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Targeted isolation of simple sequence repeat markers through the use of bacterial artificial chromosomes

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Abstract Simple sequence repeats (SSRs) are versatile DNA markers that are readily assayed and highly informative. Unfortunately, non-targeted approaches to SSR development often leave large genomic regions without SSR markers. In some cases these same genomic regions are already populated by other types of DNA markers, especially restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), and amplified fragment length polymorphisms (AFLPs). To identify SSR markers in such regions, bacterial artificial chromosome (BAC) clones can be used as intermediaries. First, one or more BAC clones in a region of interest are identified through the use of an existing DNA marker. BAC clones uncovered in this initial step are then used to create a small insert DNA library that can be screened for the presence of SSR-containing clones. Because BAC inserts are often 100-kb pairs or more in size, most contain one or more SSRs. This strategy was applied to two regions of the soybean genome near genes that condition resistance to the soybean cyst nematode on molecular linkage groups G and A2. This targeted approach to identifying new DNA markers can readily be extended to other types of DNA markers, including single nucleotide polymorphisms.

Key words Bacterial artificial chromosome · Simple sequence repeats · Microsatellites · Soybean cyst nematode · Genetic mapping

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Introduction

DNA markers are powerful tools for genomic analysis, positional gene cloning, marker-assisted selection, and studies of genome evolution. Markers based on length variation of simple sequence repeats (SSRs) or "microsatellite" DNA sequences have proven especially versatile. SSRs consist of 2–5 nucleotide core units such as (CA), (ATT), or (ATGT) that are tandemly repeated in the genome (Litt and Luty, 1989). The regions flanking a microsatellite are generally conserved among genotypes of the same species and PCR primers to the flanking regions can be used to amplify the SSR-containing DNA fragment. Length polymorphism is created when PCR products from different alleles vary in length as a result of variation in the number of repeat units in the SSR, and can then be analyzed by acrylamide or agarose-gel electrophoresis to resolve contrasting alleles. The high level of informativeness and co-dominance of microsatellite markers, their widespread occurrence in eukaryotic genomes, and easy amplification via standard PCR technology, make SSRs the current marker of choice in many species. In the past few years, dense microsatellite-based linkage maps have become available in some species including humans (Hudson et al. 1995) and mice (Dietrich et al.

1996), though SSR maps of lower marker density remain the norm for agriculturally important species such as swine (Alexander et al. 1996), soybean (Cregan et al. 1998), and maize (Senior et al. 1996).

Despite the advantages of microsatellites markers, the high cost of development has been a major impediment to the development of SSR-based linkage maps. SSR marker development not based upon pre-existing DNA sequence data is time-consuming and tedious. Typically, a genomic library of size-selected DNA fragments is probed with one or more radiolabeled SSR motifs. Positive clones are then isolated and sequenced to verify the presence of a microsatellite and to allow PCR primer selection from the flanking DNA. Further testing is then required to determine the level of polymorphism of the SSR locus. Only at this point can new microsatellite loci be analyzed by segregation analysis and placed on framework linkage maps. SSR loci developed from genomic DNA libraries tend to map randomly throughout the genome (Dietrich et al. 1992; Rohrer et al. 1994; Akkaya et al. 1995).

The random distribution of SSR markers results in large genomic regions untagged with SSR loci. In order to target microsatellite marker development to specific regions of the genome, chromosome-specific libraries have frequently been used as the source of genomic DNA for microsatellite discovery. These efforts generally depend upon chromosome-specific flow-sorted libraries (Vooijs et al. 1993; Grimm et al. 1997) or directed microdissection of chromosomal regions (Weikard et al. 1997; Ambady et al. 1998). SSR marker development targeted to specific genomic intervals has also been based on large-insert cosmics (Malo et al. 1993; Byrne et al. 1997), yeast artificial chromosomes, and P1 clones (Chen et al. 1995; Nehls et al. 1995), as a source of microsatellite-containing DNA fragments.

The recently published SSR/RFLP (restriction fragment length polymorphism) map of soybean includes a total of more than 600 SSR loci mapped in one, two or three different mapping populations (Cregan et al. 1998). A total of 412 SSR loci were positioned in one mapping population that consisted of 240 recombinant inbred lines derived from a cross of the cultivars Minsoy and Noir 1. The resulting map, approximately 2400 centimorgans (cM) in length, contains 36 intervals of at least 20 cM in which no microsatellite loci are positioned. To increase the utility of the soybean microsatellite map, it is essential to place SSR markers in these intervals.

In separate research, bacterial artificial chromosome (BAC) libraries have been developed for many important species, including soybean (Marek and Shoemaker 1997; Danesh et al. 1998). The inserts in these libraries tend to be quite large, up to 150 kilobase pairs (kbp) or more in size (Woo et al. 1994; Wang et al. 1995). These libraries can be easily screened, either by DNA hybridization of high-density colony blots by PCR-amplification of BAC clone pools (Green and Olson 1990; Woo et al. 1994). Since the frequency of di-tri- and tetranucleotide microsatellites in plants has been estimated at one in every 21.2 kbp in dicotyledonous plants and one in every 64.6 kbp in monocots (Wang et al. 1994), a typical BAC clone would be expected to contain one or more SSR sequences. BAC clones also have an important advantage over YAC clones in that there is little possibility of contamination with eukaryotic DNA that might contain microsatellite sequences. Therefore, it should be feasible to uncover new SSRs associated with previously characterized RFLP, amplified fragment length polymorphism (AFLP), or sequenced-tagged site (STS) markers using BACs as intermediaries. Employing this strategy, two sets of soybean BAC clones were examined for the presence of microsatellites. The two sets of clones were located in strategically important regions of the genome and were identified through the use of previously mapped RFLP- or PCR-based markers. For each set of BAC clones, five or more $(ATT)_n$ and $(AT)_n$ microsatellite sequences were found, and for each linkage group three new polymorphic SSR markers were developed. Physical and genetic linkage mapping confirmed that the new SSRs mapped to the correct sites in the genome.

