Theor Appl Genet (1999) 98: 690–696

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Allele-specific hybridization markers for soybean

Received: 10 August 1998 / Accepted: 17 September 1998

Abstract Soybean (*Glycine max*) is one of the world's most important crop plants due to extensive genetic improvements using traditional breeding approaches. Recently, marker-assisted selection has enhanced the ability of traditional breeding programs to improve soybeans. Most methods of assessing molecular markers involve electrophoretic techniques that constrain the ability to perform high-throughput analyses on breeding populations and germplasm. In order to develop a high-capacity system, we have developed allele-specific hybridization (ASH) markers for soybean. As one example, restriction fragment length polymorphism (RFLP) locus A519-1 (linkage group B) was converted into an ASH marker by (1) sequencing the pA519 cloned insert, (2) designing locus-specific PCR amplification primers, (3) comparative sequencing of A519-1 amplicons from important soybean ancestors, and (4) designing allele-specific oligonucleotide probes around single nucleotide polymorphisms (SNPs) among soybean genotypes. Two SNPs were identified within approximately 400 bp of the sequence. Allele-specific probes generated a 100-fold greater signal to target amplicons than to targets that differed by only a single nucleotide. The A519-1 ASH marker is shown to cosegregate with the A519-1 RFLP locus. In order to determine ASH usefulness, we genotyped 570 soybean lines from the Pioneer Hi-Bred soybean improvement using both A519-1 SNPs. Combined haplotype diversity (D) was 0.43 in this adapted germplasm set. These results demonstrate that ASH markers can allow for high-throughput screening of germplasm and breeding populations, greatly enhancing breeders' capabilities to do marker-assisted selection.

Key words Glycine max · Soybean · Allele-specific hybridization · PCR · Marker-assisted selection

Communicated by M. A. Saghai Maroof

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Introduction

Soybean represents one of the world's most important crop plants, providing most of the world's vegetable protein and 82% of the world's edible oil (American Soybean Association 1997 Soy Stats). In 1996, the United States planted 24% of its crop area with soybean and generated a $16.3 billion harvest representing 49% of the worldwide production (American Soybean Association 1997). Genetic improvements have contributed to soybean's cost-effective production. Essentially all of the genetic gains have been made by traditional breeding methods involving hybridization and phenotypic selection. Future genetic gains will doubtlessly result from a mixture of traditional, transgenic, and genetic-marker approaches.

Soybean breeding has always relied on the phenotypic assessment of traits to make selections for superior performing varieties. With the advent of molecular markers, soybean improvement programs have begun to implement marker-assisted selection (MAS) stratagems (Concibido et al. 1996). Because molecular markers have essentially 100% heritability, using markers to select for a low heritable trait is more effective and potentially less expensive than phenotypic selection for that trait (Winter and Kahl 1995). However, the effectiveness and cost of MAS is greatly influenced by the marker system used, and it must be chosen carefully.

Molecular marker technology has changed dramatically in the past few decades (Winter and Kahl 1995;

Burow and Blake 1998). The first molecular markers used in crop improvement were isozyme polymorphisms, where differently charged proteins with the same enzymatic function were used as locus identifiers. Restriction fragment length polymorphisms (RFLPs) overcame the major limitation of isozymes, that of limited genome coverage. In soybean, RFLPs have been used to construct detailed genetic maps and to map genes controlling complex traits (Apuya et al. 1988; Keim et al. 1992; Shoemaker et al. 1992; Yu et al. 1996). Polymerase chain reaction (PCR)-based markers are replacing RFLPs because of their speed and requirement for only small amounts of crude DNA. Unfortunately, many of the PCR-based markers such as random amplified polymorphic DNAs (RAPDs; Williams et al. 1990), arbitrarily primed PCR (AP-PCR; Welsh and McClelland 1990), and DNA amplification fingerprinting (DAF; Caetano-Anolles et al. 1991) are dominant in nature. Amplified fragment length polymorphisms (AFLPs) are frequently dominant markers but have the advantage of detecting an extremely large number of loci from a single PCR reaction (Vos et al. 1995). Simple sequence repeat (SSR) markers represent one of the most useful systems due to their great information capacity and codominant nature. In soybean, SSR markers have proven to be informative in many populations (Akkaya et al. 1992), and extensive development (Akkaya et al. 1995) has made these markers the choice for MAS. All of these methods are limited by their requirement to electrophoretically separate DNA fragments.

