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The use of microsatellite DNA markers for soybean genotype identification

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Abstract Conventional morphological and pigementation traits, as well as disease resistance, have been used to distinguish the uniqueness of new soybean cultivars for purposes of plant variety protection. With increasing numbers of cultivars and a finite number of conventional characters, it has become apparent that such traits will not suffice to establish uniqueness. The objective of this work was to provide an initial evaluation of microsatellite or simple-sequence-repeat (SSR) DNA markers to develop unique DNA profiles of soybean genotypes. Microsatellites are DNA sequences such as $(AT)_n/(TA)_n$ and $(ATT)_n/(TAA)_n$ that are composed of tandemly repeated 2–5-basepair DNA core sequences. The DNA sequences flanking microsatellites are generally conserved allowing the selecti^n of polymerase chain reaction (PCR) primers that wili amplify the intervening SSR. Variation in the number of tandem repeats, "n", results in PCR product length differences. The SSR alleles present at three $(AT)_n/(TA)_n$ and four $(ATT)_n/(TAA)_n$ loci were determined in each of 96 diverse soybean genotypes. Between 11 and 26 alleles were found at each of the seven loci. Only two genotypes had identical SSR allelic profiles and these had very similar pedigrees. The gene diversity for the seven markers averaged 0.87 for all 96 genotypes and 0.74 for a subset of 26 North American cultivars. These are much higher than soybean gene diversity values obtained using RFLP markers, and are similar to the average values obtained for human microsatellite markers. SSR markers provide an excellent complement to the conventional markers that are currently used to characterize soybean genotypes.

Key words Simple sequence repeats · DNA finger printing · Gene diversity · Plant variety protection · DNA markers

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Introduction

Plant scientists have long used morphological, pigmentation, quality, or other characteristics, to classify and distinguish plant genotypes within a species. With the advent of the Plant Variety Protection Act of 1970 the developer of a cultivar could "protect" it from commercial exploitation by others. While not having the same exclusivity as a patent, plant variety protection, nonetheless, offers the owner of a cultivar some legal protection for the exclusive sale of a protected cultivar. The Plant Variety Protection Office (PVPO) of the Agricultural Marketing Service, US Department of Agriculture, is charged with granting plant variety protection certificates for new crop cultivars. Those attempting to obtain a certificate for a new cultivar must demonstrate it to be unique from "all other varieties within that species."

In the case of soybean [*Glycine max* (L.) Merr.], and most other species, traits such as flower and pubescence color, leaf shape, growth habit, maturity, and other conventional morphological traits, together with disease resistance, have been, and continue to be, used to distinguish the uniqueness of a new cultivar. As more cultivars receive protection, and thereby increase the size of the PVPO database, it becomes more difficult to distinguish new cultivars from those in the database. Moreover, since new cultivars normally arise from hybridizations between members of an elite group of genetically similar parents, the amount of genetic variability among newly developed cultivars is likely to become

even smaller. This will further complicate the task of unambiguously identifying new cultivars by the use of conventional characteristics alone. It is thus apparent that the use of molecular genetic markers would provide one solution to the problem of providing unique DNA profiles for the protection of new soybean cultivars.

Restriction fragment length polymorphism (RFLP) markers are one type of DNA marker that may be used to develop DNA profiles (Soller and Beckmann 1983). Keim et al. (1989) used 17 RFLP probes to characterize a group of 58 soybean and wild soybean (*G. soja* Sieb. and Zucc.) genotypes. Despite the relatively large number of probes, seven genotypes had identical RFLP patterns and were therefore indistinguishable. In 15 of the 17 RFLP loci used by Keim et al. (1989) only two alleles were observed. In the remaining two instances three alleles were detected. In subsequent work (Keim et al. 1992) 38 ancestral and adapted cultivars were analyzed with 132 RFLP probes. A total of 92 probes, or 69%, were polymorphic and more than two alleles were detected at only three loci. Because of the limited number of alleles per locus in soybean, the amount of information generated by individual soybean RFLP probes is also limited.

