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Development of Multiplex Microsatellite Marker Sets in Soybean [*Glycine max* (L.) Merr.]

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A B S T R A C T

Multiplex PCR (mPCR) analyzes several markers simultaneously in a single reaction. The development of an SSR multiplex system requires estimation of range of allele sizes of different markers for their grouping into multiplex sets. In the present investigation duplex, triplex, quadruplex, and pentaplex combinations of SSR markers were optimized for mPCR using four different soybean genotypes namely, JJS 335, Durga, MACS 124 and JS 90-41.Twenty SSR markers distributed across all linkage groups of soybean were used to develop 22 multiplex sets of markers. These 22 multiplex sets comprised of twelve duplex, five triplex, four quadruplex and one pentaplex assays. Each band of mPCR was considered a single genetic marker and using all mPCR marker sets bands in the size range of 100-300bp were observed. The methodology used was quite systematic, rapid, reliable, and appropriate for qualitative detection and can be extended for multiplex development in other plant species having SSR marker technology for efficient plant genetic resources management including genetic diversity analysis, cultivar identification and evolutionary studies in plants.

Introduction

Multiplex Polymerase Chain Reaction (mPCR) is a molecular biology technique which amplifies several targets in a single PCR reaction. Chamberlain *et al.,* (1988) described mPCR for the first time as a method for detection of deletion in the *Dystrophin* gene. mPCR uses several primer setswhich target multiple sequences of DNA and amplify in the same reaction tube at the same time and at the same annealing temperature. Due to simultaneous amplification, multiplex PCR is fast, simple, reliable, accurate, specific, sensitive, highly efficient, less costly and requires very minimal amount of DNA for PCR amplification. mPCR has been applied in several molecular biology

applications like linkages analysis (Reis, 1991), SSR/SNP analysis (Moykishev *et al.,* 2001;Mayden *et al.,* 2008), molecular species identification (Staudacher *et al.,* 2011), GMO identification (Mazur *et al.,* 2017) etc.

SSRs or microsatellite markers have now become the marker of choice for genetic analyses owing to their abundance, high polymorphism rate, ease to use and high reproducibility. SSRs are short tandem repeats of mononucleotides to hexanucleotides with varying length of repeat motifs. Akkaya *et al.,* (1992) reported SSRs in soybean for the first time. Thereafter, Diwan and Cregan (1997) reported the presence of microsatellites in a number of plant species and suggested their potential for numerous applications. Analyzing each SSR marker is time consuming and cumbersome which can be overcome by employing mPCR. Multiplex PCR assays have been reported in soybean particularly for screening genetically modified organism. For example, triplex and quadruplex PCR was optimized in soybean by Kutateladze *et al.,* (2013), Datukishvili *et al.,* (2015) and Lisha *et al.,* (2017). Koppel *et al.,* (2012) used pentaplex PCR for GMO detection in soybean. Sayama *et al.,* (2011) designed a panel of 304 SSR markers using quadruplex PCR. The aim of this study, however, was to develop and optimize the multiplex sets of SSR markers in soybean to test their ability for amplification in single and multiplex PCR reactions particularly for genetic diversity assessment and cultivar identification.

Materials and Methods

Plant materials

Four soybean [*Glycine max* (L.) Merr.] varieties *viz.*, JS 335, Durga, MACS 124 and JS-90-41 were selected from the polymorphism surveys of 96 Indian soybean varieties using 49 SSR markers on the basis of their unique and different amplicon sizes. These varieties have been released over different time periods for different agro-climatic regions in the country by different soybean breeding centers.

DNA extraction

Total genomic DNA was isolated from 10-15 days old young and tender leaves following CTAB (CetylTrimethyl Ammonium Bromide) method with some modifications as described by Saghai Maroof *et al.,* (1984). The quality estimation of extracted DNA was accomplished by comparing the intensity of ethidium bromide- stained DNA bands on 0.8% agarose gel. The quantity of extracted DNA was further evaluated using absorbance measurements at 260 and 280 nm through NanoDrop™1000. Final concentration was adjusted to 10ng μl^{-1} for further use in PCR.