Non-electrophoretic marker techniques to detect alleles have focused on the interaction of oligonucleotide probes with DNA targets. In the simplest form, PCR amplification primers are developed around single nucleotide polymorphisms (SNPs) where successful amplification is detected by fluorescent dyes and is allele specific (Gu et al. 1995). Primer extension has also been used such that the SNP is immediately adjacent to a primer, and colorimetrically labeled dideoxy nucleotides are used to allow a single-base polymerase extension (Nikiforov et al. 1994). A commercially available system called TAQMAN™ involves the hybridization of a fluorescently labeled probe to PCR amplicons that can be detected during the PCR reaction (Heid et al. 1996; Landegren 1996; Kalinina et al. 1997).

Originally, allele-specific hybridization (ASH) methods used allele-specific probes that were hybridized to membranes containing restricted genomic DNA (Wallace et al. 1979, 1981; Conner et al. 1983; Kidd et al. 1983; Pirastu et al. 1983; Angelini et al. 1986). This technique was limited by the requirement for large amounts of genomic DNA and the use of electrophoresis. Later, Koenraadt and Jones (1992) and Wheeler et al. (1995) used locus-specific PCR to obtain amplicons from *Venturia inaequalis* genomic DNA, blotted the amplicons onto membranes, then hybridized these with allele-specific probes to determine which allele an individual strain possessed. This method has the advantage of requiring much less genomic DNA than the earlier ASH applications.

In this report, we describe an approach to ASH analysis in soybean that is capable of non-electrophoretically detecting a single-base substitution and which is suitable for high-throughput applications. This approach requires considerable resources to develop (a comparative sequence database), but the subsequent savings in time and effort can more than compensate for the initial investment required. Our approach has been to characterize a previously mapped RFLP locus by first sequencing the RFLP probe, designing genomic PCR amplification primers, and then sequencing representative soybean genotypes to identify SNPs. A set of oligonucleotide hybridization probes is then designed that are allele-specific, and these are used on PCR amplicons from the sample region. Populations can then be screened by extracting only a small amount of genomic DNA from each individual. There is great potential for this technique as a high-throughput technology as it allows thousands of plants to be genotyped in a single generation.

Materials and methods

DNA clone sequencing and primer design

The soybean genomic clone pA519 consists of the pBS^+ vector with an insert fragment generated from a *Pst*I digest of Iowa State University soybean breeding line A81–356022 DNA (Keim and Shoemaker 1988). Probe A519 hybridizes to genomic DNA located on linkage group B of the ISU-USDA genetic map (A519-1; Akkaya et al. 1995; Shoemaker and Specht 1995). It also hybridizes to loci on linkage groups C (A519-3) and D (A519-2; Akkaya et al. 1995; Shoemaker and Specht 1995). These other two loci are polymorphic in a *G. max* × *G. soja* F_2 mapping population but not in a mapping population generated from a cross of the two *G. max* cultivars 'Clark' and 'Harosoy' (Akkaya et al. 1995; Shoemaker and Specht 1995). Approximately 350 bases at each end of the insert were sequenced with an automated sequencer (ABI model 373A), using standard T3 and T7 promoter sequencing primers. The vector sequence was removed from each of the sequences, and two flanking amplification primers were designed using OLIGO® software (version 5.0 for Windows, 1994; National Biosciences, Plymouth, Minn.) and designated A519-5′ and A519-3′ (Table 1). Primer A519-5′ds was designed as a sequencing primer downstream from primer A519-5′ using A519 sequence generated with primer A519-5′ and OLIGO® software. The melting temperature (Table 1) of each primer was calculated using the nearest-neighbor method in OLIGO® and is designated as T_d by OLIGO®. These primers were designed with a target T_d of 68°C. Primer A519-3′ is located at bases 46 through 59 of the T7 end of the A519 sequence, while A519-5′ is located at bases 21 through 39 of the T3 end of A519, and primer A519-5′ds is at bases 306 through 330 of the T3 end of A519.

