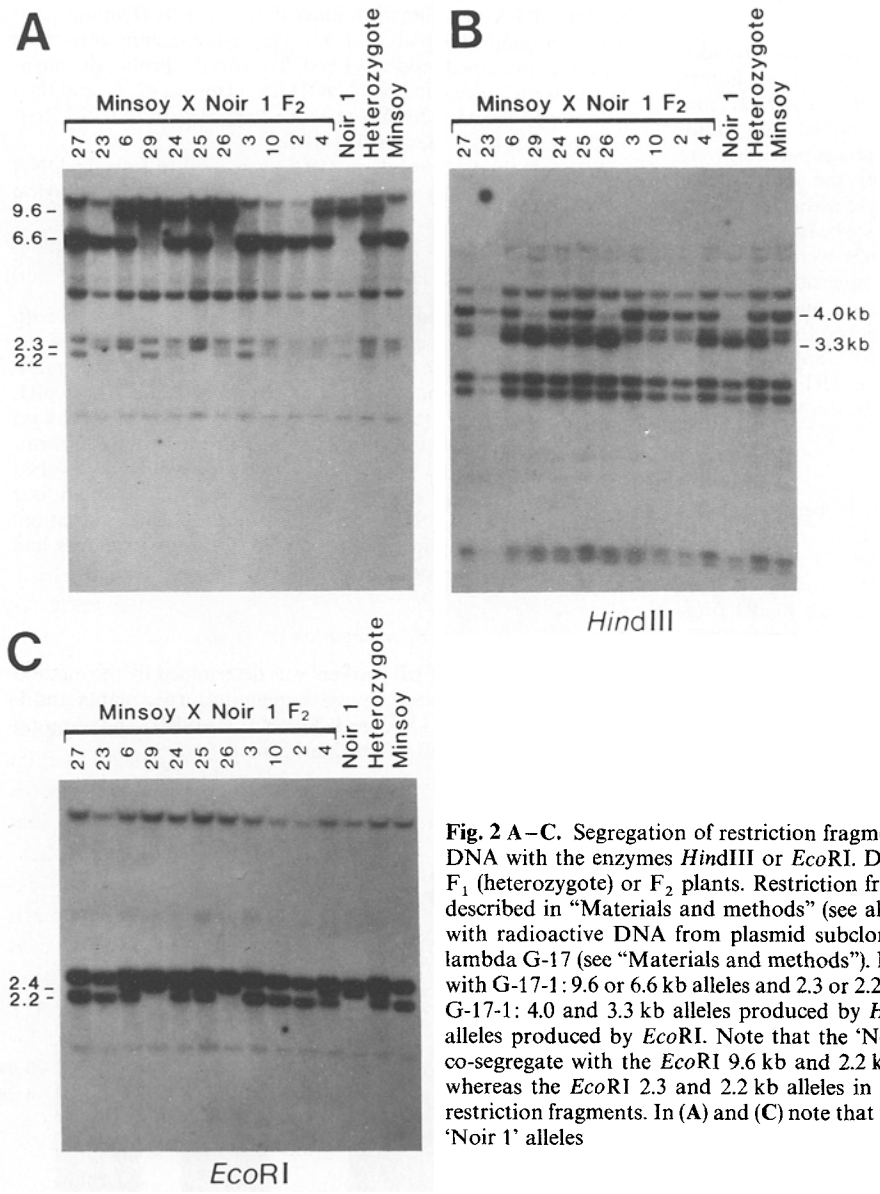

Fig. 2 A–C. Segregation of restriction fragment alleles prepared by digesting soybean DNA with the enzymes *Hin*dIII or *Eco*RI. DNA was isolated from 'Minsoy', 'Noir 1', F_1 (heterozygote) or F_2 plants. Restriction fragments were prepared and separated as described in "Materials and methods" (see also Fig. 1) and analyzed by hybridization with radioactive DNA from plasmid subclones (G-17-1 (**A** and **B**) or G-17-2 (**C**)) of lambda G-17 (see "Materials and methods"). Four polymorphisms are observed: In (**A**) with G-17-1: 9.6 or 6.6 kb alleles and 2.3 or 2.2 kb alleles produced by *Eco*RI; in (**B**) with G-17-1: 4.0 and 3.3 kb alleles produced by *Hin*dIII; in (**C**) with G-17-2: 2.4 or 2.2 kb alleles produced by *Eco*RI. Note that the 'Noir 1' and 'Minsoy' *Hin*dIII alleles in (**B**) co-segregate with the *Eco*RI 9.6 kb and 2.2 kb alleles of 'Noir 1' and 'Minsoy' in (**A**); whereas the *Eco*RI 2.3 and 2.2 kb alleles in (**A**) segregate independently of the other restriction fragments. In (**A**) and (**C**) note that the 'Minsoy' alleles co-segregate as do the 'Noir 1' alleles

Results

Previous experiments (Delanney and Palmer 1982) had suggested that two plant introductions 'Minsoy' and 'Noir 1' were widely separated during their evolution. In most of these experiments we have compared these two cultivars and progeny from a cross between them.

