Use of Cross-Species Simple Sequence Repeat (SSR) Primers for Developing Polymorphic DNA Markers

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ABSTRACT. PCR-based DNA markers are very valuable to plant breeders and the seed industries and have many uses including as tools to test varietal purity. One of the limiting factors in developing DNA markers of cotton has been the development of sufficient numbers of polymorphisms that can be evaluated as useful markers. Simple Sequence Repeats (SSRs), also known as microsatellites or Short Tandem Repeats (STRs), have proven to be a most important resource for polymorphic markers and have been used very successfully in both mam-

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malian and plant genome mapping studies. The capability for molecular fingerprinting by SSR-specific primers would be extremely valuable to seed science and accompanying industries. The present study was conducted to determine whether commercially available SSR primers from other species could be used to detect polymorphisms in cotton. Forty-four SSR primers from cotton (*Gossypium hirsutum*), soybean (*Glycine max*), sweet potato (*Ipomoea fastigiata*), banana (*Musa acuminata*), conifer (*Pinus thunbergii*) and hard pine (*Pinus radiata*), and 10 random amplified polymorphic DNA (RAPD) primer pairs were examined. Polymerase chain reaction (PCR) amplification, agarose gel electrophoresis and ethidium bromide staining were used to generate DNA fingerprints. The size range of the DNA markers varied from 40 bp to more than 1000 bp. Band separation of as low as 20 bp was achieved by use of high resolution agarose. Numbers of bands per primer combination and percentages of polymorphic bands at inter- and intra-specific levels varied widely. The SSR-generated markers from primer combinations specific to cotton produced the least number of bands (2.2 and 2.6 at inter- and intra-specific levels, respectively). The SSR primers from sweet potato produced the highest number of bands per primer combination (20.5 and 19.5 at inter- and intra-specific levels, respectively). Polymorphic markers generated by SSR primers from species other than cotton were dominant, while SSR primers specific to cotton produced both co-dominant and dominant markers. This study demonstrated the usefulness of SSR primers from diverse species in the genetic analysis of cotton. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-342-9678. E-mail address: getinfo@haworthpressinc.com <Website: http://www.haworthpressinc.com>]*

ABBREVIATIONS. PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeat; DNA, deoxyribonucleic acid; EST, expressed sequence tag; QTL, quantitative trait loci

KEYWORDS. Short tandem repeats, microsatellites, DNA fingerprinting, polymerase chain reaction, polymorphism, molecular markers

INTRODUCTION

PCR-based DNA markers are very valuable tools to plant breeders and the seed industries because: (1) they provide a rapid, cost-effective, safe and efficient way to test the genetic purity of the varieties;

(2) they can be used to estimate very precisely the genetic diversity and thus, the improvement of varieties; (3) they can be used to test selfing and outcrossing percentages within a field in any crossing program; and (4) they can be used in any genome mapping program to identify DNA markers linked to economically important quantitative trait, so that breeder can use the molecular markers in their selection program for complex quantitative traits.

Cotton (*Gossypium* sp.) ranks among the top five of agricultural crop production worldwide (MacDonald and Meyer, 1997). The spin-off industries, with revenues from fiber (textiles), cottonseed (animal feed) and oil (snacks), arguably make cotton the number one industrial crop in the world. Despite its importance to the US and world economy, the knowledge of cotton molecular mapping is not adequate due to the lack of sufficient numbers of molecular markers. Only recently have a few RFLP linkage groups been developed and assigned chromosomal locations (Reinisch et al., 1994, Shappley et al., 1996). In addition, a handful of the linkage groups have only recently been associated with agronomically important QTLs (Jenkins et al., 1998; Saha et al., 1998b).