An alternative type of DNA marker has recently come into common use by human geneticists and has been shown to be highly polymorphic. These microsatellite of simple-sequence-repeat (SSR) markers are composed of tandemly repeated 2–5-nucleotide DNA core sequences such as $(CA)_n$, $(ATT)_n$, or $(AGAT)_n$. The DNA sequences flanking SSRs are generally conserved within individuals of the same species allowing the selection of polymerase chain reaction (PCR) primers that will amplify the intervening microsatellite in all genotypes. Variation in the number of tandem repeats, "*n*", results in PCR product length differences (Tautz 1989; Weber and May 1989). Akkaya et al. (1992) demonstrated that SSRs are present in soybean and exhibit high levels of length polymorphism. Among a group of 43 soybean genotypes an average of seven alleles was detected at each of three microsatellite l ci. More recently, Cregan et al. (1994) reported one soybean SSR locus with 23 alleles. Because of the high level of polymorphism, and therefore the greater informativeness of microsatellite markers, it seemed likely that these markers would be particularly useful for developing unique DNA profiles of soybean genotypes. Such profiles would be especially valuable to unambiguously distinguish soybean cultivars in order to obtain plant variety protection. The objective of the work reported here was to assess the ability of SSR length polymorphism at a small number of loci to distinguish individuals in a group of 96 diverse soybean genotypes.

Materials and methods

Soybean genotypes and DNA isolation

A group of 91 soybean cultivars, plant introductions (PIs), and Chinese landraces, along with five wild soybean accessions, were selected to represent a diversity of soybean genotypes (Table 1). The cultivars and PIs were obtained from Dr. Randall Nelson, USDA-ARS, University of Illinois, Department of Agronomy, Urbana, Illinois, and the soybean landraces were obtained courtesy of Prof. Xu Qiaozhen, Oil Crops Research Institute, CAAS, Wuhan, China.

Five to seven plants of each soybean genotype were grown in the greenhouse for DNA isolation. Plants were inoculated with *Bradyrhizobium japonicum* strain USDA 110. DNA was isolated from the bulked leaf tissue of the plants of each genotype using the procedure described by Saghai-Maroof et al. (1984).

Table 1 Soybean cultivars, *G. max* plant introductions, *G. soja* plant introductions and Chinese landraces used in the determination of allelic variation and frequency at seven microsatellite loci

Soybean cultivars			
Adams	Amsoy	Aoda	Arksoy
Bansei	Biloxi	Bragg	CNS
Calland	Capital	Centennial	Century
Clark	Dare	Douglas	Dunfield
Dyer	Essex	Flambeau	Forrest
Goldsoy	Haberlandt	Harcor	Harosoy
Hawkeye	Hill	Hood	Illini
Jackson	Jogun	Kanro	Korean
Laredo	Lee	Lincoln	Macoupin
Mammoth Yellow	Mandarin	Mandarin (Ottawa)	Manitoba Brown
Merit	Mukden	Ogden	Palmetto
Peking	Pickett	Ralsoy	Richland
Roanoke	S-100	Seneca	Tanner
Tokyo	Tracy	Volstate	Williams
G. max plant introductions			
PI 54610	PI 65338	PI 68398	PI 80471-1
PI 81041	PI 84631	PI 84656	PI 84987A
PI 85356	PI 86063	PI 86116	PI 88310
PI 88486	PI 171442	PI 180501	PI 194654
PI 200492	PI 240664	PI 273483B	PI 349647
PI 360955A	PI 360955B	PI 398930	
G. Soja plant introductions			
PI 101404B	PI 326582A	PI 342619A	PI 378693B
PI 407287			
Chinese landraces			
Xixian	Suxie	Linzhen	Taixin Black
Tongshan	Wu	Xiangdu #3	Xiangdu #4
Aizipu	Huangmao T. J.	Qingjuan	Juhuang