Lambda and M13 clones as sources of RFLP markers

Initially we examined clones containing randomly selected segments of soybean DNA in phage lambda (see "Materials and methods") for use as probes for specific restriction fragments. Three patterns of hybridization were observed (Fig. 1). Despite initial screens to select clones which did not correspond to repeated DNA, several clones gave patterns of hybridization too complex to analyze (Fig. 1 A). With such clones, a large number of restriction fragments were labeled, and the radioactivity from these bands obscured any possible polymorphism. Others, though complex, could be analyzed (as in Fig. 1 B) and some revealed polymorphisms. (Thus in Fig. 1 B, digestion with *Hin*dIII produced a 1.95 kb fragment which is present in 'Noir 1' but not in 'Minsoy', whereas a 1.65 kb fragment present in 'Minsoy' is not found in 'Noir 1'. Both fragments appear in the F_1 hybrid.) Yet other clones yielded relatively simple patterns of restriction fragments in which polymorphisms clearly were visible (as in Fig. 1 C). Simpler patterns could be obtained by subcloning segments of these inserts into a plasmid vector (see Fig. 2).

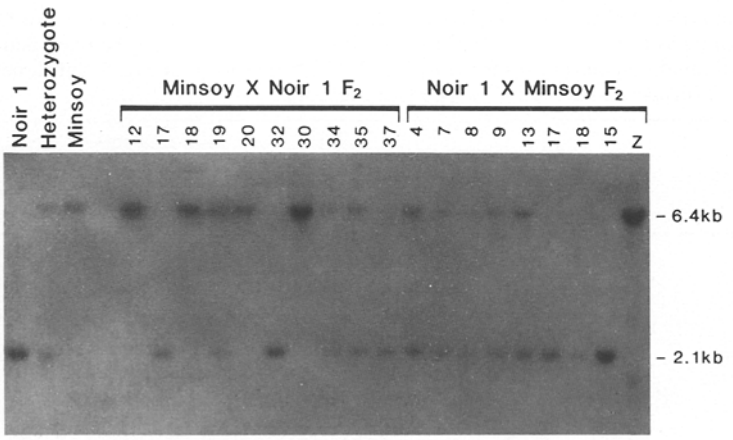

Fig. 3. Segregation of two restriction fragment alleles visualized by hybridization with radioactive DNA from an M13 clone. DNA was isolated from the soybean plants indicated, digested with *Bcl*I and restriction fragments separated for analysis as in Fig. 2 and in "Material and methods". These fragments were transferred to a nylon membrane and hybridized with radioactive DNA from the M13 clone NP-8

Many probes revealed polymorphisms when DNA was digested with each of several enzymes. Thus, in Fig. 1 C, the polymorphism is revealed by the enzyme *Hind*III as a 4.0 kb fragment from 'Minsoy' and a 3.3 kb fragment from 'Noir 1' DNA; whereas *Eco*RI produces a 9.6 kb fragment from 'Minsoy' and a 6.6 kb fragment from 'Noir 1'. These two sets of polymorphisms remain apparent when the subclone in Fig. 2 is used as the probe and co-segregate as would be expected if they represented two tightly linked loci or two aspects of a single locus. This can be seen by comparing the segregation of the 9.6 and 6.6 kb *Eco*RI fragments in Fig. 2 A with the 4.0 and 3.3 kb *Hind*III fragments in Fig. 2 B.

Most RFLP markers were not linked and therefore did not co-segregate (see below). In a few cases, a single probe revealed two polymorphisms, with one enzyme, which segregated independently in F_2 plants. An example is shown in Fig. 2 (*Eco*RI digest: 9.6 and 6.6 kb fragments should be compared with 2.3 and 2.2 kb fragments). Such examples were indicative of the complexity of the soybean genome, in which one DNA probe can hybridize with fragments of DNA on separate linkage groups, indicating that DNA homologous to this probe is interspersed throughout the genome (Goldberg 1978).