Primer screening

A total of 20 SSR markers distributed across the entire linkage map of soybean were used (Cregan *et al.,* 1999). These markers were selected on the basis of their G+C content, annealing temperature, PIC value and allele sizes observed from their PCR profile. The details of SSR markers are presented in Table 1. The allele size of each SSR primer was scored after amplification of each soybean genotype DNA with specific SSR primer in uniplex PCR. Thereafter, these 20 selected markers were finally combined to form duplex, triplex, quadruplex and pentaplex, for two, three, four and five marker combinations, respectively.

Multiplex PCR (mPCR) optimization

PCR conditions for duplex, triplex, quadruplex and pentaplex marker combinations were optimized. The master

mix for mPCR was prepared in 2ml eppendrof tube then distributed in four PCR tubes and total volume of each PCR tube was made to 25ml. PCR mixture consisted of 1X buffer, 3mM $MgCl_2$, 0.25mM dNTPs mixture (dATP, dCTP, dGTP, dTTP), 0.5U *Taq* DNA polymerase and 0.5µm of each primer for duplex, triplex quadruplex and pentaplex mPCR. The template DNA concentration for mPCR varied from 40-100ng whereas for uniplex PCR with single primer pair, the template DNA concentration was 35 ng. Amplification was carried out in a peltierthermo cycler (Model PTC-200, M/S MJ Research). All multiplex PCR conditions had the same optimal cycling condition. PCR cycling conditions consisted of initial denaturation at 94^0 C for 5 min followed by 40 cycles, each cycle consisting of denaturation at 94^0 C for 1 min, primer annealing at 55^0 C for 1min, elongation at72^0 C for 1min. Finally, elongation step of PCR was carried out at 72^0 C for 8 min. The amplification products were analyzed by electrophoresis in 3.0% high resolution agarose (metaphor) gel containing 0.5 mg/ml of ethidium bromide. 100bp DNA size standard was also loaded to assess the size of the amplicons generated by each SSR primer. The gel was run in 1x TAE buffer at 90V for 60 min. DNA bands were visualized under UV transilluminator and photographed using gel documentation system (M/S Syngen).

Results and Discussion

Multiplex PCR is a transformation of the conventional PCR method. The basic principle of multiplex PCR is the same as that of the conventional PCR, except that several specific primers are combined in a single PCR reaction. The primers are specifically combined with their corresponding DNA template, and more than one DNA fragment amplified in one reaction simultaneously. Multiplex PCR (mPCR) analyzes several markers simultaneously in a single reaction. The development of an SSR multiplex system requires selection of sufficiently different sized markers for their grouping into multiplex sets. The quality, quantity and DNA extraction procedure form a critical factor in mPCR amplification. The DNA for mPCR of four soybean samples was extracted using CTAB method and was then quantified at A_{260}/ A_{280} nm absorbance. The concentration of DNA samples ranged from 1.7 to 2.0ng/ µl which indicated high quality of DNA preparation. Absorbance ratio exceeding this range indicates poor quality of the extracted DNA, which might create problems during PCR.

During mPCR the specificity and sensitivity of PCR is affected by many factors, such as the purity of the DNA, primer design, concentration of the corresponding primers, ratio of primers and annealing temperature. To optimize the multiplex sets of primers, equal concentration of component primers of a set was used in multiplex PCR so as to prevent generation of excessive quantities of primer dimers. No amplification was found in the multiplex reaction when increasing the annealing temperature to 70^0 C. Keeping the annealing temperature at55^0 C provided the reproducible PCR products. Annealing temperature when changed slightly (±2^0 C), PCR did not result in satisfactory output. Primers with the allele size difference of more than 30 bp were considered more suitable for mPCR. Combination of different SSR markers for duplex, triplex, quadruplex and pentaplex PCR were optimized using four soybean varieties namely, JS335, Durga, MACS 124 and JS 90-41.