In order to overcome this deficit in linkage analysis, more DNA markers need to be found. However, many of the current techniques are encumbered by physical and logistical limitations, such as special gel apparatus (AFLPs, Vos et al., 1996; Saha et al., 1998a), radioactive handling and disposal (RFLPs, Reinisch et al., 1994, Shappley, 1996), prior sequence knowledge (PCR, Piotrowski and Oecking, 1998), and reliability (RAPDs, Lanner et al., 1996). Simple Sequence Repeats, also known as microsatellites or Short Tandem Repeats (STRs), have proven to be very important sources for polymorphic markers and have been used very successfully in both mamalian and plant genetic studies (Moore et al., 1991; Kondo et al., 1993; Lagercrantz et al., 1993). We offer a simple, reproducible, PCR-based technique that takes advantage of a large pool of commercially available Simple Sequence Repeat (SSR) primers to generate large numbers of polymorphisms. The significance of our results is that these SSR primers are normally used for developing markers within species because they are developed based on the DNA sequences from a specific plant genome and are considered conserved within the species. Thus, there have been very few reports in other crops and no reports in cotton on the use of SSR primers from outside the species. Here we report the

first use of cross-species SSR to amplify polymorphic markers from the cotton genome. Our results suggest that cross-species SSR primers could be very useful in many crops, similar to our results in cotton, to generate sufficient numbers of polymorphic DNA markers. Over several thousands of SSR primers are now commercially available from different crops and they can be very valuable tool in fingerprinting individuals, based on unique bands and band patterns.

MATERIALS AND METHODS

Plant Materials

Six cotton inbred lines consisting of two sets of tetraploid parents, "TM-1" (*Gossypium hirsutum*), "Pima 3-79" (*G. barbadense*) and their (inter-specific) hybrid, "PD-3-14", "Simian-2" (both *G. hirsutum*) and their (intra-specific) hybrid, were used in a primary screening for polymorphisms. The TM-1 and Pima 3-79 parents were selected because they are morphologically and taxonomically distinct, are easily crossed, and TM-1 is considered as the genetic standard of *G. hirsutum* and Pima 3-79 is a double haploid of *G. barbadense*. The PD-3-14 and Simian-2 parents were included because they are very diverse inbred lines of *G. hirsutum* with good fiber characteristics and are part of an ongoing QTL study.

DNA Extraction

Genomic DNA samples used for *in vitro* PCR amplification were extracted from young green leaves from greenhouse-grown individual plants according to Saha et al. (1997). If not used immediately, DNA samples were stored at $-20°C$.

PCR Materials and Conditions

A total of 37 SSR primer pairs from cotton (*G. hirsutum*) (13), soybean (*Glycine max*) (9), sweet potato (*Ipomoea fastigiata*) (2), banana (*Musa acuminata*) (4), conifer (*Pinus thunbergii*) (7) and hard pine (*Pinus radiata*) (2), and 10 RAPD primer pairs were randomly chosen and screened. The SSR primers specific to cotton were obtained from Dr. Sam Reddy, Texas A & M University, and were developed from a cotton genomic library enriched in $(GA)_n$ repeats

(Reddy et al., 1998). The SSR primers, from species other than cotton, MapPairs™, were purchased from Research Genetics (Huntsville, AL). The RAPD primers were obtained from Operon Technologies (Alameda, CA).

GeneAmp PCR reagent kits (Perkin Elmer, Norwalk, CT), consisting of AmpliTaq DNA polymerase, GeneAmp 10X buffer II, $MgCl_2$ solution and GeneAmp dNTPs (dATP, dTTP, dGTP, dCTP), GeneAmp PCR System 2400 and MicroAmp 8-Strip reaction tubes, were used for the *in vitro* amplification. Concentrations of PCR materials given in the protocol supplied by the company were used as standard for all the primer pairs. The reaction mixture consisted of 250 ng template DNA, 200 µM each dNTP, 2 µl primers, 2.5 mM $MgCl_2$, 2.5 units AmpliTaq DNA polymerase and ddH_2O to make the volume to 100 µl. The samples were run for 40 thermal cycles each of denaturation at 94°C (15 s), annealing at 55°C (30 s), and extension at 72°C (60 s). A "hot start" at 94°C (90 s) and a final extension at 72°C (7 min) were used for all reactions. The same conditions were used for SSR and RAPD PCR, so that a comparison could be made. If not electrophoresed immediately, PCR products were stored at −20°C.