To avoid this type of complexity as well as the complex patterns of hybridization shown in Fig. 1 A (or B), we used smaller probes, fragments of DNA cloned into M13 phage. These could be made highly radioactive by primer extension and were simple to prepare, therefore reducing the time required to screen for polymorphisms (see "Materials and methods"). Many of these revealed extremely simple patterns of restriction fragments as in the example shown in Fig. 3.

Table 1 summarizes the probes currently available which hybridize with RFLP markers. For each probe, we indicate which enzyme(s) can be used to reveal polymorphism in the genomic DNA. In many cases (more than half those tested) more than one enzyme could be used to reveal the polymorphism. Whereas the RFLP in Fig. 3 reveals a very simple pattern of restriction fragments, more complex patterns were occasionally observed. These could be simplified to an extent where they could be analyzed by the following procedure (suggested by David Kirk (Harper et al. 1987)): The genomic DNA was digested by enzymes specific for four nucleotides and then electrophoresed until only large fragments (>0.7 kb) remained on the gel. This resulted in a simpler pattern of fragments. Among the fragments which hybridized with the probe were polymorphic fragments whose low radioactivity suggested that they were less abundant than other fragments (Fig. 4). Clearly these probes are homologous with repeated DNA, yet by examining only larger fragments, a simpler pattern is seen and polymorphisms are not obscured. As we shall discuss below, these probes appear to be useful in distinguishing between different cultivars. They may be similar to the hypervariable probes described by Jeffries et al. (1985) for human RFLP markers.

Polymorphism arising from rearrangement of genomic DNA

Length of the restriction fragment produced from a particular segment of DNA will be altered when the distance changes between the sites of cleavage by a specific restriction enzyme. This may occur by the gain or loss of a single restriction site (e.g., by mutation) or by a rearrangement of the DNA bringing the two sites closer together or increasing their separation. Changes in a specific cleavage site will alter only the fragments produced by that specific enzyme, whereas rearrangement of the DNA will alter the size of fragments produced by several different restriction enzymes. This is because insertions, deletions, substitutions or inversions alter the spatial arrangement of large segments of DNA containing many different restriction sites. As noted in Table 1 more than half of the probes which reveal polymorphism show such changes with fragments produced by several different

Table 1. Probes defining RFLP markers with different enzymes. Subclones (17-1, 17-2, 21-2, 21-3, 21-4, 26-27) are shown beneath lambda clones 17, 21 and 26. An additional polymorphism was shown with λG-11 between 'Minsoy' and 'Noir 1' on the one hand and Sooty on the other for *Hin*dIII, *Taq*I and *Hin*fI. Additional polymorphisms for λG-17 and λG-21 are shown in Fig. 5. Subclone 17-1 identifies an additional RFLP marker with *Eco*RI: 'Minsoy' 2.3 kb. 'Noir 1' 2.2 kb (see Fig. 2A)

A Lambda probes

Probe	*Bgl*II	*Dra*I	*Eco*RI	*Hin*dIII	*Hin*fI	*Taq*I	Enzymes showing polymorphisms
λG-8							
G-8-15	–	–	+	–	–	–	1
λG-17							
G-17-1	+	–	+	+	+	–	4
G-17-2	+	+	+	–	+	+	5
λG-21							
G-21-2	[a]	–	+	+	–	+	3
G-21-3	–	+	nd	–	nd	nd	1
G-21-4	+	+	+	+	+	+	6
λG-26							
G-26-27	–	–	–	+	+	+	3

B Simple M13 probes

Probe	*Bcl*I	*Bgl*II	*Dra*I	*Eco*RI	*Hin*dIII	Enzymes showing polymorphisms
NC-4	–	–	–	+	–	1
NE-10	+	–	–	+	–	2
NF-31	–	–	–	+	–	2
NG-2	+	+	nd	–	–	2
NG-24	+	+	nd	+	+	4
NH-18	nd	–	nd	nd	+	1
NH-23	nd	–	nd	nd	+	1
NJ-10	+	–	nd	+	–	2
NJ-11	nd	–	nd	+	–	1
NK-6	–	–	nd	+	nd	1
NN-8	+	+	–	+	–	3
NN-21	+	+	–	–	+	3
NO-9	–	+	+	nd	–	2
NO-15	–	–	–	+	–	1
NP-8	+	nd	+	–	–	2
17/15	+	nd	nd	+	+	3
20	–	–	–	+	–	1
36	–	–	–	+	–	1
38	+	–	+	+	–	4
47	–	–	–	–	+	1
58	–	–	–	+	–	1
63	–	–	+	–	+	2
69	+	+	+	+	–	4
75	+	–	–	–	–	1
79	–	–	–	–	+	1
100	+	+	+	+	–	4
109	+	–	nd	–	–	1
121	+	nd	+	+	–	3
146	–	+	+	+	+	4
147	–	–	–	+	–	1
253	–	–	+	–	–	1
363	–	–	–	+	–	1
369	–	–	+	–	+	2
373	+	+	–	+	–	3
378	–	–	+	–	+	2
379	+	–	–	–	+	2
464	–	–	–	+	–	1