Materials and methods

Plant materials

Two soybean cultivars were used as sources of DNA for the construction of BAC libraries: Faribault (Danesh et al. 1998) and Williams 82 (Marek and Shoemaker 1997). Several additional soybean cultivars and one plant introduction (PI) were used to create mapping populations for the map placement of SSR and other DNA markers. One mapping population was derived from a cross of soybean cultivar Evans and PI 209332 (Concibido et al. 1996). A second mapping population was derived from a cross of the soybean cvs Minsoy and Noir 1 (Mansur et al. 1993) and consisted of 240 F_7-derived recombinant inbred lines. To characterize SSR polymorphism during the process of marker development, the following soybean cultivars were assayed: Clark, Harosoy, Jackson, Williams, Amsoy, Archer, Fiskeby V, Minsoy, Noir 1, and Tokyo.

Construction of BAC libraries

One BAC library (referred to as 'UMN") consisting of approximately 30 000 clones with an average insert size of 120 kbp (three genome equivalents) was generated from the DNA of soybean cultivar Faribault (Danesh et al. 1998). This library was created by partial *Eco*RI digestion of high-molecular-weight genomic DNA and ligation into the vector pECSBAC4 (Frijters et al. 1997). A second BAC library (referred to as "USDA-ISU") consisting of approximately 40 000 clones and an average insert size of 150 kbp (4–5 genome equivalents) was generated from the DNA of soybean cultivar Williams 82 (Marek and Shoemaker 1997). Briefly, this involved partial *Hin*dIII digestion of high-molecular-weight genomic DNA and ligation into the vector pBeloBAC11 (Kim et al. 1996).

Identification of specific BAC clones

The UMN BAC library was screened with several RFLP markers known to map on molecular linkage group (MLG)-G of soybean, very close to the soybean cyst nematode resistance gene, *rhg1* (Concibido et al. 1996). The RFLP clones were radiolabeled and hybridized to high-density filters as described (Danesh et al. 1998). (The preparation of these filters was carried out in the laboratory of Dr. Rod Wing, Clemson University, and with the generous assistance of Dr. David Frisch.) Positive clones were verified by digestion with the restriction enzyme originally used to map the RFLP locus, followed by a comparison of the hybridizing fragments. End- and sub-clones were generated as described (Danesh et al. 1998). Linkage mapping of BAC end- and sub-clones also demonstrated that these BAC clones were located at the expected chromosomal positions and that there were no chimeras within the genomic inserts.

Three-dimensional pools of the USDA-ISU BAC library were prepared as described by Marek and Shoemaker (1997). Briefly, each clone in the library was represented in three DNA pools: a specific full-plate pool, a row pool, and a column pool. The full-plate pools were generated by combining the DNA from all 384 clones in each individual microtiter dish. Row pools were generated by combining the DNA from all of the wells in row 1 from blocks of 20 plates, and repeating this procedure with all the wells in row 2, and so on. Column pools were generated in a like manner with all the wells in column A and repeating this procedure with the remaining columns (through column P). To make library screening more efficient, DNA super-pools were made by combining aliquots of the individual pools, as described by Marek and Shoemaker (1997). The USDA-ISU BAC library DNA pools were screened using oligonucleotide primers designed based upon sequence data from a soybean cDNA clone encoding the enzyme aspartokinase-homoserine dehydrogenase (AK-HSDH) gene (Weisemann et al. 1992; Matthews et al. 1998) which was previously positioned in MLG A2 within 0.6 cM of the *rhg4* locus (Webb et al. 1995). The BAC clones selected in this manner were assembled into a contig, as suggested by Marek and Shoemaker (1997).

Sub-cloning of BACs

BAC clones identified in the preceding steps were then used as a source of DNA for the construction of small-insert libraries suitable for screening SSR markers. Preparations of BAC DNA were made as described previously (Marek and Shoemaker 1997; Danesh et al. 1998). BAC-DNA from each of the targeted regions (MLG-G and MLG-A2) were then pooled into two sets according to genome location. Set 1 consisted of two clones, UMN-I18 and UMN-K9, and Set 2 consisted of eight clones, USDA-ISU BAC-6E3, -7B18, -29M4, -56G2, -57A5, -86G8, -86118, and -98H8. Approximately equal amounts of each BAC clone (0.25–0.5 μg) were combined to create each BAC pool.

Random sub-cloning of the two sets of BAC clones was undertaken in an effort to find all the $(ATT)_n$ and $(AT)_n$ SSRs present. One difficulty that has been observed in SSR discovery from genomic libraries is the presence of an SSR too close to the cloning site to allow sufficient sequence data to design a PCR primer (Cregan et al. 1994). A second difficulty is that many SSRs remain undetected because they are not present in DNA fragments that are in the size range of 500–700 base pairs (bp) typically used for the creation of the genomic libraries that accommodate single-pass sequence determination (Cregan et al. 1994). To minimize the likelihood of these potential problems, each set of BAC clones was digested with three different sets of restriction endonucleases and the resulting fragments were cloned without size selection, as described below.