The template DNA concentration was optimized because without appropriate concentration no amplification was observed. Template DNA concentration of 30ng generated weak bands when this

concentration was used with two or more than two primers, so the concentration of template DNA was increased which resulted in corresponding increase in the intensity of the observed DNA bands. Genomic DNA concentration in the range of 40 to 100ng was used for different mPCR reactions which were 40ng for duplex, 60ng for triplex, 80ng for quadruplex and 100ng for pentaplex PCR.

On the basis these parameters, up to five SSR primers were included in a set. Sometimes samples could not be amplified with given marker set of duplex, triplex, quadruplex and pentaplex PCR (datanot shown) and hence were eliminated to be part of the set. Finally, a set of twenty-two marker combinations (Table 2) was selected on the basis of their successful amplification in the four individual soybean genotypes in a reaction. Out of these 22 sets, 12 multiplex sets were duplex, five triplex, four quadruplex and onepentaplex. Thesesets of multiplexed markers would enable detection of genetic diversity with high specificity in very short time.

DNA profiles of four soybean genotypes were generated and the observed band sizes ranged between 100 – 360bp (Table 2). Representative profiles for duplex, triplex, quadruplex and pentaplex PCR are shown in Figure 1 to 4, respectively. It can be seen that the combination of markers SATT 244 and SATT 031 in duplex PCR amplified bands of sizes 170bp and120bp; 150bp, 180bp and 120bp; 170bp and 120bp; 150bpand 120bp, respectively for JS 335, Durga, MACS 124, and JS 90-41 soybean genotypes (Fig. 1). Similarly, the marker set of SATT 373 and SATT 177 in duplex PCR produced 220bp and 120bp bands for JS 335, 270bp and 100bp for Durga, 220bp and 100bp for MACS 124, and 270bp and 110bp for JS 90-41 (Table 2). In the triplex mPCR, SSR marker set SATT 373, SATT 335, SATT 309 generated bands in the size of 220bp, 160bp and 140bp for JS 335; and 270bp, 150bp and 140bpfor Durga. The band sizes were 220bp, 150bp and 140bp for MACS 124; and 270bp, 160bp and 140bp for JS 90-41 using this set of triplex markers (Fig 2). In quadruplex mPCR the set of four markers SATT 031, SATT 244, SATT 285 and SATT 268 was used which amplified fragments of sizes 120bp, 170bp, 200bp and 360bp for JS 335 (Fig 3). Band sizes of 120bp, 150bp, 180bp, 200bp and 340bp were amplified in Durga using this quadruplex set. Bands of sizes 120bp, 170bp, 200bp and 300bp for MACS 124 and 120bp, 150bp, 240bp and 320bp for JS 90-41were produced using this quadruplex set. In the pentaplex PCR, markers namely SATT 177, SATT 309, SATT 285, SATT 335 and SATT 268 were used which produced bands of sizes 120bp, 140bp, 200bp, 160bp and 360bp for JS 335; 110bp, 140bp, 200bp, 150bp, and 340bp for Durga;100bp, 140bp, 200bp, 150bp and 300bp for MACS 124; and 110bp, 140bp, 240bp, 160bp and 320bp for genotype JS 90-41 (Fig 4).

In this study, mPCR has been optimized and established for the simultaneous detection of upto five marker loci in one reaction. Compared with regular PCR, mPCR shows many merits, including specificity, low cost, high efficiency, and reproducibility. Therefore, it can be applied in qualitative and quantitative studies, genetic diversity analyses, and identification of soybean cultivars and would result in saving time for marker detection and reducing the reagents' cost.

Table.1 Details of multiplex primers used for multiplex PCR in soybean [*Glycine max* (L.)Merr.]