Gel Electrophoresis

High resolution agarose (Sigma, St. Louis, MO), 2% in 1X TBE buffer, was used to separate fragments. This type of agarose gel is very easy to use in comparison to polyacrylamide gel and has the capacity of separating very small size DNA markers (Senior et al., 1998). Amplified PCR products (20 µl/sample) were mixed with 6X bromophenol blue loading buffer and electrophoresed in a minigel at 90 volts for 90 minutes. A 20 bp DNA ladder (20-1000 bp) (Sigma) was run for size determination of the marker bands. For staining, ethidium bromide was added to the tank buffer and also into the gel before pouring. Gels were observed on a UV light box and photographed with Polaroid film. Only the bands which appeared on two replicate gels were included in the analyses for both SSR-generated and RAPD markers.

RESULTS

Only six of 10 RAPD primer pairs amplified bands, averaging 11 bands per primer pair for both *Gossypium hirsutum* and *G. barbadense* genotypes (Table 1). Approximately 1.5% of these bands were

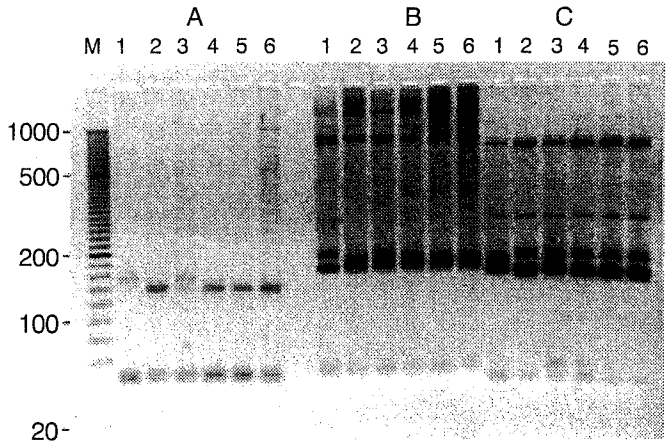
TABLE 1. Comparison of PCR-Based DNA Markers.

Type of Marker	Crop	DNA Markers/ Primer Combination		% Polymorphic DNA Markers		Size (bp)
		Interspecific	Intraspecific	Interspecific	Intraspecific	
RAPD	N.A.	11.8	11.3	19.7	1.5	120-<1000
SSR	Cotton	2.2	2.5	57.1	9.4	40-220
SSR	Soybean	15.0	14.1	18.5	18.9	40-<1000
SSR	Conifer	10.3	8.9	19.4	8.1	60-<1000
SSR	Hard Pine	19.0	18.5	15.8	10.8	40-<1000
SSR	Banana	12.3	13.3	24.5	1.9	100-<1000
SSR	Sweet Potato	20.5	19.5	19.5	7.7	80-<1000

polymorphic intraspecific markers in *G. hirsutum* accessions, while 20% were polymorphic interspecific markers differentiating between *G. hirsutum* and *G. barbadense*. Lazo et al. (1994) reported six to eight bands per RAPD primer combination, of which 34% were polymorphic between *Gossypium hirsutum* and *G. barbadense*. Representative RAPD studies have found 35% polymorphic bands in soybean (Lin et al., 1996) and 34% in maize (Beaumont et al., 1996). The lower values in our study could reflect the few genotypes screened. In addition, the quantitative differences could arise because of the higher annealing temperature used as well as the use of different RAPD primers.

The identification of the DNA markers based on cross-species SSR primers was far more complex. The SSR-generated markers exhibited both co-dominance (Figure 1) and dominance (Figure 2). The SSR-generated markers, generated from cotton-derived SSR primers, provided by Dr. Sam Reddy, were generally co-dominant. However, we observed in another study that the markers generated by cotton-derived SSR primers from Research Genetics were 13% polymorphic, of which more than 60% were dominant (Saha et al., 1998a). Polymorphic markers generated by SSR primers from species other than cotton were all dominant (Table 1). However, we do not know if the polymorphic bands amplified with cross-species SSR primers actually contain SSRs in cotton. We can only state that they are polymorphic DNA markers and, as such, will be very useful for genetic analysis based on our preliminary results from the parents and their hybrid. The

FIGURE 1. DNA fingerprints generated by different cotton SSR primer pairs (A = CM-42, B = CM-68, C = CM-162). Lanes: **M**–20 bp marker, **1**–Pima 3-79 (*Gossypium barbadense*), **2**–TM-1 (*G. hirsutum*), **3**–Interspecific F_1 hybrid, **4**–PD-3-14 (*G. hirsutum*), **5**–Simian-2 (*G. hirsutum*), **6**–Intraspecific F_1 hybrid. Note co-dominance of bands separating around 200 bp. Also note the similarities in band patterns among all *G. hirsutum* accessions.