Each BAC pool was digested with the restriction endonuclease *Sma*I in a total volume of 450 μl, consisting of 10 units of *Sma*I (New England Biolads, Beverly, Mass.) 20 mM Tris-Acetate, 10 mM Mg-acetate, 50 mM K-acetate (NEBuffer 4), and 0.1 mM bovine serum albumin (BSA) at 25°C for 1 h. The digest was divided into three aliquots of 150 μl each and 5 μl of 10 × NEBuffer 4, 0.5 μl of 10 mM BSA, and 42.5 μl of water were added to each. In addition, *Alu*I (8 units) and EheI (10 units) were added to the first aliquot; *Rsa*I (10 units) and *Nae*I (10 units) to the second; and *Hin*cII (10 units), *Xmn*I (6 units), *Ecl*136II (12 units) to the third, followed by digestion at 37°C overnight. Ten units of calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Beverly, Mass.) were added to each digest, which were held at 37°C for 15 min, followed by an additional incubation at 56°C for 15 min. Another 10 units of CIP were added to each digest followed by identical 15-min incubations at 37°C and 56°C, in turn followed by denaturation at 75°C for 10 min. A 100-μl aliquot from each of the resulting digests was pooled and precipitated with NH_4-Acetate/70% ethanol. The pellet was re-suspended in 40 μl of water. A total of 5 μl of this solution was used in a ligation reaction with 0.4 μg of pBluescript + KS (Stratagene, La Jolla, Calif.), 10 units of *Sma*I, 8 units of T4 ligase, 10 mM ATP, 1 × NEBuffer 4 in a total volume of 20 μl. The ligation reaction was allowed to continue for 60 h at 20°C, followed by denaturation of the ligase at 65°C for 15 min, after which 54 μl of water, 6 μl of 10 NEBuffer 4, and 10 units of *Sma*I were added. Digestion was allowed to proceed at 25°C for 2 h to ensure that any pBluescript + KS that did not contain an insert would be linearized. Competent cells of *Escherichia coli* XLII Blue (Stratagene, La Jolla, Calif.) were transformed as per the manufacturer's instructions.

Screening for the presence of $(AT)_n$ and $(ATT)_n$ SSRs

Transformed cells were plated on LB medium containing 0.1 mg/ml of ampicillin. X-gal (20 mg/ml) and IPTG (200 mg/ml) were added to the medium to allow blue/white color selection to identify transformed cells carrying plasmids with genomic inserts (Sambrook et al. 1989). Between 1000 and 1200 tranformants were screened on a 88-mm Petri plate for the presence of an insert containing a desired microsatellite as described previously by Cregan et al. (1994). Briefly, this procedure included colony lifts with a nitrocellulose membrane and growth on LB agar for 8 h, with additional growth for 12–15 h on LB agar containing chloramphenicol. This was followed by standard denaturation, neutralization, and vacuum-oven baking (Sambrook et al. 1989). Membranes were first screened with a $(TAA)_{10}$ oligonucleotide probe in which all the adenine residues were ^{32}P labeled. The probe was stripped and the membranes re-screened with a $(AT)_{15}$ oligonucleotide probe in which all the adenine residues were ^{32}P labeled. Selected colonies were picked onto microtiter plates followed by two additional cycles of screening and purification.

Characterization of SSRs

The length of the genomic inserts of selected clones from BAC Set 1 was estimated by PCR and agarose-gel electrophoresis. Template DNA was derived from a single colony lysed in 100 μl of 0.1% Tween-20 and heated for 15 min at 95°C. A total of 5 μl of this lysate was used in a 50-μl PCR amplification reaction with T3 and T7 primers. Products were separated on a 1.0% agarose gel. Plasmids of all clones were isolated using the QIAwell 8 Plasmid Kit of QIAGEN Inc. (Valencia, Calif.). Genomic inserts were sequenced using the ABI Prism dRhodamine Terminator Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) with SK and/or KS primers. When necessary to obtain the complete sequence, data were obtained from both ends of the clone. In those instances where clones were sequenced from both ends, a consensus sequence was determined by analysis with Auto-Assembler software (Perkin-Elmer, Applied Biosystems, Foster City, Calif.). After consensus sequences were determined, all sequences from the set of BAC clones were compared using Auto-Assembler software in order to detect duplicate sequences. Those consensus sequences containing a dinucleotide repeat of 20 or more base pairs, or a trinucleotide repeat of 21 or more base pairs, were considered to possess an SSR. This definition of the minimum length for a microsatellite is one that is widely used and generally accepted (Beckman and Weber 1992; Cregan 1992; Lagercrantz et al. 1993; Morgante and Olivieri 1993; Bell and Ecker 1994; Wang et al. 1994).

Primer selection and testing

For each unique microsatellite that was identified and for which sufficient reliable flanking sequence data were available, PCR primers to the flanking regions were selected using the primer selection program OLIGO (National Biosystems, Inc., Plymouth, Minn.). Primers were selected to function under the standard PCR conditions used in the development of over 600 soybean SSR loci (Cregan et al. 1998) with an optimal annealing temperature of 47°C, low 3′ end stability, a G-C clamp towards the 5′ end of each primer, minimal primer-dimer formation, and a product size between 100 and 300 bp.

PCR primers were initially tested in amplification reactions using two different templates: (1) the plasmid carrying the BAC subclone originally sequenced to determine the presence of an SSR, and (2) genomic DNA of either soybean genotype Faribault (BAC Set 1) or Williams (BAC Set 2). Those primer sets producing a single discrete product with the genomic DNA as a template were further examined in amplification reactions using the ten diverse soybean cultivars referred to above. These reactions, which included alpha-^{32}P, were conducted as described by Akkaya et al. (1995). Products were separated on a sequencing gel followed by drying and overnight exposure to X-ray film. Those primer sets producing a single product with each of the ten genotypes, and for which there was at least one product length difference among the ten genotypes, were assigned a locus name. Microsatellite markers based on AT-repeats were given designations starting with "BARC-Sat_", and those based on ATT-SSRs were given the designation starting with "BARC-Satt". To estimate the informativeness of each locus, a gene-diversity score was calculated as described by Rongwen et al. (1995) based upon the frequency of allele sizes in the ten diverse soybean genotypes.