S. No.	Marker name	Linkage group	Chromosome no.	G+C%	Forward primer	Reverse primer
1	SATT031		16		GCGTGGCACCCTTG ATAAATAA	GCGCACGAAAGTTTT TCTGTAACA
2	SATT002	D2	17	F-22.73%, R-27.78%	TGTGGGTAAAATAG ATAAAAAT	TCATTTTGAATCGTTG AA
3	SATT082	D2	17	F-30.00%, R-40.00%	AATTCATTTAGGGA GTTGAT	CTAGCCAATGTCATA TGACT
4	SATT177	A2	8	F-42.86%, R-50.00%	CGTTTCATTCCCAT GCCAATA	CCCGCATCTTTTTCAA CCAC
5	GlySATT 180	C1	4	F-57.1%, R-33.3%	TCGCGTTTGTCAGC	TTGATTGAAACCCAA CTA
6	GlySATT 184	D1a	1	F-40.7%, R-42.4%	GCGCTATGTAGATT ATCCAAATTACGC	GCCACTTACTGTTACT CAT
7	SATT197	B1	11	F-50.00%, R-32.00%	CACTGCTTTTTCCCC TCTCT	AAGATACCCCCAACA TTATTTGTAA
8	SATT244	J	16	F-39.29%, R-35.71%	GCGCCCCATATGTT TAAATTATATGGAG	GCGATGGGGATATTT TCTTTATTATCAG
9	GlySATT 267	D1a	1	F-50.00%, R-35.00%	CCGGTCTGACCTAT TCTCAT	CACGGCGTATTTTTAT TTTG
10	SATT268	E	15	F-36.00%, R-40.00%	TCAGGGGTGGACCT ATATAAAATA	CAGTGGTGGCAGATG TAGAA
11	SATT285	J	16	F-29.63%, R-37.93%	GCGACATATTGCAT TAAAAACATACTT	GCGGACTAATTCTAT TTTACACCAACAAC
12	GlySATT 300	A1	5	F-47.8%, R-60.0%	GCGCCCACACAACC TTTAATCTT	GCGGCGACTGTTAAC GTGTC
13	SATT308	M	7	F-57.7%, R-54.5%	GCGTTAAGGTTGGC AGGGTGGAAGTG	GCGCAGCTTTATACA AAAATCAACAA
14	SATT309	G	18	F-52.88%, R-41.67%	GCGCCTTCAAATTG GCGTCTT	GCGCCTTAAATAAAA CCCGAAACT
15	GlySATT 335	F	13	F-50.00%, R-45.45%	CAAGCTCAAGCCTC ACACAT	TGACCAGAGTCCAAA GTTCATC
16	SATT 337	K	9	F-22.73%, R-27.78%	GCGTAAATCTGATA TATGTTACCACTGA	GCGTAATACGCAAAA CATAATTAGCCTA
17	SATT373	L	19	F-34.78%, R-48.00%	TCCGCGAGATAAAT TCGTAAAAT	GGCCAGATACCCAAG TTGTACTTGT
18	GlySATT 449	A1	5	F-38.5%, R-40.9%	GCGTGCTTCTTATA TTAGGTGTTAGT	GCGCATTGGAGTTTTT GCTTTT
19	GlySATT 586	F	13	F-54.5%, R-43.5%	GCGGCCTCCAAACT CCAAGTAT	GCGCCCAAATGATTA ATCACTCA
20	GlySATT 588	K	9	F-45.8%, R-43.5%	GCTGCATATCCACT CTCATTGACT	GAGCCAAAACCAAAG TGAAGAAC

Table.2 Multiplex sets of SSR markers in genotypes of soybean [*Glycine max* (L.)Merr.]