[a] Expected differences were too small to detect
nd: not determined

Table 1 (Continued)

C Complex M13 probes								
Probe	*Alu*I	*Ava*II	*Dde*I	*Hae*III	*Hin*fI	*Mbo*I	*Msp*I	*Taq*I
NI-19	+	+	−	−	−	+	−	−
NJ-5	−	−	−	+	+	−	−	−
NJ-9	−	+	−	nd	nd	−	−	nd
NJ-14	−	+	+	+	+	−	−	−
NJ-21	−	+	−	+	−	−	−	−
NK-17	−	+	+	+	nd	−	+	−
NN-2	−	−	−	+	−	−	−	−
NN-7	−	−	−	+	−	−	−	nd

In addition, NE-9 is a complex probe which shows polymorphism with *Dra*I.
224 is a complex probe which shows polymorphism with *Dra*I, *Eco*RI and *Hin*dIII.
262 is a complex probe which shows polymorphism with *Eco*RI

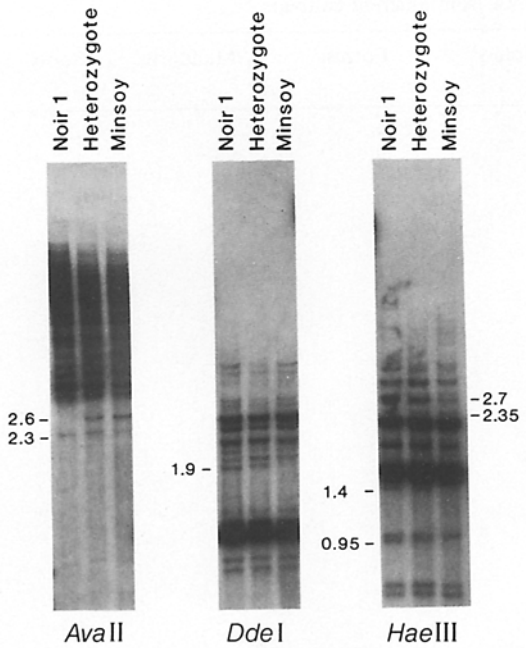

Fig. 4. Example of a restriction fragment length polymorphism in DNA from 'Minsoy', 'Noir 1', or F_1 plants hybridized with a probe homologous to repeated DNA. DNA was prepared and digested with the enzymes shown, most of which are specific for four nucleotides. In this procedure, a number of small fragments were produced which were allowed to migrate off of the gel. The remaining larger fragments (greater than 0.7 kb) could be separated and polymorphisms were observed (indicated by the fragments whose size is shown) when these fragments were hybridized with the radioactive probe, NJ-14. This technique was suggested to us by Dr. David Kirk who had used it to analyze polymorphic repeated DNA from Volvox

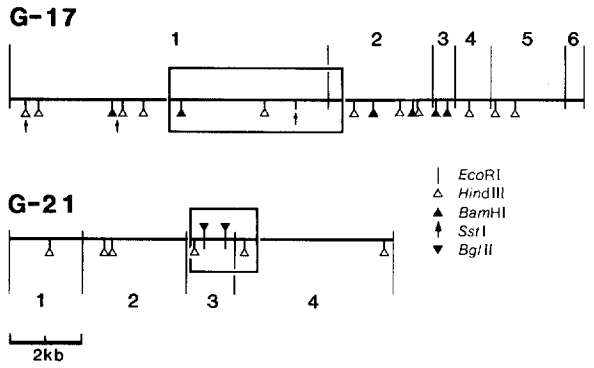

Fig. 5. Restriction maps of the λ probes G-17 and G-21. *Eco*RI fragments *1* and *2* of *G-17* and *2* and *4* of *G-21* wer used as probes to detect restriction fragment length polymorphisms between 'Minsoy' and 'Noir 1' DNA. The *boxes* contain regions of DNA in which differences (rearrangements) occur which cause the polymorphisms (see text)

restriction enzymes suggesting that rearrangement of the DNA is involved. Two of these, analyzed in detail, involve restriction fragments homologous to the DNA in lambda clones G-17 and G-21.