SSR amplification from BAC templates

SSR sequences were also characterized using the BAC clones from which they originated as templates. To test BAC clones from the UNM library, primer sets were used to amplify 15 pg of BAC template DNA employing the amplification conditions described in Mudge et al. (1997). The following modifications were made for Satt610 and Sat_141: reactions were run in the absence of radiolabeled nucleotides, the reaction volumes were scaled to 15 μl and products were analyzed by 3.5% Metaphor agarose (FMC BioProducts, Rockland Me.) electrophoresis.

Genomic DNA of cultivar Williams and individuals BACs from the USDA-ISU library were also screened by PCR to identify the BACs that generated specific SSR markers. PCR reactions were carried out in a 50-μl reaction volume containing PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100], 1.5 mM $MgCl_2$, 0.15 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 0.15 μM of each primer, 1.0 U of *Taq* polymerase enzyme and 1 μl of a 1:1000 dilution of BAC DNA (approximately 100 pg). In the case of Satt632, amplification conditions were as described by Mudge et al. (1997). A touchdown PCR procedure was employed in reactions using primers to Sat_157 and Sat_162. The thermocycling program had an initial 30 s denaturation step at 95°C, followed by 11 cycles of 30 s each of denaturation (94°C) annealing, and extension (68°C). The annealing temperature was reduced by 1°C intervals from 57 to 46°C from cycles 1 to 11. This was followed by 22 cycles each of denaturation, annealing, and extension of 94°C, 46°C and 68°C. The program terminated with a 4°C soak. PCR products were separated on 3.0% Agarose3:1 (Ameresco, Solon, Ohio) and visualized by staining with ethidium bromide.

Genetic mapping of SSRs and BAC ends

The newly developed SSR markers described in this study were placed on medium-density DNA marker maps of soybean. Those originating from BAC clones on MLG-G were mapped in a population derived from a cross between Evans and PI 209332. Out of an original mapping population of 98 $F_{4:5}$ lines, a sub-set of 17 lines recombinant in the marker interval (between RFLP markers Bng122 and C006) near *rhg1* on MLG-G were used to pinpoint the exact location of the newly identified SSRs. A total of 21 DNA markers have previously been located in this 7.5-cM region of the soybean genome (N. D. Young et al., unpublished observations). The SSR markers originating from BAC clones on MLG-A2 were mapped in the 240 recombinant inbred lines of the University of Utah Minsoy × Noir 1 mapping population. A total of more than 630 DNA markers now reside on this map, including 48 DNA marker loci on University of Utah linkage group U03, which is the equivalent of MLG-A2 (Cregan et al. 1998).

In each case, genomic DNA from individuals in the appropriate mapping population was used as a template for PCR-amplification reactions. In the case of the Evans × PI 209332 $F_{4:5}$ lines, PCR reactants and conditions were as described by Mudge et al. (1997). The analysis of the Minsoy × Noir 1 recombinant inbred lines was completed as described by Cregan et al. (1998). Mapping data for both populations were analyzed using Mapmaker version 2.0 (settings: LOD score = 3.0 and a 0.4 recombination fraction) (Lander et al. 1987).

Results

Identification and characterization of BAC clones

In previous mapping studies, the genomic region proximal to *rhg1* on MLG-G was found to be poorly populated with DNA markers, and the markers located there tended to be relatively low in polymorphism (Shoemaker and Olson, 1993; Concibido et al. 1996). Two SSR markers (BARC-Satt038 and BARC-Satt130), that flanked *rhg1* by 0.8 cM on the distal side and 15 cM on the proximal side, were identified in this region by random screening of soybean genomic DNA (Mudge et al. 1997). SSRs more tightly linked to the gene, particularly on the proximal side, are imperative for marker-assisted breeding and high-resolution mapping. Therefore, BAC clones that corresponded to RFLP markers located in this genomic region represented an attractive source of potential SSRs.

Screening the UMN BAC library with RFLP clone Bng122D uncovered one BAC clone, UMN BAC-I18, approximately 110 kbp in length. Bng122D is known to be located 4 cM proximal to *rhg1* (Fig. 1 A). Comparison of the genomic fragment size that corresponds to Bng122D and the corresponding fragment from UMN BAC-I18 indicated that this clone was located on MLG-G (Danesh et al. 1998). Screening the UMN BAC library with RFLP marker D0140D uncovered a second clone, UMN BAC-K9, approximatley 100 kbp in length. D0140D has been previously mapped to a locus 3.5 cM proximal to *rhg1* (Fig. 1 A). Genetic mapping of sub- and end-clones from this BAC clone confirmed that it, too, was located on MLG-G (Danesh et al. 1998).