Multiple x set no.	No. of markers	SSR marker names	Genotype band sizes (bp)			
			JS335	**Durga**	**MACS124**	**JS-90-41**
1	2	Gly SATT, 300, SATT 002	250, 140	270, 140	250, 140	250, 145
2	2	SATT 373, SATT 177	220,120	270,110	220,100	270,110
3	2	SATT 197, SATT 373	170, 220	170, 270	140,220	170, 270
4	2	Gly SATT 267, Gly SATT 184	24,140	240,180	230,180	240,160
5	2	Gly SATT 449, Gly SATT 588	250,180	240,180	250,150	260,180
6	2	SATT 268, SATT 373	360, 220	340,270	300,220	320,270
7	2	SATT 308, SATT 268	150,360	170,340	170,300	170,320
8	2	SATT 285, SATT 268	200,360	200,340	200,300	240,320
9	2	SATT 244, SATT 031	170,120	150,180,120	170,120	150,120
10	2	SATT 308, SATT 082	150,110	170,110	170,110	170,110
11	2	SATT 309, SATT 285	140, 200	140,200	140,200	140,240
12	2	SATT 180, SATT 586	120,200	110,250	100,250	110,230
13	3	SATT 082, SATT 002, SATT197	110,140,170	110,140,170	110,140,140	110,145,170
14	3	SATT 373, SATT 335, SATT177	220,160,120	270,150,110	220,150,100	270,160,110
15	3	SATT 309, SATT 285, SATT268	140,200,360	140,200,340	140,200,300	140,240,320
16	3	SATT 373, SATT 335, SATT 309	220,160,140	270,150,140	220,150,140	270,160,140
17	3	SATT 285, SATT 335, SATT 177	200,160,120	200,150,110	200,150,100	240,160,110
18	4	SATT 309, SATT 373, SATT335, SATT 268	140,220,160, 360	140,270,150, 340	140,220,150, 300	140,270,160, 320
19	4	SATT 309, SATT 244, SATT285, SATT 268	140,170,200, 360	140,150,180, 200,340	140,170,200, 300	140,150,240, 320
20	4	SATT 177, SATT 285, SATT335, SATT 268	120,200,160, 360	110,200,150, 340	100,200,150, 300	110,240,160, 320
21	4	SATT 031, SATT 244, SATT285, SATT 268	120,170,200, 360	120,150,180, 200,340	120,170,200, 300	120,150,240, 320
22	5	SATT 177, SATT 309, SATT 285, SATT 335, SATT 268	120,140,200, 160,360	110,140,200, 150,340	100,140, 200, 150,300	110,140,240, 160,320

Fig.1 Multiplex PCR amplification in four soybean [*Glycine max*(L.)Merr.] genotypes (1: JS-335, 2: Dugra (JS-72-280), 3: MACS-124, 4: JS-90-41). M is 100 base pair DNA marker. A: duplex multiplex PCR using primer combinations SAT244 and SATT031, B: uniplex PCR amplification with SSR primer SATT244 and C: uniplex PCR amplification using SSR primer SATT031

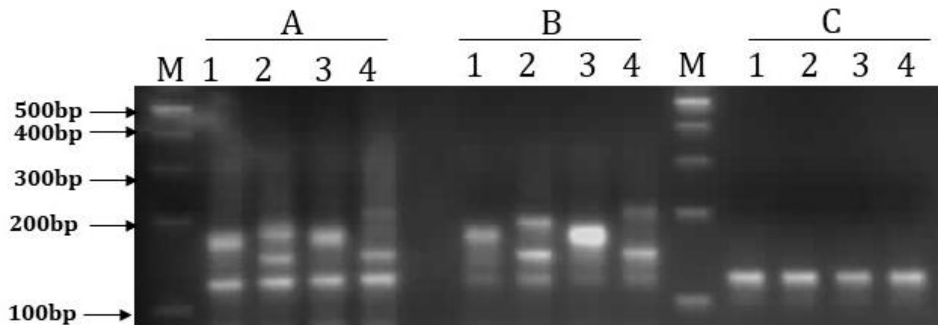


Fig.2 Multiplex PCR amplification in four soybean [*Glycine max* (L.)Merr.] genotypes (1: JS-335, 2: Dugra (JS-72-280), 3: MACS-124, 4: JS-90-41). M is 100 base pair DNA marker. A: triplex multiplex PCR using primer combinations SATT373, SATT335 and SATT309, B: uniplex PCR amplification with SSR primer SAT373 and C: uniplex PCR amplification using SSR primer SATT335 and D: uniplex PCR amplification using SSR primer SATT309