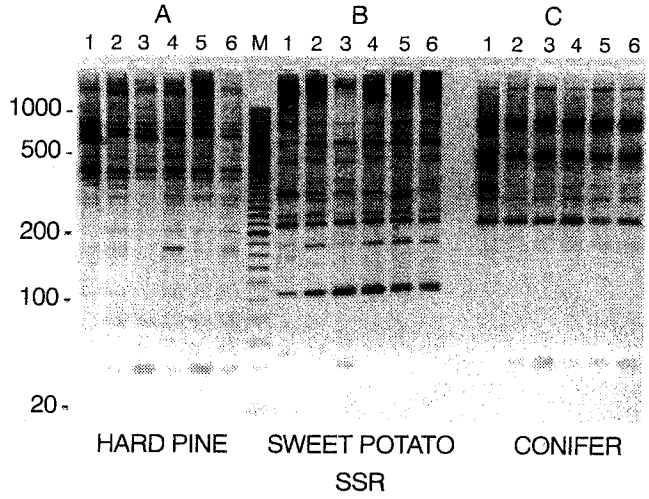


SSR primers from outside the Malvaceae consistently generated more bands than the cotton SSR primers (Table 1, Figure 2 vs. Figure 1). The SSR primers from sweet potato and hard pine generated around 20 bands per primer pair, and those from other species averaged about 10 bands per primer pair, compared to 1 to 2 bands per primer pair generated by the cotton SSR primer combinations (Table 1).

DISCUSSION

PCR-based markers have many advantages, especially ease of generation and analysis, but vary in their applicability. For example, microsatellites are generally co-dominant (van de Ven and McNicol, 1996) while RAPDs are usually dominant (Thomas and Scott, 1993). The use of non-radioactive techniques (e.g., silver staining, fluorescence, luminescence) has reduced the regulatory problems and exposure hazards of generating radioactive RFLP and AFLP markers, al-

FIGURE 2. DNA fingerprints generated by across taxa (hardpine [NZRP6], sweet potato [Ib3/36] and conifer chloroplast [Pt 87268]) SSR primer pairs. Lanes: **M**–20 bp marker, **1**–Pima 3-79 (*Gossypium barbadense*), **2**–TM-1 (*G. hirsutum*), **3**–Interspecific F_1 hybrid, **4**–PD-3-14 (*G. hirsutum*), **5**–Simian-2 (*G. hirsutum*), **6**–Intraspecific F_1 hybrid. Note dominance of bands separating around 600 bp. Note also the increased numbers of bands compared to Figure 1.



though the use of polyacrylamide gels still increases detection time. The use of high resolution agarose in cross-species SSR analysis combines the ease of setup and analysis of agarose gels with the separation power of polyacrylamide gels, while eliminating the added time and the need for special skills associated with preparation of polyacrylamide sequencing gels (Senior et al., 1998).

Repetitive DNA markers, a successful approach in mammalian genome mapping, have only recently been applied to plant studies (Walton, 1994). In particular, small unit repeat families of genomic DNA, known as short tandem repeats (STRs), microsatellites or simple sequence repeats (SSRs), have been the most useful due to the high amount of variation in these units.

Microsatellite loci are amplified via PCR either by utilizing primer pairs which flank the repeat sequence (SSR) (van de Ven and McNicol, 1996) or from within the repeat sequence itself (Inter-SSR) with (Charters et al., 1996; Nelson et al., 1996) or without (Nelson et al., 1996) primer anchors.

Although SSRs have been amplified across mammalian genera (Moore et al., 1991; Kondo et al., 1993), microsatellites have generally been considered to be conserved within plant species and thus of limited use between related species (van de Ven and McNicol, 1996).