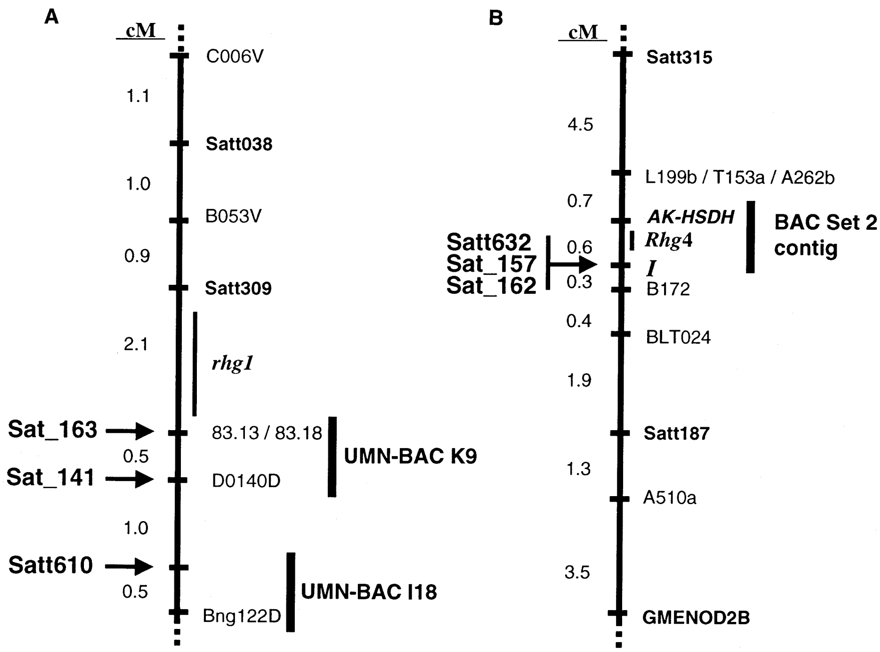

Fig. 1 **A** Genetic linkage maps in a target region on soybean MLG G composed of RFLP markers and two SSR loci (BARC-Satt038 and BARC-Satt309) in the region near the soybean cyst nematodes resistance gene, *rhg1*. Distances are given in centimorgans. Locations of the UMN BAC clones described in this study are shown, as are the positions of the newly developed SSR loci BARC-Sat_141, BARC-Sat_163, and BARC-Satt610. **B** Genetic linkage map in a target region on soybean MLG A2 composed of RFLP and SSR markers (BARC-Satt315, BARC-Satt187, and GMENOD2B) in the region near the cyst nematode resistance gene, *Rhg4*. Distances are given in centimorgans. The locations of the aspartokinase-homoserine dehydrogenase (AK-HSDH) and *l* loci are indicated, as is the approximate position of the contig of the USDA-ISU BAC clones making up BAC Set 2. The map positions of the newly developed SSR loci BARC-Satt632, BARC-Sat_157, and BARC-Satt162 are also shown

The region of MLG A2 near the *Rhg4* locus has long been of interest because of the presence of the *l* locus that controls seedcoat color and which has been closely associated with the *Rhg4* locus conferring resistance to the soybean cyst nematode (Matson and Williams 1965). Although a number of RFLP and SSR loci have been mapped to this region (Fig. 1 B), SSRs more closely associated with *Rhg4* would be very useful for high-resolution mapping. Additionally, it was important to have confidence in the precision of locus positions, and SSR markers provide a high degree of information content and locus specificity without the ambiguity typically associated with hybridization (RFLP-type) technologies. Screening of three-dimensional pools of the USDA-ISU BAC library using primers designed for the cDNA encoding AK-HSDH identified a total of eight clones in the USDA-ISU BAC library. Based upon restriction fingerprinting these were assembled into a contig of approximately 260 kbp in length. The position of this contig with reference to previously mapped loci in the region is shown in Fig. 1 B.

Identification and characterization of SSR markers

Rather than search for SSR sequences from each individual BAC clone, two pools of BAC clones were created. BAC Set 1 consisted of clones UMN-118 and UMN-K9 from *rhg1* region on MLG G and BAC Set 2 consisted of the eight USDA-ISU clones from the *Rhg4* region on MLG A2. The logic behind pooling clones was that the steps of sub-cloning and SSR identification are relatively tedious and complex. Furthermore, any SSR markers that were developed could be unambiguously associated with a specific BAC clone(s) with a relatively small number of PCR reactions.

Discovery of SSRs and microsatellite marker development

A total of 24 plasmid clones putatively containing an $(ATT)_n$ or $(AT)_n$ were identified from BAC Set 1, while 22 such clones were selected from the library created from BAC Set 2. The length of the PCR-amplified BAC-derived inserts from BAC Set 1 ranged from approximately 130 to 1300 bp. The sequence analysis of these 46 clones resulted in the recovery of 14 unique microsatellites (Table 1). This included four $(ATT)_n$, seven $(AT)_n$, one imperfect $(AT)_n$, and two compound SSRs. Nine of the 14 microsatellites were found in more than one clone. One$(AT)_{10}(CT)_{11}$ SSR from BAC Set 1 and one $(ATT)_{17}$ from Set 2 were each present in six different clones.

A total of 12 primer sets (Table 1) were synthesized and each was employed in PCR amplification reactions using the corresponding plasmid clone and Faribault or Williams genomic soybean DNA as a template. In the case of BAC Set 1, four of the five primer sets tested produced a single discrete product with Faribault soybean, while one produced multiple bands and was discarded. Subsequent testing of the primer sets on a panel of ten genotypes revealed that one was monomorphic while three produced single products with each genotype, showed allele size differences, and were therefore assigned locus names (Table 2). Of the seven primer sets derived from SSR-flanking regions from BAC Set 2, four produced multiple products with the genomic DNA or Williams soybean as a template and three were assigned locus names. Primers to locus BARC-Sat_163, distinct from the previously described locus BARC-Satt163 (Lange et al. 1998), were used in amplification reactions with genomic DNA of ten diverse soybean genotypes and the parents of one of the mapping populations, Evans and PI 209332. Separation on a DNA sequencing gel revealed four alleles among the ten genotypes (Fig. 2A). Products ranged in size from approximately 208 bp for Fiskeby and Minsoy to 233 bp for genotypes Jackson, Archer, and Tokyo. A fifth allele, with an estimated length of 242 bp, was present in PI 209332. Similar amplification reactions using primers to the BARC-Sat_162 locus

Table 1 Sequencing results of clones from BAC Set 1 and 2 selected to contain an $(ATT)_n$ or $(AT)_n$ microsatellite, the number of clones containing the identical SSR, and the forward and reverse primer sequences derived from their flanking regions