Genomic DNA preparation

Soybean [*Glycine max* (L.) Merrill] plants were grown by Pioneer Hi-Bred, International, Johnston, Iowa. Genomic DNAs were extracted from leaves of about 25 plants of each genotype following the

Table 1 Sequences of the PCR/sequencing primers used for A519

Primer name	Sequence (5′ → 3′)	T_d [a] (°C)
A519-5′	CCCCGTTGCCAGAAACAAG	68.3
A519-3′	CCAAAGAGTAAGAGGTTGCTGACA	68.0
A519-5′ds[b]	CTTGATGCCTATTTGTTTCTAAACC	66.7

[a] T_d is the oligonucleotide melting temperature calculated using the nearest-neighbor method
[b] A519-5′ds is an internal sequencing primer, 3′ to primer A519 5′

technique of Keim and Shoemaker (1988). Eight of the ten *G. max* genotypes used were ancestral cultivars which have contributed significantly to the North American germplasm (Gizlice et al. 1994): 'A. K. Harrow,' 'CNS,' 'Jackson,' 'Lincoln,' 'Mandarin Illinois' 'Ogden,' 'S100,' and 'Tokyo.' The other two genotypes studied were the cultivar 'BSR101' and the plant introduction 437.654, which are the parents of the recombinant inbred line (RIL) mapping population utilized in this study (Keim et al. 1994, 1997).

Genomic DNA amplification, amplicon sequencing, and sequence alignment

Genomic DNAs from the ten genotypes were screened for allelic diversity using PCR. The PCR reactions consisted of 20 m*M* TRIS-HCl, pH 8.4 (Gibco BRL Life Technologies, Gaithersburg, Md.), 50 m*M* KCl (Gibco BRL Life Technologies, Gaithersburg, Md.), 2.5 m*M* $MgCl_2$ (Gibco BRL Life Technologies, Gaithersburg, Md.), 50 μ*M* dATP (Promega Corporation, Madison, Wis.), 50 μ*M* dCTP (Promega Corporation, Madison, Wis.), 50 μ*M* dGTP (Promega Corporation, Madison, Wis.), 50 μ*M* dTTP (Promega Corporation, Madison, Wis.), 0.02 U/μl *Taq* DNA polymerase (Gibco BRL Life Technologies, Gaithersburg, Md.), 0.2 μ*M* primer 519-5′, 0.2 μ*M* primer 519-3′, and 2 ng/μl genomic template. The reactions were amplified in a MiniCycler (MJ Research, Waterloo, Mass.) with the following steps: (1) 94°C for 5 min, (2) 30 s at 94°C, (3) 45 s at 60°C, and (4) 90 s at 72°C. Steps 2 through 4 were repeated 40 times, followed by a final step (5) 72°C for 10 min. The amplicons were purified using QIAquick™ PCR Purification Kit (QIAGEN, Chatsworth, Calif.). The amplicons were sequenced using primer 519-5′ on a model 373A ABI automated sequencer.

The ten sequences were then compared by aligning them and searching for polymorphisms. The alignment was done using PC/GENE® (IntelliGenetics, Mountain View, Calif.). Primer 519–5′ds was used to generate additional sequences from the same ten amplicon samples used for the original sequencing. The additional sequences were used to extend the original sequences generated from primer 519-5′. The sequences were then realigned. The polymorphisms were confirmed with these additional sequencing data.

Probe design

Oligonucleotide probes were designed by placing the polymorphic nucleotide as centrally located as possible and targeting a melting temperature (T_m) of 32°C (Fig. 1) using the [2(A + T) + 4(G + C)] method as calculated by OLIGO® software (Fig. 1).