Detection of SSRs

Soybean DNA (cv Williams) fragments of approximately 500 bp in length, obtained by digestion with the restriction enzyme *Sau*3AI (U.S. Biochemical Corp., Cleveland, Ohio), were cloned into the *Bam*HI site of Bluescript II KS + (Stratagene, LaJolla, Calif.) and transformed in *Escherichia coli* host strain XL1-Blue followed by blue/white color selection. Selected colonies were picked into microtiter plates containing LB medium with ampicillin (50 µg/ml) and tetracycline (10 µg/ml). The library was screened by colony hybridization. Procedures outlined by Sambrook et al. (1989) were followed throughout. Filters were hybridized to a ^{32}P-labeled $G(AT)_{11}$ and subsequently to a $CCC(AAT)_{10}$ oligonucleotide probe. The $G(AT)_{11}$ was labeled using a reaction mix containing a $(AT)_{11}$C oligonucleotide template, a $G(AT)_3$ primer, Klenow polymerase, dTTP, and alpha-^{32}PdATP. In the case of the $CCC(AAT)_{10}$ probe the reactants were similar except that a $(ATT)_{10}$GGG template and a $CCC(AAT)_2$ primer were used. After labeling, the reactants were filtered through a BioSpin 6 chromatography column (Bio-Rad, Richmond, Calif.) for removal of unincorporated nucleotides. Hybridization conditions were as described by Rostas et al. (1986). Membranes were washed

twice for 1/2 h at 38 °C in 2*x* SSC in the case of the $G(AT)_{11}$ probe, and at 45 °C for $GGG(AAT)_{10}$ probe, and then exposed to X-ray film.

Sequencing of microsatellite-containing clones

Plasmid DNA of clones putatively containing a $(AT)_n/(TA)_n$ or $(ATT)_n/(TAA)_n$ SSR were isolated using a standard protocol supplied by Applied Biosystems International (ABI, Foster City, Calif.). Sequencing was performed with an Applied Biosystems International 370A DNA Sequencer from both the T3 and T7 primer sites of pBluescript II KS+ using a *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (ABI, Foster City, Calif.).

PCR primer selection and synthesis

Sequence data of clones containing SSRs were analyzed using Primer Detective (Primer Design Software Program, CLONTECH Laboratories, Inc., Palo Alto, Calif.) to select primers flanking the SSR that would produce a PCR product between 100 and 250 bp in length. Primers were synthesized on an ABI 391 DNA Synthesizer and purified using an oligonucleotide purification column (ABI, Foster City, Calif.).

PCR amplification of microsatellite loci

PCR amplifications were performed with each of the 96 soybean genotypes using primers to three $(AT)_n/(TA)_n$ and four $(ATT)_n/(TAA)_n$ SSR loci (Table 1). Reaction mixes contained 30 ng of soybean genomic DNA, 1 mM of Mg^{2+}, 0.15 µM of 3′ and 5′ end primers, 100 µM of each nucleotide, 0.1 µl of 3000 Ci/mmol [alpha-^{32}P] dATP, 1*x* PCR Buffer containing 50 mM of KCl, 10 mM of Tris-HCl pH 8.3, 1 unit of *Taq* DNA polymerase (U.S. Biochemical Corp., Cleveland, Ohio) in a total volume of 10 µl. Cycling consisted of 25 s denaturation at 94 °C, 25 s annealing at 45 °C, and 25 s extension at 68 °C for 32 cycles on a MJ Research model PTC-100 thermocycler (MJ Research, Watertown, Mass.). PCR products (3.0 µl/lane) were separated on a DNA sequencing gel containing 6% polyacrylamide, 8 M urea and 1*x* TBE, at 60 W constant power for 2–3 h. A and G sequencing reactions of M13 ssDNA were used as molecular-weight standards to determine the exact nucleotide length of the denatured PCR products. Following drying, gels were exposed to a Kodak XAR-5 film (Eastman Kodak).

Determination of microsatellite allele lengths

The signal produced on X-ray film by radiolabeled PCR products containing microsatellites normally shows more than a single band (Weber and May 1989). These shadow bands are a common phenomenon and have been attributed to "polymerase slippage" associated with *Taq* polymerase and the PCR amplification of microsatellites. To determine allele length we used the most intense band for comparison with the M13 length standards. In those instances when two or more bands were of equal intensity the uppermost (highest molecular weight) band was used to estimate allele length.