Cross species amplification has been found in plants (Lagercrantz et al., 1993; Brown et al., 1995; van de Ven and McNicol, 1996), but the number of useful primers decreased, as did the number of alleles detected, when crossing genera boundaries (Brown et al., 1996; Taramino et al., 1996; van de Ven and McNicol, 1996). For example, van de Ven and McNicol (1996) found three to 15 alleles per Sitka spruce SSR primer pair, and 0-47% polymorphisms. However, when used to amplify DNAs from other spruce or pine species, only one to two alleles were found per SSR primer pair and 0% polymorphisms. In contrast, when internal repeat sequences (Inter-SSR) were used as primers, 72% produced polymorphisms from various eukaryotic DNAs, including plant and animal representatives (Gupta et al., 1994).

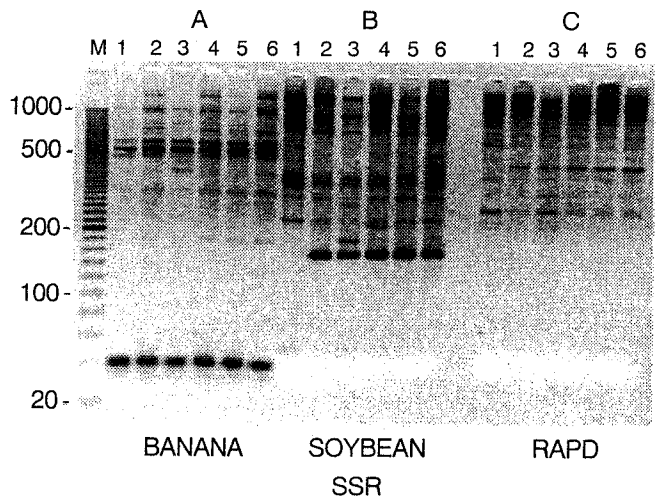
Results presented here, confirmed by the various distantly related SSR primer pairs as well as by repeatability between gels, consistently showed greater numbers of amplified bands as well as polymorphisms when cross-family SSR primers were used. We have not attempted to confirm the presence of SSRs by sequencing as our interest is strictly to deliver numbers of polymorphisms for evaluation as potential QTL markers. Confirmation of the presence of repeats would have great ramifications both from chromosome architectural as well as evolutionary viewpoints. Whether unique to cotton or not, this reversal of the plant SSR paradigm offers increased opportunities to quickly generate useful DNA markers using existing SSR primers especially considering the fact that large numbers of SSR primers are readily available from private industries (e.g., Research Genetics, Huntsville, AL, USA, offers over 1000 primer pairs from 12 plant species). Accordingly, very little time and cost will be required for the use of cross-species SSR primers. The considerable number of combinations offers an opportunity for finding the unique bands and patterns that can identify individuals, populations, seed lots, cultivar lines and other areas that are of importance to seed industries (Walton, 1994).

CONCLUSIONS

There have been very few reports in other crops and no reports in cotton of the application of cross-species SSR amplification for developing polymorphic DNA markers. Our results confirm that SSR markers from other species outside the Malvaceae can provide significant numbers of intra- and inter-specific DNA markers for use in cotton linkage studies, genotype fingerprinting, marker-assisted breeding and gene isolation. Cross-family SSR primers have the potential to provide more bands for analysis than RAPD primers (Figure 3) in cotton.

Of interest to molecular geneticists is the use of distantly related SSR primers to generate higher numbers of amplified bands and greater percentages of polymorphisms, thereby increasing the potential of the molecular fingerprint. Cross family and cross order primer pairs may provide another productive means to increase the number of polymorphic DNA markers necessary to fill the demands of molecular genetics, as well as the needs of population geneticists and seed

FIGURE 3. Comparison of DNA fingerprints generated from across taxa (banana [Ma3/127] and soybean [SoyRPRP1]) SSR primer pairs and RAPD [OPP-1, OPP-2] primers. Lanes: **M**–20 bp marker, **1**–Pima 3-79 (*Gossypium barbadense*), **2**–TM-1 (*G. hirsutum*), **3**–Interspecific F_1 hybrid, **4**–PD-3-14 (*G. hirsutum*), **5**–Simian-2 (*G. hirsutum*), **6**–Intraspecific F_1 hybrid. Note differences in number of bands generated between soybean SSR and the RAPD primers.



technologists, without the added time and expense of identifying new SSR primer sequences. Finding SSR-generated markers will be further accelerated as more plant SSR primers become commercially available.

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