Amplicon and dot blot membrane preparation

'A. K. Harrow', 'BSR 101', 'CNS', 'Lincoln', 'Mandarin Illinois', 'Ogden', P. I. 437.654, and 'S100' genomic DNAs were used individually as templates for PCR amplification with primers 519-5′ and 519-3′. The resulting amplicons were quantified by agarose gel analysis by comparison with known quantities of *Hind*III-digested lambda phage DNA. Each of these DNAs was diluted to concentrations of 1 ng/μl, 0.1 ng/μl, and 0.01 ng/μl with denaturing solution (0.6 *M* NaCl, 0.4 *M* NaOH).

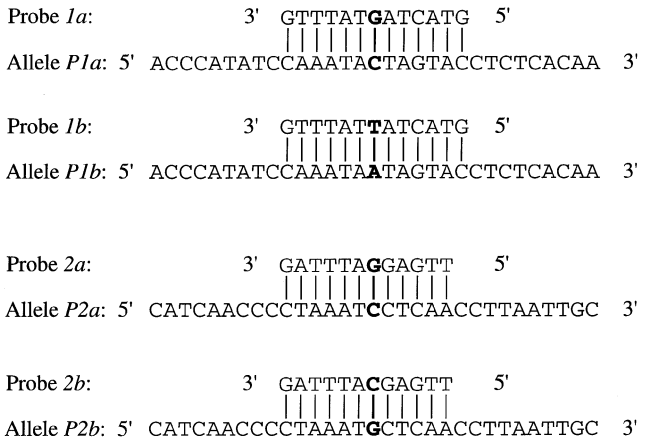

Fig. 1 Allele-specific probes and their target sequences. Polymorphic bases are shown in *bold*

A 9 × 13-cm rectangle of Whatman 3-mm chromatography paper (Whatman, Maidstone, UK) was moistened with denaturing solution and placed on the manifold base of a HYBRI·DOT 96-well filtration manifold (Gibco BRL Life Technologies, Gaithersburg, Md.). An 8 × 12-cm rectangle of Hybond™-N + (Amersham, Arlington Heights, Ill.) was moistened with denaturing solution and placed on top of the Whatman paper. The manifold was then assembled.

One hundred microliters of each DNA solution was placed into adjacent wells, resulting in 100, 10, or 1 ng of total DNA in each well; the dilution series was replicated four times. A vacuum was applied to the manifold until all the liquid was drawn from the wells. The manifold was disassembled, and the membrane placed for 2 min, DNA-side up, onto Whatman 3-mm paper that was saturated with denaturing solution. The membrane was then transferred for 10 min, DNA-side up, to Whatman paper saturated with 0.5 *M* TRIS-Cl, pH 7.5, and 1.5 *M* NaCl. The membrane was baked for 1 h at 85°C and exposed to 200 μJ of UV radiation (UV Stratalinker™ 1800, Stratagene, La Jolla, Calif.).

Probe labeling, hybridization, and probe visualization

The probe was radioactively labeled by kinasing with [γ^{32}P] ATP: a solution of 2.5 μl distilled, deionized water, 2 μl of 5 × Forward Reaction buffer (Gibco BRL Life Technologies, Gaithersburg, Md.), 2.5 μl of a 2 μ*M* probe solution, 2 μl [γ^{32}P]ATP (370 kBq/μl, Gibco BRL Life Technologies, Gaithersburg, Md.), and 1 μl T4 polynucleotide kinase (10 U/μl solution, Gibco BRL Life Technologies, Gaithersburg, Md.) was incubated at 37°C for 45 min. Five milliliters of hybridization/wash buffer (0.75 *M* Na, 0.5 *M* PO_4, 1.0 m*M* Na_2 EDTA, and 1% sarkosyl) was added to the radioactive probe solution. The solution was then layered on top of the membrane and allowed to hybridize overnight at room temperature, with gentle shaking. The radioactive probe solution was then decanted and the blot rinsed with 5 ml of hybridization/wash buffer, which was subsequently decanted. Two 15-min washes, two 30-min washes, and one 60-min wash with 5–10 ml of hybridization/wash buffer each wash, with gentle shaking, were applied to the membrane. The membrane was then wrapped in Saran Wrap™ (Dow Chemical Company, Indianapolis, Ind.) and exposed to X-ray film (Konica Medical Corporation, Newark, N.J.) for 18 h.