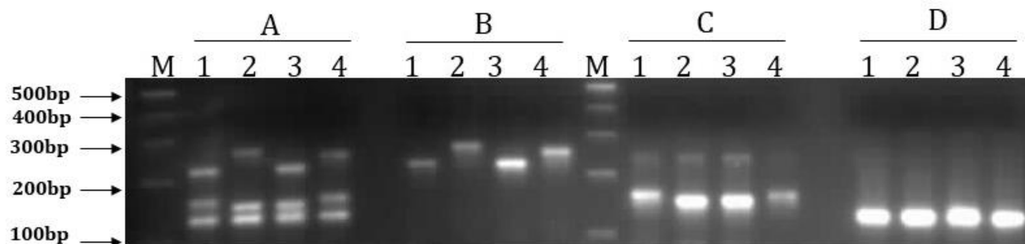


Fig.3 Multiplex PCR amplification in four soybean [*Glycine max* (L.)Merr.]genotypes (A: JS-335, B: Dugra (JS-72-280), C: MACS-124, D: JS-90-41). M is 100 base pair DNA marker. Lane 1: quadruplex multiplex PCR using primer combinations SATT031, SATT244, SATT285 and SATT268 and lanes 2 to 4 are uniplex PCR amplifications using primers SATT031, SATT244, SATT285 and SATT268, respectively

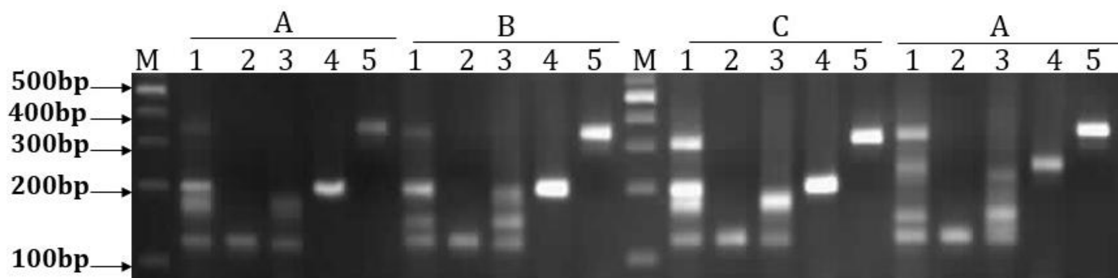
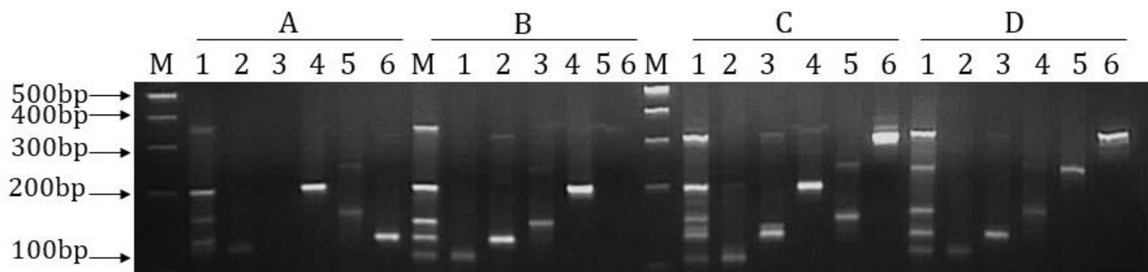


Fig.4: Multiplex PCR amplification in four soybean [*Glycine max* (L.)Merr.] genotypes (A: JS-335, B: Dugra (JS-72-280), C: MACS-124, D: JS-90-41). M is 100 base pair DNA marker. Lane 1: pentaplex multiplex PCR using primer combinations SATT177, SATT309, SATT285, SATT335 and SATT268 and lanes 2 to 6 are uniplex PCR amplifications using primers SATT177, SATT309, SATT285, SATT335 and SATT268, respectively



There are certain limitations of mPCR like multiple primer sets may lead to cross hybridization with each other and there is the possibility of mis-priming with other templates, self-inhibition among different sets of primers, low amplify ication efficiency, primer dimer formation, over lapping of amplicons sizes of template DNA etc. which may hinder the use of mPCR in many applications.

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