DNA fragments from these two lambda clones were subcloned into plasmids and a restriction map of the original lambda clones was prepared (Fig. 5). In both G-17 and G-21 two subclones were found each of which hybridized with restriction fragment length alleles. The adjoining DNA segments G-17-1 and G-17-2 both hybridized with restriction fragments which were polymorphic between 'Minsoy' and 'Noir 1'. When these subclones were used to examine F_2 plant DNA, we found that the markers hybridizing with G-17-1 and G-17-2 segregated together as if they were completely linked (see Fig. 2, A and C). This rearrangement is being analyzed further. However, preliminary data comparing the genomic fragments to the 'Forrest' clones indicate that the rearrangement occurred in the region indicated by the box in Fig. 5 and consists of either a deletion-insertion or a substitution. Analysis of the rearrangement involving the sequence in clone G-21 is more complicated. When the two, non-adjacent clones, G-21-2 and G-21-4 are used as probes, both detected polymorphisms and these segregated together in F_2 plants (the polymorphisms were completely linked). However, G-21-3, contains a region common to a highly repeated subsequence and therefore could not be used to analyze the polymorphism. The data from Southern blots using G-21-2 and

Table 2. Linkage between RFLP markers. Linkage between marker 1 and 2 with a recombination frequency (r ± SE) is given, together with the chi-square associated *P* value for deviation from expected values for obtaining the result by chance

Marker 1	Marker 2	r	*P*
38	G8-15	0.19±0.05	6.0×10^{-8}
G8-15	NE-9B	0.32±0.08	5.3×10^{-3}
NN-21	NE-9B	0.19±0.07	8.7×10^{-4}
69	NG-24	0.12±0.04	3.3×10^{-7}
69	100	0.28±0.07	3.3×10^{-2}
NE-10A	109	0.22±0.07	1.8×10^{-2}
262	NE-10B	0.35±0.09	2.2×10^{-4}

G-21-4 indicate that the rearrangement involves a region included in the box in Fig. 5. Preliminary data indicate that the rearrangement must be either an inversion of this region or a substitution by another sequence. Final analysis of both of these rearrangements (G-17 and G-21) must await cloning of each allele from 'Minsoy' and 'Noir 1' DNA.

Linkage analysis

To date, some 27 RFLP markers have been analyzed in F_2 progeny plants for linkage (see Figs. 2 and 3). Up to 50

Table 3A. Restriction fragments displayed by different simple probes with DNA from different cultivars

Probe	Enzyme	Polymorphic bands (kb)	'Minsoy'	'Noir 1'	'Forrest'	'Mandarin'	'Sooty'
λG-8-15	*Eco*RI	8.4	+	−	+	+	−
		4.2	−	+	−	−	+
λG-11-1	*Hin*dIII	6.6	−	−	−	−	+
		5.9	+	+	+	+	−
λG-17-1 A	*Eco*RI	9.6	−	+	+	+	−
		6.6	+	−	−	−	+
B	*Eco*RI	2.3	+	−	−	−	+
		2.2	−	+	+	+	−
λG-21-2	*Hin*dIII	2.4	−	+	+	+	−
		2.2	+	−	−	−	+
λG-21-4	*Hin*dIII	3.5	+	−	−	−	+
		3.2	−	+	+	+	−
λG-26-27	*Hin*III	1.9	−	+	−	−	−
		1.6	+	−	+	+	+
NE-10A	*Bcl*I	5.7	+	−	+	−	+
		5.0	+	−	−	−	+
NF-31	*Eco*RI	5.0	+	−	+	+	−
NG-2A	*Bcl*I	1.5	+	−	+	+	+
		0.7	−	+	−	−	−
NN-8*	*Bgl*II	25.0	−	−	−	−	+
		9.0	+	+	+	+	−
		7.4	+	−	−	−	+
		5.5	−	+	+	+	−
NN-21	*Bcl*I	3.5	−	+	+	+	+
		2.6	+	−	−	−	−
NP-8	*Bcl*I	6.4	+	−	+	−	−
		2.1	−	+	−	+	+
pGly3 (Glycinin)	*Hin*dIII	4.4	+	−	+	−	+
		3.9	+	+	−	+	+
pJCX* (Lectin)	*Hin*dIII	5.2	−	−	−	−	+
		4.4	+	+	+	+	−
		1.6	+	−	+	+	+
		1.0	−	+	+	+	+
17/15	*Bcl*I	3.2	+	−	−	+	+
69	*Eco*RI	9.2	+	−	−	−	+
		7.6	−	+	+	+	−
		4.3	+	+	+	+	−

Table 3A.