Calculation of gene diversity

Weir (1990) suggested the term gene diversity be used to describe the relative value of a RFLP probe with respect to the amount of polymorphism it detects in a group of homozygous individuals from a self-fertilizing species. Thus,

$$\text{Gene Diversity} = 1 - \Sigma P_{ij}^2$$

where P_{ij} is the frequency of the *j*th RFLP pattern for probe *i* and is summed across patterns. The value of gene diversity is very similar to "heterozygosity" which is commonly used to describe the informativeness of a genetic marker in out-crossing species. Anderson et al. (1993) indicated that gene diversity is essentially the same as the polymorphism information content (PIC) as used by Botstein et al. (1980). Keim et al. (1992) referred to gene diversity as a polymorphism index.

The calculation of simple matching coefficients

A simple matching coefficient was calculated for each of the possible comparisons of the 96 soybean genotypes using the allele lengths at the seven SSR loci. The simple matching coefficient is equal to the number of identical alleles shared by two genotypes divided by seven, i.e., the number of comparisons.

Results and discussion

Microsatellite allele sizing

A number of microsatellite loci with $(AT)_n$ and $(ATT)_n$ core motifs were identified. Three loci with $(AT)_n$, and four with $(ATT)_n$, cores (Table 2) were used to profile the 96 soybean genotypes. Data from the SATT2 locus (Fig. 1) are typical of the four loci with $(ATT)_n$ core motifs and serve to illustrate the sizing of microsatellite alleles. Observation of the smaller alleles in the lanes on the left side of Fig. 1 indicates that each lane contains two major bands. While the presence of two bands in lanes 8 through 11 is not discernible in the figure, two distinct bands are apparent when these products are run further down the gel. Thus, alleles at SATT2 locus were sized based upon the migration of the slower migrating of the two bands. The upper band in each product was compared with the adjacent M13 ladder to determine its exact size. It was then possible to select the series of genotypes with the different allele sizes shown in Fig. 1. Eleven of the twelve alleles identified at the SATT2 locus are shown. This group of genotypes was then used as

Table 2 Seven soybean microsatellite loci identified from a DNA library of Williams soybean, including the microsatellite locus, the core motif and number of repeat units (*n*), and the sequence of primers (5′ to 3′) used in the PCR amplification

Locus	Core motif and number of repeats in cv Williams soybean (*n*)	Sense primer	Anti-sense primer
SAT1	$(AT)_{17}$	ctggtggactattgatacgacc	aactgcgaagatactaccctcc
SAT36	$(AT)_{19}$	aaagtcataagtggcactccaagtt	gaacataacaataataaatatagctc
SAT43	$(AT)_{20}$	aaattctgttcattgtccgtc	cattttaatatcccgagtagg
SATT1	$(ATT)_{24}$	agtacatagatattaaagtct	aaatgatgaacgtgaattatt
SATT2	$(AAT)_{16}$	aataatgtggaaactaaatgg	taatgtgcctatccttgtctt
SATT5	$(TAA)_{21}$	tatcctagagaagaactaaaaaa	gtcgattaggcttgaaata
SATT9	$(AAT)_{12}$	attactagagaaattagttta	cttactagcgtattaacctt

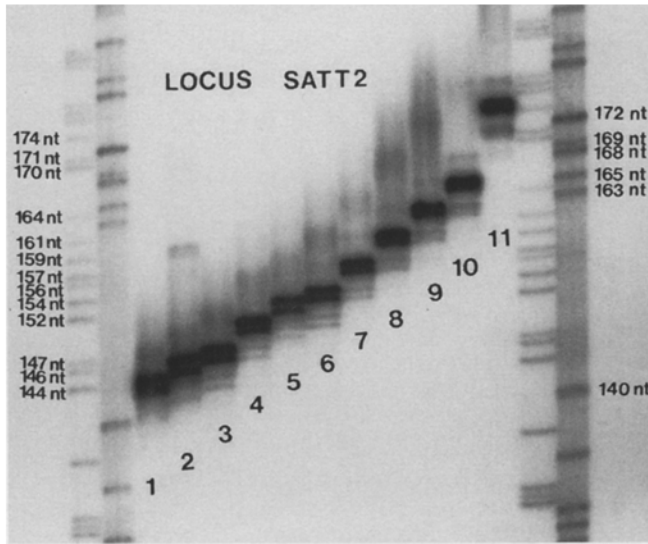

Fig. 1 The SATT2 microsatellite locus showing 11 of the 12 alleles detected among 96 soybean genotypes. The denatured DNA fragments in *lanes 1 through 11* are derived from PCR products (microsatellite alleles) from the soybean genotypes indicated with the estimated allele size (in basepairs) *in parentheses*: *1* Williams (145), *2* Mammoth Yellow (147), *3* Haberlandt (148), *4* S-100 (151), *5* CNS (153), *6* Tanner (154), *7* PI 342619A (157), *8* Aoda (160), *9* Fiskeby V (163), *10* PI 101404B (166), and *11* PI 349647 (175)

standards with which to determine the SATT2 allele present in the remainder of the 96 genotypes.