Results

In order to perform comparative sequencing of the A519-1 locus in soybean germplasm, we designed genomic amplification primers from pA519 clone sequence data. The cloned insert sequence was determined using vector-specific primers (T3 and T7). The genomic amplification primers A519-5′ and A519-3′ (Table 1) were designed and then used to amplify the A519-1 locus from genomic DNAs of 'A. K. Harrow', 'BSR 101', 'CNS', 'Jackson', 'Lincoln', 'Mandarin Illinois', 'Ogden', P. I. 437.654, 'S100', and 'Tokyo'. The use of these primers resulted in an amplicon of approximately 1400 base pairs from each of the soybean genotypes (data not shown).

Nucleotide sequence polymorphisms among the ten genotypes were identified by sequencing the amplicons on the 5′ side of the sequence (T3 vector side) using the A519-5′ primer. Sequence comparisons revealed two differences: polymorphism 1 (*P1*), a C/A substitution at base 343 of the A519-5′ sequence, and polymorphism 2 (*P2*), a C/G substitution at base 399 of the A519 5′ sequence (Fig. 2). In both cases, the more frequently observed allele was designated the *a* allele.

The genotypic distribution of nucleotide differences reveals a high degree of disequilibrium between *P1* and *P2*. In all of the sequenced genotypes except BSR101, the *a* alleles are in coupling phase (Fig. 2). The observed allelic combinations result in three of four possible haplotypes with only the *P1b*/*P2a* haplotype not represented. Haplotype I (*P1a*/*P2a*) is the most frequently observed, haplotype II (*P1b*/*P2b*) is the second most frequently observed, and haplotype III (*P1a*/*P2b*) is the rarest haplotype among the ten genotypes sequenced.

Four allele-specific oligonucleotide probes were designed to allow non-electrophoretic detection of specific haplotypes (Table 2). Four identical membranes were prepared by dotting 100 ng, 10 ng, and 1 ng of purified amplicons from eight genotypes onto each membrane. Each probe was radioactively labeled by kinasing with [γ^{32}P]ATP and then hybridized to one of the membranes. The membranes were exposed to X-ray film to visualize the extent to which the various probes hybridized to the membranes. All four probes hybridized well with their perfectly complementary (target) sequences and poorly with single-base mismatched (nontarget) sequences, as measured by spot intensity (Fig. 3).

Probe specificity was determined by comparing the hybridization intensities of target and nontarget spots. For example, the probe 1a 100-ng signal for 'CNS' (allele *1b*) is essentially equivalent to the 1-ng signal for 'A. K. Harrow' (allele *1a*). We therefore estimate that the nonspecific hybridization for probe 1a is about 1/100. Likewise, the nonspecific hybridization for probes 1b, 2a, and 2b is estimated to be 1/100, 1/50, and 1/50, respectively.

The genetic map location of the A519-1 amplicon and allele-specific probes on linkage group B was confirmed in a segregating population. The ASH probes were used to genotype 72 recombinant inbred lines (RILs) from the well-characterized mapping population generated from 'BSR 101' and PI437.654 (Keim et al. 1997). At the *P1* SNP, 'BSR101' has allele *a*, while PI437.654 has allele *b*. Each RIL allele was determined by amplifying genomic DNA with primers A519-5′ and A519-3′, dot-blotting, and hybridizing with probes 1a and 1b. Only 2 of the 72 ASH scores differed from the RFLP scores used to identify the map location of A519 (data not shown). In 1 case, the ASH genotype was allele *a*, while the corresponding RFLP genotype was heterozygous. This could be due to the sampling of multiple individuals within the F_6-derived RIL prior to DNA extraction for RFLP genotyping, while DNA from a single plant was used for ASH genotyping. The other RIL had allele *b* when determined by RFLP analysis and allele *a* when determined by ASH analysis.