Probe	Enzyme	Polymorphic bands (kb)	'Minsoy'	'Noir 1'	'Forrest'	'Mandarin'	'Sooty'
109*	*Bcl*I	5.5	+	−	+	+	−
		5.0	−	+	−	−	−
		2.3	−	−	−	−	+
		0.9	−	+	−	−	−
121	*Bcl*I	6.7	−	+	+	−	−
		4.7	+	−	−	+	+
142	*Eco*RI	3.0	+	+	+	−	−
		2.4	−	−	−	+	+
146*	*Hin*dIII	7.7	+	−	−	−	−
		6.2	+	−	+	+	+
		4.7	−	+	−	−	−
		4.4	−	−	−	−	+
373	*Bcl*I	3.0	+	−	−	−	+
		2.5	−	+	+	+	−
464	*Eco*RI	3.2	+	+	+	+	−
		2.4	−	+	+	−	−
		0.9	−	+	+	−	+

* Probes hybridizing with an extra restriction fragment in digest of DNA from the cultivar 'Sooty'

Table 3B. Restriction fragments displayed by different complex probes with different cultivars

Probe	Enzyme	Polymorphic bands (kb)	'Minsoy'	'Noir 1'	'Forrest'	'Mandarin'	'Sooty'
NE-9	*Dra*I	2.0	−	−	−	−	−
		1.9	−	+	+	−	+
		1.2	*[a]	+	−	−	+
		1.1	−	−	−	−	+
		1.0	*[a]	+	−	+	+
NI-19	*Mbo*I	2.4	−	+	−	−	+
		2.1	+	−	+	+	−
		1.5	+	+	−	−	+
NJ-14	*Hae*III	3.4	−	−	−	−	+
		2.7	+	−	−	−	−
		2.35	+	−	−	−	−
		1.4	−	+	−	+	−
		0.95	−	+	+	−	−
pS-5H1.3 (Tgm)	*Taq*1	2.0	−	−	−	−	+
		1.9	−	−	+	+	−
		1.5	−	+	+	+	+
		1.0	−	−	+	−	−
		0.78	+	−	−	+	+
224	*Dra*I	4.4	−	+	−	+	+
		2.7	−	−	−	+	+
		2.5	−	+	+	+	+
		2.3	+	−	−	−	+
262	*Eco*RI	15.5	−	+	−	−	+
		12.5	+	−	+	−	+
		8.8	−	+	−	+	+

[a] Ambiguous, i.e., possible faint radioactive bands on Southern transfers

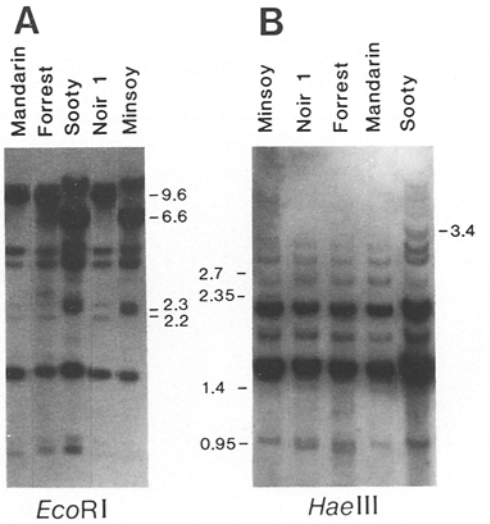

Fig. 6 A, B. Comparison of RFLP alleles between different cultivars of soybean. DNA was extracted from 'Mandarin', 'Forrest', 'Sooty', 'Noir 1', and 'Minsoy'. The DNA was digested with (**A**) *Eco*RI or (**B**) *Hae*III. After separating the restriction fragments (as in Figs. 2 or 4), the fragments, on Southern transfers, were hybridized with radioactive probe DNA: **A** G-17-1, a plasmid subclone derived from the lambda clone G-17 (see Figs. 1 and 2); **B** NJ-14, an M13 clone (see Fig. 4). Note the weakly radioactive fragments in (**B**) which characterize and distinguish each of the five cultivars from each other: 'Minsoy' has fragments of length 2.3 and 2.7 kb; 'Noir 1' has a 0.95 kb and a 1.4 kb fragment; 'Forrest' has a 0.95 kb fragment; 'Mandarin' has a 1.4 kb fragment; and 'Sooty' has a 3.4 kb fragment