Allele sizes were then determined at the other six loci (Table 3). For example, at the SAT43 locus a total of 21 different alleles were identified among the 96 soybean genotypes. Twenty of the twenty-one are shown in Fig. 2 ranging in size from 157 bp (lane 1) to 203 bp (lane 20). Likewise at the SAT1 locus (Fig. 3) 26 alleles were detected ranging in size from 231 to 289 bp in length. The remaining four loci showed similarly high levels of allelic length polymorphism (Table 3).

Di-nucleotide versus Tri-nucleotide core motifs and fingerprinting

There are a number of "ghost" bands present in the PCR products from the SAT43 and the SAT1 loci. This phenomenon is known to be particularly prevalent with dinucleotide microsatellites and has been shown to be less of a problem with tri- and tetra-nucleotide SSRs (Edwards et al. 1991). Our results support the conclusion that fewer ghost bands ("stuttering") are associated with tri-nucleotide microsatellites as compared to loci with 2-bp core motifs. Many comparisons of genotypes were required, particularly at the SAT1 locus, to be certain of the exact allele length of each. In contrast, it was much less difficult to unambiguously determine the genotype of each soybean line at the four loci with tri-nucleotide core motifs. Thus, for purposes of fingerprinting, it is our conclusion that SSR loci with trinucleotide core motifs are preferable to those with dinucleotide cores because unambiguous allele sizing is significantly less difficult.

The informativeness of microsatellite markers

The values of gene diversity associated with each of the SSR markers were extremely high, ranging from 0.71 to 0.95 with a mean value of 0.87 when calculated based upon the full set of 96 genotypes (Table 3). Even when the subset of genotypes consisting only of cultivars was used, much allelic variation still remained. As many as ten alleles were present among this group of lines and gene diversity ranged from 0.52 to 0.88 with a mean of 0.74. This contrasts to the low level of polymorphism usually detected by single-locus RFLP probes in soybean (Keim et al. 1989, 1992). Calculations from data presented by Keim et al. (1989) indicated that the most informative of the 17 RFLP probes used in a group of 58 diverse soybean and wild soybean genotypes has a gene diversity value of 0.55. The 16 remaining probes had values of less than 0.50. While it is difficult to quantify, the apparent genetic diversity of the 58 genotypes used

Table 3 The number of alleles, the range of allele sizes, and the gene diversity of seven microsatellite loci assayed in 96 soybean genotypes

Locus	All genotypes			Non-ancestral cultivars[a]	
	Range of allele sizes (bp)	No. of alleles	Gene diversity	No. of alleles	Gene diversity
SAT1	221–289	26	0.95	9	0.80
SAT36	117–197	26	0.93	7	0.83
SAT43	157–203	21	0.93	10	0.88
SATT1	117–159	12	0.71	5	0.52
SATT2	145–175	11	0.83	5	0.60
SATT5	133–190	15	0.85	7	0.77
SATT9	155–236	19	0.87	8	0.81

[a] A total of 26 cultivars developed in North American soybean breeding programs as a result a hybridizations between original ancestral cultivars and including the cultivars: Adams, Amsoy, Bragg, Calland, Capital, Centennial, Century, Clark, Dare, Douglas, Dyer, Essex, Forrest, Harcor, Harosoy, Hawkeye, Hill, Hood, Jackson, Lee, Lincoln, Merit, Pickett, Tracy, Volstate, and Williams