A

Polymorphism 1 (*P1*) sequences (5'→3'):

Allele *a*: acccatatcc aaata**C**tagt acctctcaca

Allele *b*: acccatatcc aaata**A**tagt acctctcaca

Polymorphism 2 (*P2*) sequences (5'→3'):

Allele *a*: catcaacccc taaat**C**ctca accttaattg

Allele *b*: catcaacccc taaat**G**ctca accttaattg

B

Genotype	*P1* Allele	*P2* Allele	Haplotype
A. K. Harrow	*a*	*a*	I
BSR101	*a*	*b*	III
CNS	*b*	*b*	II
Jackson	*a*	*a*	I
Lincoln	*a*	*a*	I
Mandarin Illinois	*b*	*b*	II
Ogden	*a*	*a*	I
P. I. 437.654	*b*	*b*	II
S100	*a*	*a*	I
Tokyo	*a*	*a*	I

Fig. 2 A The allele sequences for polymorphism 1, at base 343 from the 5′ end of the A519 insert, and polymorphism 2, at base 399 from the 5′ end of the A519 insert. Polymorphisms are shown in *uppercase*, *bold letters*. **B** The genotypic distributions of the alleles for each of the polymorphisms and the haplotype of each genotype

Table 2 Allele-specific probes used on A519 amplicons

Probe name	Sequence (5′ → 3′)	T_m[a] (°C)
1a	GTACTAGTATTTG	34.0
1b	GTACTATTATTTG	32.0
2a	TTGAGGATTTAG	32.0
2b	TTGAGCATTTAG	32.0

[a] T_m was calculated using the [2(A + T) + 4(G + C)] method

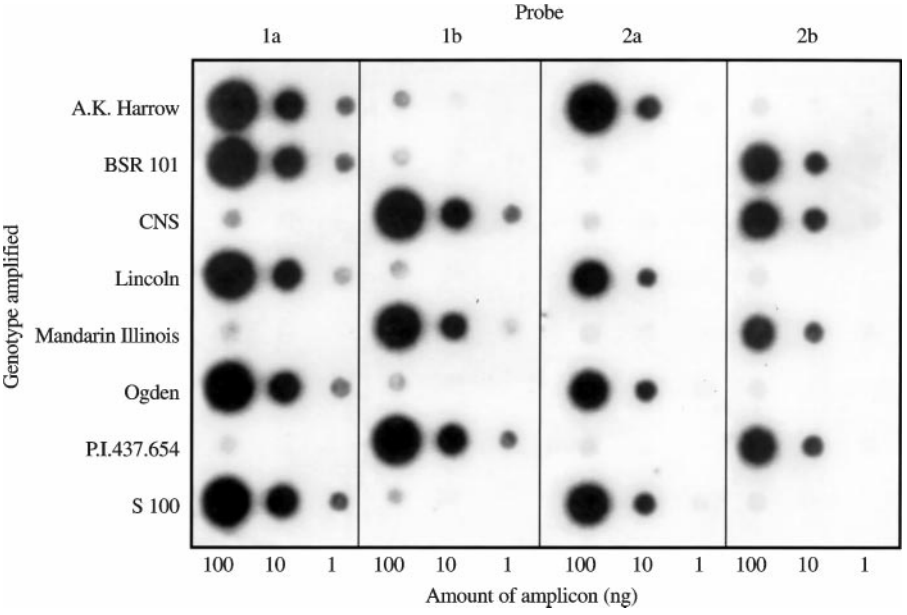

Fig. 3 Autoradiogram of allele-specific probes hybridized to varying amounts of PCR-amplified target sequence. Four membranes were dotted identically with PCR product obtained by amplifying each of the eight genotypes with primers A519-5′ and A519-3′. One hundred, ten, or one nanogram of PCR product was dotted onto the membranes, as indicated *below* each column. A radioactive probe was hybridized to a membrane, followed by exposure to X-ray film

This may be the result of seed contamination or sample misidentification at some point between the RFLP and ASH analyses. Because this particular RIL has manifested discrepancies between RFLP and ASH markers at 8 of 29 other loci tested (D. Webb, personal communication), it seems likely that seed mixture or contamination has occurred in this case. These discrepancies aside, it is clear that the ASH A519-1 map location and that of the RFLP marker are the same, both on linkage group B of the public soybean genetic map (Akkaya et al. 1995, Shoemaker and Specht 1995, Keim et al. 1997). The true genetic map location is important to confirm due the extensive genome duplication observed in soybean and the other RFLP loci detected with pA519.