Unique SSR	No. of clones containing the identical unique SSR	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
BAC Set 1			
$(ATT)_{10}$[a]	5	gcggtaatggccatttattgtgaa	gcggaggcaaatgttaatgaatttgtt
$(ATT)_{12}$[a]	2	ccctccgcaagcaataattaatct	gcggaatgcttccattttat
$(AT)_{10}$ $(CT)_{11}$	6	cgcaatcaaagacctgtt	gccttggctatttcctta
$(AT)_{21}$	2	gcggtatatatgtttgcaagacatatt	gcggaatctcgcccaggaggaactt
$(AT)_{44}$	2	tcggcttaattctgagcatgt	gctctaaggcttttctcgtctcag
BAC Set 2			
$(ATT)_{12}$	2	gcgatggcaatgaatgatcactt	gcgtcgcactaatgaatagt
$(ATT)_{17}$	6	gggctatgaagggaatggaaagga	cccatattgaagatttgaagtaat
$(AT)_{18}$	1	gcggttttgcaagatgtgatgagt	gcgcgtacgcaaaatttatattca
$(AT)_{21}$	3	gcgccaatagtcaaacgtatataa	gcggtactcataatctaacgagagtat
$(AT)_{25}$	2	ccgtggttggaattaaaaaagaaga	gcggcttggacaaatgtgaaat
$(ATT)_{34}$	1	gggaagtgggatagcttatag	ggcggccgctctagaac
$(AT)_{36}$	1	gcgtggtttttcgctggatata	gcgcatttcgtaacatatttttcac
$(AT)_{16}(AG)_{32}$	1	Poor Sequence	
$(AT)_3AC(AT)_{18}$	1	SSR too close to the end of the clone to allow primer design	

[a] The $(ATT)_{10}$ and $(ATT)_{12}$ SSRs from BAC Set 1 were in one case found in the same clone separated by a distance of 397 bp

Table 2 Results of the PCR-amplification and sequencing-gel separation of products amplified using primers selected from the SSR flanking regions, the SSR locus name if assigned, and the gene diversity of the named lici

Unique SSR	PCR amplification results	Assigned locus name	Gene diversity
BAC Set 1			
$(ATT)_{10}$	Single products, monomorphic		
$(ATT)_{12}$	Single products, polymorphic	BARC-Satt610	0.32
$(AT)_{10}(CT)_{11}$	Single products, polymorphic	BARC-Sat_141	0.62
$(AT)_{21}$	Single products, polymorphic	BARC-Sat_163	0.74
$(AT)_{44}$	Multiple products		
BAC Set 2			
$(ATT)_{12}$	Multiple products		
$(ATT)_{17}$	Single products, polymorphic	BARC-Satt632	0.54
$(AT)_{18}$	Single products, polymorphic	BARC-Sat_157	0.48
$(AT)_{21}$	Multiple products		
$(AT)_{25}$	Multiple products		
$(AT)_{34}$	Multiple products		
$(AT)_{36}$	Single products, polymorphic	BARC-Sat_162	0.74
$(AT)_{16}(AG)_{32}$			
$(AT)_3AC(AT)_{18}$			

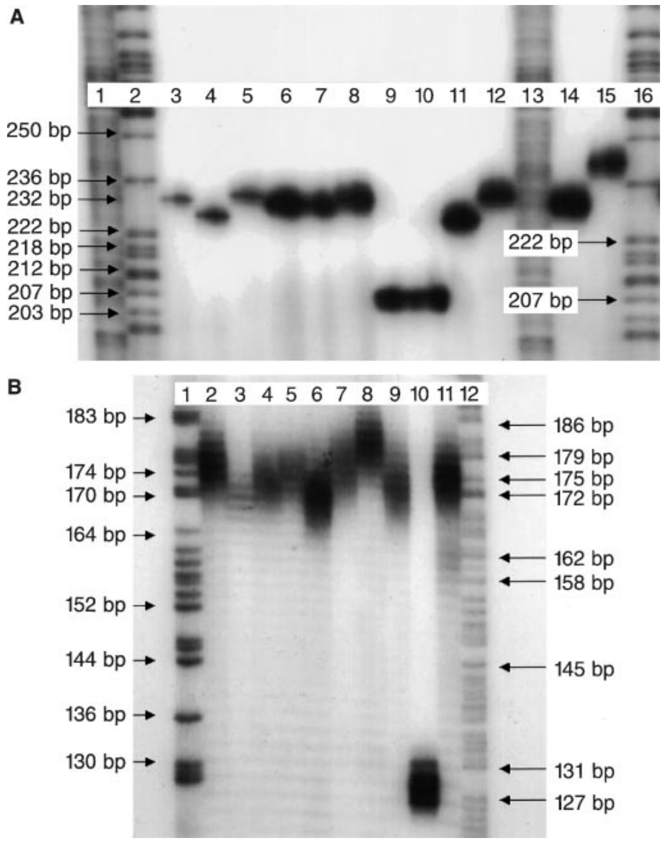

Fig. 2A Allelic diversity at the soybean SSR locus BARC-Sat_163 among a standard set of ten genotypes (*lanes 3–12*) and the Evans (*lane 14*) and PI 209332 (*lane 15*) parents of a mapping population, as determined on a DNA sequencing gel. *Lanes 1, 2, 13, and 16* contain DNA size standards derived from sequencing reactions of M13 single-stranded DNA. *Lanes 1 and 13* are the combined reactions using ddTTP, ddGTP, and ddCTP terminators and *lanes 2 and 16* contain the sequencing reaction with the ddATP terminator. Fragment sizes in basepairs (*bp*) are indicated. **B** Allelic diversity at the soybean SSR locus BARC-Sat_162 among a standard set of ten genotypes (lanes *2–11*) that include Minsoy (*lane9*) and Noir 1 (*lane 10*), parents of the University of Utah recombinant inbred line mapping population, as determined on a DNA sequencing gel. *Lanes 1 and 12* contain DNA size standards derived from sequencing reactions of M13 single-stranded DNA. *Lane 1* contains the sequencing reaction with the ddATP terminator and lane *12* the combined reactions using ddTTP, ddGTP, and ddCTP terminators. Fragment sizes in basepairs (*bp*) are indicated

