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Molecular characterization of seed quality in soybean [*Glycine max* (L.) Merill]

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Abstract
Soybean [*Glycine max* (L.) Merill] cultivars varies in storability period at ambient storage. In this study, 11 cultivars of soybean varying in storability (good and poor), seed hardiness and seed testa colour (black and yellow) were characterized with 9 SSR markers. Polymorphisms detected by SSR markers were zero to 11%. Cultivars with black seed coat colour showed better storability than the yellow seed coated cultivars indicating possible association of black seed coat colour with good storability. Genetic similarity coefficients obtained through SSR data analysis in all the soybean cultivars. SSR markers SATT 453, SATT 618, SATT 400, SATT 143 and SATT 286 produced specific allelic bands making them candidate markers for linkage with seed storability and testa colour. SATT 184, SAT_170 had linkage with seed hardiness and SATT 162 and SATT 523 had linkage with seed testa colour. SSR markers data grouped the cultivars broadly into three major clusters that had no congruence with seed storability, seed hardiness and seed testa colour.

Keywords: Soybean, seed storability, seed hardiness, testa colour and SSR candidate marker

Introduction
Seeds are uniquely equipped to survive as viable regenerative organisms until the time and place are right for the beginning of a new generation; however, like any other form of life, they cannot retain their viability indefinitely and eventually deteriorate and die. Therefore maintenance of seed viability and vigour from harvest till next sowing, which may be for one planting season or more, is crucial for the success of agriculture and crop production. Soybean [*Glycine max* (L.) Merill] seed reaches its maximum potential for germination and vigour at physiological maturity (Crookston 1978) [3], which then gradually declines till harvest, followed by a more rapid decline thereafter. Soybean seed is classified as "poor storer" (Justice and Bass 1978) [7], when compared to other grain crops. Germination is often reduced below the minimum standards prior to planting time under warm and humid or hot climate (Nkang and Umoh 1996) [11]. Loss of germination potential is more acute in tropical and sub-tropical regions compared to temperate environments (Bhatia 1996) [1]. A number of seed characters such as seed size, percent hard seededness, seed coat thickness and permeability, hull percentage, oil content etc., are associated with seed quality in soybean and were shown to be under genetic control (Verma and Ram 1987) [17]. These traits are being utilized in breeding programs to improve seed quality and seed longevity in soybean. Significant progress has been made in soybean genomics to target important genes, which has provided a deeper insight into the soybean genome structure and organization. Many reports about the construction of soybean genetic linkage maps using various markers have been published (Keim *et al.*, 1990) [8]. Based on the construction of soybean genetic maps, quantitative trait loci (QTL) for a number of agronomic traits in soybean have been mapped (Hyten *et al.*, 2004) [5]. Pawar *et al.* (2017) [13] reported SATT 453, SATT 618, SATT 400, SATT 143 and SATT 286 SSR markers are associated with seed storability and testa colour SATT 184 and SAT_170 are associated with seed hardiness. Another set of two SSR markers (SATT 162 and SATT 523) has been reported to be significantly associated with seed testa colour in soybean. However, applicability of these markers is yet to be ascertained in the Indian soybean cultivars. Therefore, an attempt was made to characterize a set of good and poor seed quality soybean cultivars with SSR markers. Possible association of testa colour with seed longevity was also studied.

Material and Methods

Plant material: The experimental material consisted of eleven soybean cultivars collected from the Agricultural Research Station, Kasbe Digraj, Sangli. All the cultivars was characterize based on the DNA amplification in the State Level Biotechnology Center, MPKV. Rahuri, District Ahmednagar.

DNA Extraction

Total genomic DNA was isolated from fresh young leaves following the CTAB (cetyl trimethyl ammonium bromide) procedure as described by Saghai Maroof *et al.,* (1984) [15] with some modifications. Quantification of DNA was accomplished by analyzing the DNA on 0.8% agarose gel stained with ethidium bromide using diluted uncut lambda DNA as standard. Final concentration was adjusted to 50ngµl^{-1} for further uses in PCR analysis.

PCR amplification

A total of 9 SSRs primer pairs, distributed across the integrated linkage map of soybean (Cregan *et al.,* 1999) [2] were used. The details of SSR markers, their sequences and motifs are given in table 2. DNA was amplified by PCR using our previously standardized method (Sahu *et al.,* 2012) [16] in a total volume of 10 µl containing 2X PCR assay buffer, 1.5 mM MgCl2, 100 µM of each dNTPs, 12 ng each of forward and reverse primers, 0.2 units of Taq DNA polymerase and 25 ng of genomic DNA template. Amplification reaction initiated with a 5-minute pre-denaturation steps at 94 °C followed by 35 cycles of DNA denaturation at 94 °C for 30 seconds, primer annealing at 50-55 °C for 30 seconds and DNA extension at 72 °C for 7 minutes was performed after 35 cycles. Amplified PCR products was separated on 2.0% of agarose gel at a volage of 90V for the period of 45 minutes to 1 hour in 1X TBE buffer stained with ethidium bromide. The gel was visualized in UV transilluminator and photograph taken using Syngen make gel documentation system.