plants were compared (see "Materials and methods"). Each of the alleles segregated in a Mendelian fashion. In those cases where a fragment was absent from one parent (e.g., see 17/15, Table 3 A below), the alleles were treated as if a dominant allele were segregating. The linkage data are given in Table 2. Eleven RFLPs comprised four linkage groups. Other RFLP markers were either unlinked or too loosely linked to determine linkage with this number of plants.

Use of RFLP markers in tissue culture

We have examined the RFLP patterns of DNA from suspension cell cultures prepared from stem or leaf tissue taken from 'Minsoy', 'Noir 1', and heterozygote plants. Of nine probes tested against 'Noir 1' tissue culture DNA, all showed the same fragments as in intact plants. Of 29 tested against 'Minsoy' DNA all but one showed the same pattern of fragments as the intact plant. The one deviant had a fragment not previously seen in the intact plant. Of 19 tested against the heterozygote cell line, all but two had the same pattern as the intact plant. One of the two deviant patterns had a new fragment in addition to those expected; the other had lost a fragment corresponding to the 'Minsoy' allele.

RFLP patterns of other cultivars

We expected that other cultivars would show RFLP patterns that were not observed with either 'Minsoy' or 'Noir 1'. Twenty-seven different probes were used to examine DNA from the five cultivars 'Minsoy', 'Noir 1', 'Mandarin', 'Sooty' and 'Forrest'.

Examples of comparisons between the five cultivars using different probes are shown in Fig. 6. Figure 6 A compares the cultivars using a simple probe. As can be seen only two alleles are observed. In contrast, the complex probe in Fig. 6 B identifies several polymorphisms, different combinations of which are found in different cultivars. In all, 22 probes were used which identified simple patterns of restriction fragments (Table 3 A). In the majority of these (18 of the 22 in Table 3 A), the five cultivars showed an RFLP pattern indicating that only alleles found either in 'Minsoy' or in 'Noir 1' were found in the other cultivars. However, for different loci (identified by different probes), the allele types were distributed differently between different cultivars and thus the overall pattern of alleles could be used to distinguish different cultivars. In four cases (* in Table 3 A) new alleles were found. All of these new allele types were found in the cultivar 'Sooty' known to contain a transposable element responsible for the change seen with the lectin probe (pJCX-1, Table 3 A). In five cases, complex probes, such as NJ-14, 224 or pS-5H1.3, revealed several polymorphisms which discriminated between all five cultivars (Table 3 B). These data suggest that single probes which give complex RFLP patterns can be used to distinguish between a variety of cultivars, whereas a number of probes which give simple RFLP marker patterns (two alleles) can be used to distinguish different cultivars.

Discussion

We have examined about 300 clones of soybean DNA for usefulness as probes to detect restriction fragment length polymorphisms (RFLP). Of these, almost 60 or about 20%, detected RFLP markers. We have found that M13 clones can be prepared rapidly and that primer extension using these clones yields highly radioactive probes. Two types of M13 probes were obtained: those yielding a simple pattern of restriction fragments which probably correspond to low copy DNA and those which hybridize to a large number of restriction fragments and probably correspond to repetitive (multi-copy) DNA (Jefferies et al. 1985; Harper et al. 1987). When different cultivars were examined, only two RFLP alleles were found for most of the simple probes. However, the cultivars differed with respect to which one of the two alleles were found. For example, in Table 3 A the five probes, G-8-15, NP-8, NN-21, 17/15, and NG-2A can be used to differen-

tiate the cultivars 'Forrest', 'Mandarin', 'Minsoy', 'Noir 1' and 'Sooty' from each other.

Probes corresponding to repeated DNA reveal more polymorphisms, perhaps because they analyze a large number of variable DNA fragments. A single such probe (e.g., NJ-14, Table 3 B) can differentiate all five cultivars. Thus, both simple and complex probes should be extremely useful in tracing contributions of different cultivars to germplasm prepared in breeding programs. This is similar to studies of mammals and birds in which a single complex probe has been used to differentiate a number of related individuals (Jefferies et al. 1985; Burke and Bruford 1987; Nakamura et al. 1987; Vassart et al. 1987).