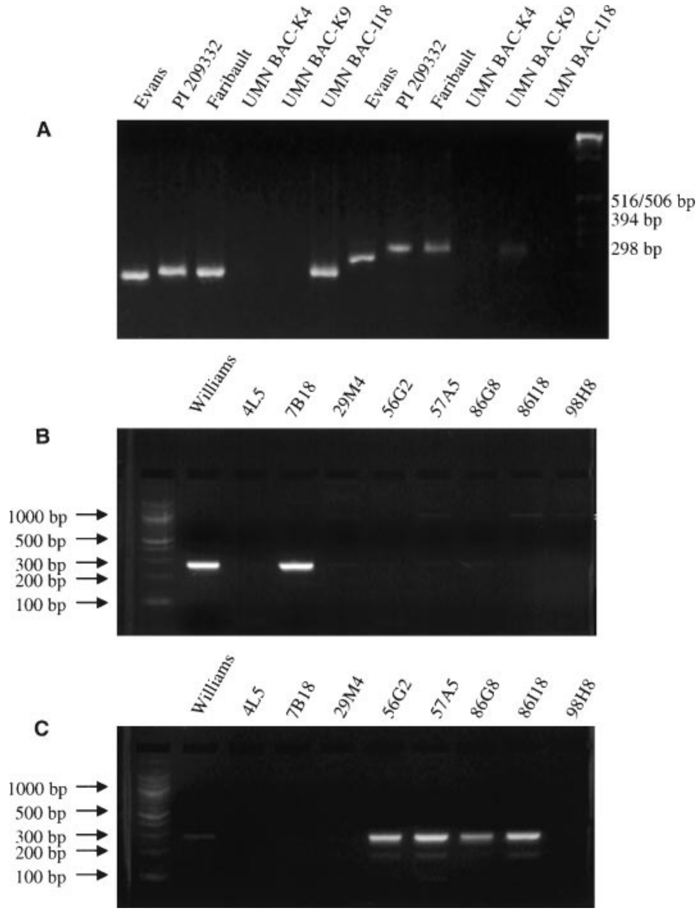

Fig. 3A Amplification products from UMN BAC clones using SSRs BARC-Satt610 and BARC-Sat_141. BAC clones UMN BAC-K9 and BAC-118 are described in detail in the text. UMN BAC-K4 is another clone located in this same region of the soybean genome (Danesh et al. 1998), but not overlapping any of the SSRs described in this study. *Lanes 1–6* were amplified with BARC-Satt610; *lanes 7–12* with BARC-Sat_141. *Lane 13* contains a 1-kbp molecular-weight ladder (Gibco/BRL). **B** Amplification products from USDA-ISU BACs using primers to SSR BARC-Satt632. *Lane 1* contains a 1-kbp DNA Ladder (New England BioLabs, Beverly, Mass.). *Lane 2* contains the PCR product from Williams soybean and lanes *3–10* products from BAC clones 6E3, 7B18, 29M4, 56G2, 57A5, 86G8, 86118, and 98H8, respectively. **C** Amplification products from USDA-ISU BACs using primers to SSRBARC-Sat_157. *Lane 1* contains 1-kbp DNA Ladder (New England BioLabs, Beverly, Mass.). *Lane 2* contains the PCR product from Williams soybean and *lanes 3–10* products from BAC clones 6E3, 7B18, 29M4, 56G2, 57A5, 86G8, 86I18, and 98H8, respectively

derived from BAC Set 2 produced four different allele sizes with the panel of ten soybean genotypes (Fig. 2B). The gene diversity of the six loci developed from the two sets of BAC clones ranged from 0.32 to 0.74 (Table 2).

Confirmation that SSRs originate from BAC clones

To confirm that these SSRs originated from the corresponding BAC clones, PCR reactions using the original BAC-clone DNA as a template were performed. In these experiments, BARC-Satt610 produced a product only with UMN BAC-I18, while BARC-Sat_141 produced a product with UMN BAC-K9 (Fig. 3A). BARC-Sat_163 also produced a product only with UMN BAC-K9 (data not shown). As expected, the size of these SSR products was identical to the products obtained when Faribault genomic DNA was used as a template. Likewise, BARC-Satt632 produced a product only with USDA-ISU BAC 7B18 as a template (Fig. 1B), BARC-Sat_157 produced products with USDA-ISU BAC clones 56G2, 57A5, 86G8, and 86118

(Fig. 3C), and BARC-Sat_162 yielded amplification products with all eight of the USDA-ISU BACs (data not shown). In each case, the size of the PCR products obtained from the BAC clones appeared identical to that produced from genomic DNA of Williams soybean.

Linkage mapping of SSR markers

The final step in confirming the genomic origin of new SSR markers was their positioning on genetic linkage maps. For SSR markers BARC-Satt610, BARC-Sat_141 and BARC-Sat_163, linkage mapping was performed with a sub-set of $F_{4:5}$ lines that represent recombinants in the genomic region near *rhg1*. In this analysis, BARC-Satt610 was found to have a single recombinant with RFLP marker Bng122D, the clone originally used to uncover UMN BAC-118, placing BARC-Satt610 nearer to *rhg1* (Fig. 1A). BARC-Sat_141 was found to co-segregate with RFLP marker D0140D, the marker used to uncover UMN BAC-K9, but showed one crossover with two other RFLPs, 83.13 and 83.18, which are subclones isolated from UMN BAC-K9 (Fig. 1A). BARC-Sat_163 was found to co-segregate with subclones 83.13 and 83.18, but to have one crossover with D140D and BARC-Sat_141 (Fig. 1A).

SSR markers BARC-Satt632, BARC-Sat_157 and BARC-Sat_162 were each mapped in the population of 240 F_7-derived recombinant inbred lines from the cross of Minsoy and Noir 1 which is segregating for seedcoat color controlled by the *l* locus. No recombination was observed between the *l* locus and any of the three SSR loci developed from BAC Set 2. Thus, each of the SSR loci mapped to the same position as the *l* locus (Fig. 1B).