The frequencies of A519-1 ASH alleles and haplotypes in a broad collection of soybean germplasm were determined. We chose to analyze 570 soybean lines from the Pioneer Hi-Bred breeding program to determine the applicability of this system to a major soybean breeding program. A519-1 amplicons from each line were probed with each of the four ASH probes to ascertain the relative frequencies of the various alleles and A519-1 haplotypes. At *P1*, the allele frequencies were 88.26% for *1a* and 11.74% for *1b*. At *P*2, the frequency of allele *2a* was 72.87% versus 27.13% for allele *2b*. The haplotype frequencies of I, II, and III are 72.91%, 11.67%, and 15.42%, respectively. One line was heterozygous at both loci, which made its haplotype determination impossible. The Nei's diversity indices were calculated to be 0.21, 0.40 and 0.43 for *P1*, *P2*, and the combined haplotypes, respectively. The diversity index values directly indicate the percentage of populations for which this marker would be informative in a random mating design.

Discussion

Using the A519-1 genetic locus as an example, we have demonstrated the feasibility of detecting DNA-based genetic variation in soybean germplasm without using electrophoretically based technologies. Molecular markers have the potential for improving the efficiency of soybean breeding, but high throughput methods are necessary for large breeding programs. Allele-specific hybridization has the potential for higher throughput than more commonly used technologies. The data presented here demonstrate that a single nucleotide polymorphism between alleles can be unambiguously detected in soybean by ASH. This procedure allows for the screening of thousands of lines, accommodating conditions where high throughput is desired. The PCR-based technology also allows for minute amounts of original genetic material to be used, enabling MAS to be applied early in the breeding cycle, thus conserving resources and time. This technique has the potential to be a powerful tool for soybean breeders. Additionally, recent advances in microtechnology have resulted in the development of the "DNA chip", where hundreds of oligonucleotides are covalently attached to small silicon dioxide chips; these chips are then used in hybridization experiments (Lamture et al. 1994; Stimpson et al. 1995; O'Donnell-Maloney and Little 1996). The power and throughput of ASH would be greatly increased by the incorporation of these new approaches, enhancing breeders' MAS capabilities.

The level of variation observed for the A519 ASH marker was comparable to that of most RFLP markers. Keim et al. (1992) reported that in adapted soybean germplasm, RFLP markers had diversity values of less than 0.5 with an average of 0.32. The

haplotype diversity value of 0.43 is at the high end of the range observed in RFLPs. The potential to increase the haplotype value with further study is very likely. The A519 amplicon itself was approximately 1400 bp, but only 400 nucleotides were examined for polymorphisms in this study. Additional sublocus differences could potentially generate additional haplotype discrimination. While disequilibrium among subloci was high, it wasn't complete. This suggests that many more than two haplotypes are possible and that regions separated by a greater distance, but still within a single locus amplicon, may be more useful. It is unlikely that ASH diversity would ever approach that observed in SSR markers, but non-electrophoretic approaches using SSRs also seem unlikely. Soybean breeders now have a greater choice of marker systems, each with its own strengths and weaknesses, for their particular applications.

Acknowledgements We thank L. Shi and T. Zhu for the initial characterization of pA519. The experiments performed for this publication comply with the USA's current statutes.

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Note added in proof. The NCBI GenBank accession numbers for the *Glycine max* sequences utilized in this paper are as follows: 'A. K. Harrow' (AF125924), 'BSR101' (AF125933), 'CNS' (AF125930), 'Jackson' (AF125925), 'Lincoln' (AF125926), 'Mandarin Illinois' (AF125931), 'Ogden' (AF125927), P.I. 437.654 (AF125932), 'S100' (AF125928), 'Tokyo' (AF125929), A81-356022 T3 end (AF125934), and A81-356022 T7 end (AF 125935)

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