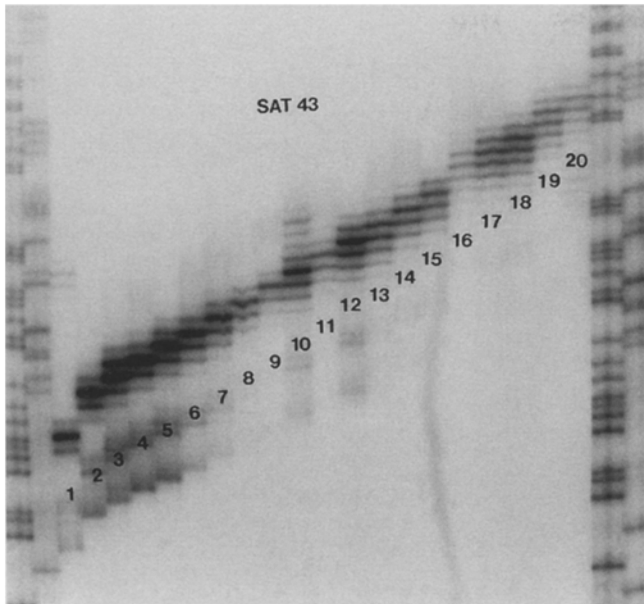

Fig. 2 The SAT043 microsatellite locus showing 20 of the 21 alleles detected among 96 soybean genotypes. The denatured DNA fragments in *lanes 1 through 20* are derived from PCR products (microsatellite alleles) from the soybean genotypes indicated with the estimated allele size (in basepairs) *in parentheses*: *1* PI 101404B (157), *2* PI 80471-1 (163), *3* Taixin Black (165), *4* Forrest (167), *5* Peking (169), *6* Calland (171), *7* Illini (173), *8* Laredo (175), *9* Haberlandt (177), *10* Flambeau (179), *11* Kanro (181), *12* PI 86063 (183), *13* Goldsoy (185), *14* Capital (187), *15* PI 342619A (189), *16* Bragg (193), *17* Lee (195), *18* PI 273483B (197), *19* Biloxi (201), and *20* PI 240664 (203)

by Keim et al. (1989) and that used in the current study are fairly similar. In the later work by Keim et al. (1992) the mean gene diversity of 132 RFLP probes was 0.32. It is therefore our conclusion that microsatellite markers are much more informative than RFLPs in soybean.

A similar disparity in the informativeness of RFLP versus microsatellite markers has been noted in humans (NIH/CEPH Collaborative Mapping Group 1992). Here it has been reported that while only 20% of the 1 676 markers on the human genetic linkage map were microsatellites (most of the remainder were RFLP loci), the SSRs represented more than 58% of the loci with heterozygosities greater than 0.70.

Weissenbach et al. (1992) described the identification and mapping of 814 newly identified human $(CA)_n/(GT)_n$ microsatellites. The mean level of heterozygosity of these "highly polymorphic" markers was about 0.75 which is less than the mean gene diversity of 0.87 of the seven soybean SSRs reported here. The $(AT)_n/(TA)_n$ SSRs, SAT1, SAT36 and SAT43, were chosen from a group of approximately 20 $(AT)_n/(TA)_n$ microsatellites which we have identified. These loci appeared to be particularly polymorphic based upon a standard initial screening of seven diverse soybean genotypes. Thus, it might be anticipated that these markers would be particularly polymorphic. However, the four $(ATT)_n/(TAA)_n$ loci were simply the first four such SSRs we had identified and were not selected for inclusion with the anticipation of an unusually high level of polymorphism. It is therefore reasonable to suggest that gene diversity values of 0.80, or greater, are common for soybean microsatellite loci.

Distinguishing soybean genotypes using microsatellite markers

Simple matching coefficients between the 4 560 combinations of the 96 soybean genotypes were calculated to assess the ability of the seven SSR markers to distinguish