Of 27 RFLP markers analyzed for linkage, 11 were contained in four possible linkage groups. Assuming that these markers were chosen at random, we estimate (from a fit to a Poisson distribution) that some 60–70 groups of markers exist linked by a recombination frequency of less than 25%. This would imply that 150 evenly spaced markers should yield a map in which each marker is separated from the next by 10 recombination units. Such a map would allow a detailed genetic analysis of the existing soybean germplasm. To accumulate this many markers, it is necessary to have a large amount of DNA preferably from one set of F_2 plants. Our results with stem and leaf tissue culture suggest that we can use tissue culture from F_2 plants to provide an unlimited source of the DNA required for analyzing crosses. This resource parallels the use of human cell cultures in providing material for analysis and construction of a genetic map (White et al. 1985). Previous work in our laboratory developed a method for removing chromosomes from F_1 hybrid cells in tissue culture by treatment with the herbicide CIPC (Roth and Lark 1984). As yet we do not know if treatment with CIPC leads to somaclonal change (Scowcroft 1985). If not, we should be able to use this methodology to map RFLP markers to individual chromosomes. Clearly, with an appropriate effort, it should be possible to have a comprehensive map of soybean within three to four years.

Our results show that a high frequency of polymorphism is caused by genome rearrangement. We have made a preliminary analysis of two such changed: one of these is consistent with either an inversion or a fragment substitution (G-21); the other may be a fragment substitution or a deletion-insertion (G-17). Previous work by Vodkin et al. (1983) and by Rhoades and Vodkin (1985) has shown that the presence or absence of lectin was due to the deletion-insertion of an element (resembling a transposon) into the lectin gene. It would appear that soybean, like other plants, may have a large number of such changes. Further analysis of other rearrangements should indicate whether such changes occur by common mechanisms or involve common elements.

At most loci, we have found only two alleles for those RFLP markers corresponding to simple probes. This was true for the five different cultivars examined ('Forrest', 'Mandarin', 'Minsoy', 'Noir 1' and 'Sooty'). This is in contrast to maize in which different cultivated varieties exhibit a large number of alleles and RFLP markers are much more abundant (Burr et al. 1983; Helentjaris et al. 1986a). One explanation for the difference is that cultivated soybean arose from a gene pool restricted to a few plants. If so, we might expect that the ancestral wild species *G. soja* (Hymowitz and Newell 1980, 1981) and *G. gracilis* (Hadley and Hymowitz 1973) would carry different alleles. However, preliminary experiments indicate that this is not the case (P. Keim and R. Shoemaker, unpublished data). The data in Table 3 demonstrate that different cultivars may carry either a 'Minsoy' or 'Noir 1' allele at any locus and that the three cultivars, 'Forrest', 'Mandarin' and 'Sooty' have different mixtures of these two allelic types. This indicates an unexpectedly high degree of mixing of the gene pools in this inbred plant. We have found (see * in Table 3A) that the cultivar 'Sooty' is characterized by several distinct alleles, which set it apart from 'Forrest', 'Mandarin', 'Minsoy' and 'Noir 1'. This cultivar is one of only a very few of the cultivated varieties (*G. max*) which lack lectin (Pull et al. 1978; Vodkin and Raikhel 1986). This is in contrast to the wild soybean, *G. soja*, in which almost half of the taxonomic varieties are lectin negative (Stahlhut and Hymowitz 1980; Stahlhut et al. 1981). Thus the cultivar 'Sooty' appears to belong to a minor group of cultivars which may have shared a common ancestry separate from that of lectin positive taxonomic varieties. Despite this, 'Sooty' shares many RFLP alleles with 'Forrest', 'Mandarin', 'Minsoy', and 'Noir 1'. Either cultivated soybean varieties have been crossed repeatedly during the 3,000 years in which the plant has been associated with man, or the variation which we have seen can arise repeatedly within isolated cultivars.

If the rearrangements described here arise repeatedly within isolated cultivars, they may be due to some form of site specific recombination. Evidence in support of this idea (to be published elsewhere) has been obtained in experiments with tissue culture. The occurrence of site specific recombination with high frequency would imply that soybean, a plant restricted to self pollination, has evolved a system for generating specific forms of genetic variation.

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The results in Figs. 1 and 2 and some of the data in Tables 1 and 2 will be submitted to the University of Utah by Barbara Frazier in partial fulfillment of the M.S. degree in biology.

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Note added in proof

With the help of T. Malcalma, we have expanded the number of linked markers to include 42 markers in 12 linkage groups (see *Linkage analysis*).