Discussion

Potential applications of SSRs derived from BAC clones

In previous work, we constructed an integrated set of RFLP/SSR maps with more than 600 SSR loci. Despite this large number of marker loci, the map still contained a number of intervals of at least 20 cM in which no SSR markers were located (Cregan et al. 1998). Nonetheless, nearly all of these intervals have at least one, and often several, RFLP markers present. Based on the results of our present study, it now seems feasible to fill these gaps in the SSR map using pre-existing RFLP marker loci and BAC clones as intermediaries in the search.

Our success in finding SSRs based on BAC clones also provides opportunities for more effective marker-assisted breeding. Since many of the populations used in variety development involve crosses between closely related parents, DNA markers with higher levels of genetic diversity are very desirable. Before this study, for example, the region near *rhg1*, though well populated by RFLP markers, still lacked any tightly linked SSR marker proximal to the resistance gene. The use of BACs already known to map in this region enabled us to identify three immediately applicable and highly informative SSR markers which we have already begun to apply in our breeding program. Likewise, while a number of RFLP loci were previously positioned in the *Rhg4* region, the closest SSR loci were approximately 6 cM distal (BARC-Satt315) and 3 cM proximal (BARC-Satt187) to this soybean cyst nematode resistance locus (Fig. 1B). The availability of three polymorphic SSR loci in extremely close proximity to *Rhg4* will facilitate the identification of genotypes carrying resistance to SCN at this locus. A preliminary survey of soybean genotypes used as sources of resistance to the soybean cyst nematode, such as Peking, PI 88788, PI 90763, PI 209332, and PI 437654, indicated that each carries alleles at the BARC-Sat_162 locus that distinguish them from a number of common soybean cultivars that are susceptible to soybean cyst nematode attack (P. B. Cregan et al., unpublished observations).

BAC clones can clearly be used as intermediaries to seek out DNA marker types other than SSRs. For example, BACs in known regions of a genome could be used in a search for single-nucleotide polymorphisms (SNPs). In fact, techniques remarkably similar to those described here would be very suitable in a search for SNP markers in a targeted genomic region with a BAC clone as the starting point. As the field of genomics moves more toward more chip-based technology (Hacia et al. 1996), BAC clone-based searches for SNP markers could be extremely powerful.

Finally, for successful map-based cloning, it is necessary to have a high density of molecular markers covering a genomic region of interest. SSRs are generally more useful then other classes of molecular markers because SSRs can be used directly for PCR-based mapping in nearly any segregating population. This is especially important in plants with complex and highly duplicated genomes, such as soybean (Shoemaker et al. 1996). For example, we have used BAC-specific SSR markers from a pool of BACs on MLG-J to provide greater confidence in the mapping of those BAC clones (Shoemaker and Marek, unpublished observations). The SSRs provided a high degree of information content and locus specificity without the ambiguity that was associated with hybridization-based (RFLP) markers derived from the BACs.

Distribution of SSRs on BAC clones

A total of 14 unique SSRs were isolated from subclones derived from the two sets of BAC clones. For BAC Set 1, which was derived from BAC clones UMN-I18 and UMN-K9 whose total length was 210 kbp, five microsatellites were found, which translates to one $(ATT)_n$- or $(AT)_n$-SSR per 42 bkp. Nine unique $(ATT)_n$- and $(AT)_n$-containing microsatellites were found in BAC Set 2 which has an estimated contig length of 260 kbp. This is an average of one $(ATT)_n$- or $(AT)_n$-SSR every 28.9 kbp. Calculations from Wang et al. (1994) indicate one $(ATT)_n$- or $(AT)_n$-SSR every 34.6 kbp in 726 GenBank sequences of dicotyledonous plants that totalled 1.416 Mb in length. These data included 101 soybean sequences totalling 199.7 kbp in which an $(ATT)_n$ or $(AT)_n$ microsatellite was present every 22.2 kbp. In the current work, we detected an average of one SSR per 33.6 kbp which is a somewhat lower frequency than that reported by Wang et al. (1994). This difference may result from the small and perhaps unrepresentative sample of genomic DNA analyzed herein or from the fact that we did not detect all SSRs in the two sets of BAC clones. Because a number of the microsatellite was detected only once (Table 1) it is possible that a more thorough examination of subclones from the two sets of BACs would detect additional SSRs. Another explanation for the apparent lower frequency of microsatellite detection observed here is that the sequence data analyzed by Wang et al. (1994) may not have been representative of the soybean genome as a whole. This could be a result either of the relatively small sample of sequence data analyzed by Wang et al. (1994) or to the fact that the GenBank sequences they surveyed were generally from coding regions or were closely associated with coding regions. Alternatively, the *rhg1* and *Rhg4* flanking regions may contain fewer microsatellites than the soybean genome as a whole.

The level of polymorphism of the six new microsatellite loci developed here was slightly lower than the mean level of polymorphism of SSR loci developed randomly from genomic DNA clones. The mean gene diversity value of 450 soybean SSR loci containing $(ATT)_n$ core motifs was 0.56 and the mean of 60 $(AT)_n$-containing loci was 0.72 (P.B. Cregan, unpublished data). These values are higher than the mean of 0.43 and 0.64 for the two $(ATT)_n$ and the four $(AT)_n$ loci, respectively, that are reported here (Table 2).

The procedure described in this paper represents an efficient approach for targeted SSR marker development. A number of intervals can be targeted in tandem in one library by combining BAC DNA from many clones. After a robust microsatellite marker is successfully developed, the specific BAC clone from which it was derived can be readily determined. We are currently developing SSR markers targeted to many of the intervals in the soybean SSR/RFLP map for which no microsatellites markers are currently available.

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