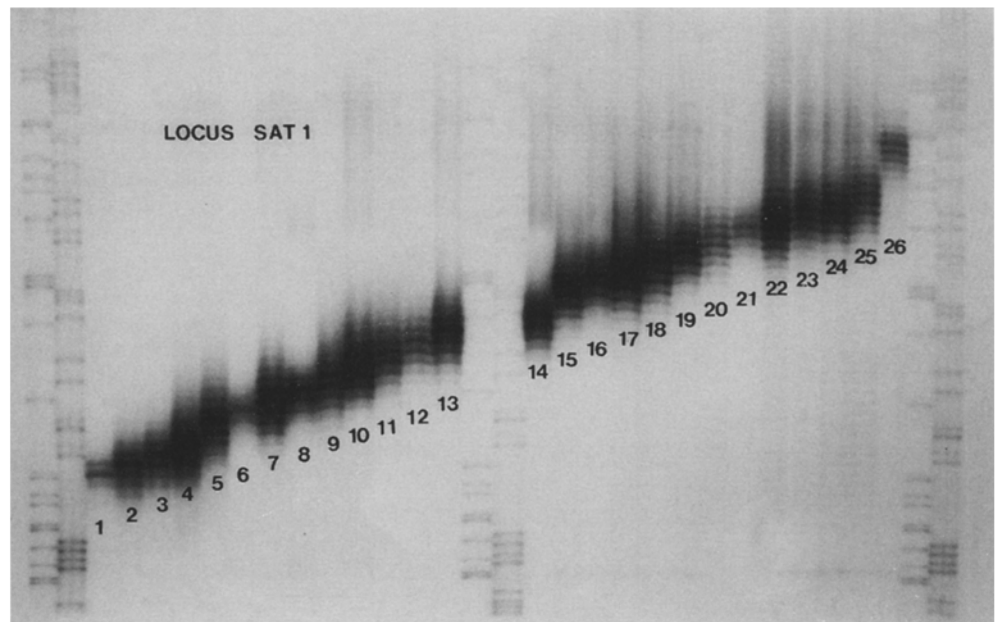

Fig. 3 The SAT1 microsatellite locus showing the 26 alleles detected among 96 soybean genotypes. The denatured DNA fragments in *lanes 1 through 26* are derived from PCR products (microsatellite alleles) from the soybean genotypes indicated with the estimated allele size (in basepairs) *in parentheses*: *1* Huangmao T. J. (221), *2* Ralsoy (223), *3* PI 342619A (225), *4* Xiangdu #3 (227), *5* Fiskeby V (231), *6* PI 65338 (233), *7* Palmetto (235), *8* PI 180501 (237), *9* Jackson (239), *10* PI 54610 (241), *11* Kanro (243), *12* PI 349647 (245), *13* PI 273483B (247), *14* Dunfield (249), *15* Haberlandt (255), *16* PI 85356 (257), *17* Mandarin (259), *18* Mandarin (Ottawa) (261), *19* S-100 (265), *20* Illini (267), *21* Centennial (269) *22* Mammoth Yellow (271), *23* Calland (273), *24* Suxie (275), *25* PI 84987A, and *26* Ogden (289)

them. The matching coefficient equalled 1.0 only in the comparison of the cultivars Lee and Pickett. The pedigree of Lee is S-100 × CNS and that of Pickett is [(D49–2491)^6 × (Dorman)] × (Lee^4 × Peking). D49-2491 was derived from the same F_2 plant as Lee. The coefficient of parentage of Lee and Pickett is 0.838 (Carter et al. 1993). The estimate of the coefficient of parentage of two soybean cultivars assumes no genetic relatedness among the Far Eastern ancestral cultivars used in North American soybean breeding. Carter et al. (1993) considered this assumption as faulty and calculated a more realistic estimate of genetic relatedness referred to as "genetic similarity". By this estimate they reported Lee and Pickett to be 93.4% identical. Clearly, these two cultivars are closely related and it is therefore not surprising that they are indistinguishable with only seven markers.

Conclusions

The set of microsatellite markers used here provides a positive assessment of the ability of SSR markers to produce unique DNA profiles of soybean genotypes. With careful selection of microsatellite loci for high levels of polymorphism it is very likely that 10 or 15 loci may be adequate to distinguish closely related cultivars arising from similar pedigrees. In the next 2 years we plan to identify and map between 250 and 300 new SSR loci. It is our intention to select a number of highly informative loci with gene diversity values exceeding 0.8 which can be used to provide the basis for a soybean DNA profile system. Such a system should be an excellent complement to morphological and other conventional markers that are currently used to obtain plant variety protection for new soybean cultivars.

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