Table 1: SSR Markers, their sequence with linkage group (LG), cM position in linkage group, motif and annealing temperature

Sr. No.	Primer	Forward sequence	Reverse sequence	LG	cM Position in LG	Core motif	Temp. (°C)	Characters
1	SATT 453	GCGGAAAAAAAACAATAAACAACA	TAGTGGGGAAGGGAAGTTACC	B_1	123.96	(ATT)13	57	Storability & testa colour
2	SATT 618	GCGGTGATATTACCCCAAAAAAATGAA	GCGCTAGTTTCTAGTGGAAAGATGGAT	M	101.06	(ATT)13	61.9	Storability
3	SATT 400	TTGGTCATCAAACTGTTA	CATAAGGGGTCCCACTCTA	G	63.28	(ATT)11	56	Storability
4	SATT 143	GTGCCACAAATTTAAAATTACTCA	TCCCTCCCTTTTGATTTACAC	L	30.19	(ATT)18	52	Storability
5	SATT162	GGGAAGAAGTATATGCTACATCAA	GGGTTAATTTTTATCTTCTAATAGTTT	I	86.74	(ATT)16	57	Black testa colour
6	SATT 286	GCGGCGTTAATTTATGCCGGAAA	GCGTTTGGTCTAGAATAGTTCTCA	C_2	101.75	(ATT)17	60	Storability
7	SATT 523	GCGATTTCTTCCTTGAAGAATTTTCTG	GCGCTTTTTCGGCTGTTATTTTTAACT	L	27.92	(ATT)15	56	Black testa colour
8	SATT 184	GCGCTATGTAGATTATCCAAATTACGC	GCCACTTACTGTTACTCA	D1a	17.52	(ATT)13	55	Seed hardiness
9	SAT_170	GCGGATTGATTTAATTAAGTGTGATTAA	GCGCCGATGATCATGAATTAGAATAACA	I	75.00	(AT)26	55	Seed hardiness

Table 2: Characteristics of the amplification products with SSR primer among cultivars

Sr. No.	Primers	Total number of amplifications	Monomorphic amplifications	Polymorphic amplifications	Percent polymorphism	PIC value
1	SATT 453	10	10	0	0	0.4995
2	SATT 618	10	10	0	0	0.4775
3	SATT 400	11	11	0	0	0.7493
4	SATT 143	7	7	0	0	0.7216
5	SATT162	11	0	11	100	0.7714
6	SATT 286	11	11	0	0	0.7387
7	SATT 523	11	11	0	0	0.5528
8	SATT184	10	10	0	0	0.4914
9	SAT_170	10	10	0	0	0.3673
	Total amplifications	91	80	11		
	Average amplifications	10.11	8.88	1.22	11.11	

SSR allele scoring and data analysis

The presence or absence of SSR fragment in each accession was recorded for all the polymorphic SSR markers. The SSR bands appearing without ambiguity were scored as 1 (present) and 0 (absent) for each primer. The size of the amplified product was calculated on the basis of its mobility relative to molecular mass of marker (100 bp DNA ladder). The genetic similarity among cultivars was estimated based on Jaccard's similarity coefficient. The resulting similarity matrix was further analyzed using the un weighted pair-group method arithmetic average (UPGMA) clustering algorithm for construction of dendrogram; the computations were carried out using NTSYSpc version 2.02 (Rohlf 1998) [14].

Results and Discussion

Identification and validation of candidate markers for seed longevity, seed storability and seed hardiness in soybean: The construction of soybean genetic linkage maps using different markers have been published in many reports. Based on the construction of soybean genetic maps quantitative trait loci (QTL) for a number of agronomic traits in soybean have been mapped. A few SSR markers could clearly discriminate between the good and the poor storer *viz.*, SATT 453, SATT 618, SATT 400, SATT 143 and SATT 286 produced specific allelic bands (117 to 251 bp) with respect to storability and testa colour. SATT 184 and SAT_170 molecular marker produce 168 to 180 bp allelic bands for seed hardiness. Total nine SSR primers either related to seed

coat colour and/or storability and seed hardiness of soybean seeds SATT 453, SATT 618, SATT 400, SATT 143, SATT 286 SATT 184 and SAT_170 were screened to observe the differences in the allelic size of the fragment in cultivars *viz.*, KDS-726, KDS-753, KDS-980, KDS-344, JS-9305, KDS-980, DS-228, NRC-129, NRC-130, NRC-131 and JS-335. All the 9 SSR primer showed very distinct allelic size fragments in good and poor storer, seed storability and seed hardiness.

SATT 453

The preset amplification was observed distinct band corresponding 280 bp was observed in soybean cultivars *i.e.* from KDS-753, KDS-980 and NRC-129, NRC-130, NRC-131, JS-335, DS-228, KDS-726, NRC-132 and KDS-344 soybean cultivars. While, in JS-9305 cultivars no amplification was found (Plate 1) The SATT 453 primer has been reported to produce specific allelic bands with respect to storability and testa colour (Jagdish *et al.*, 2013) [6].

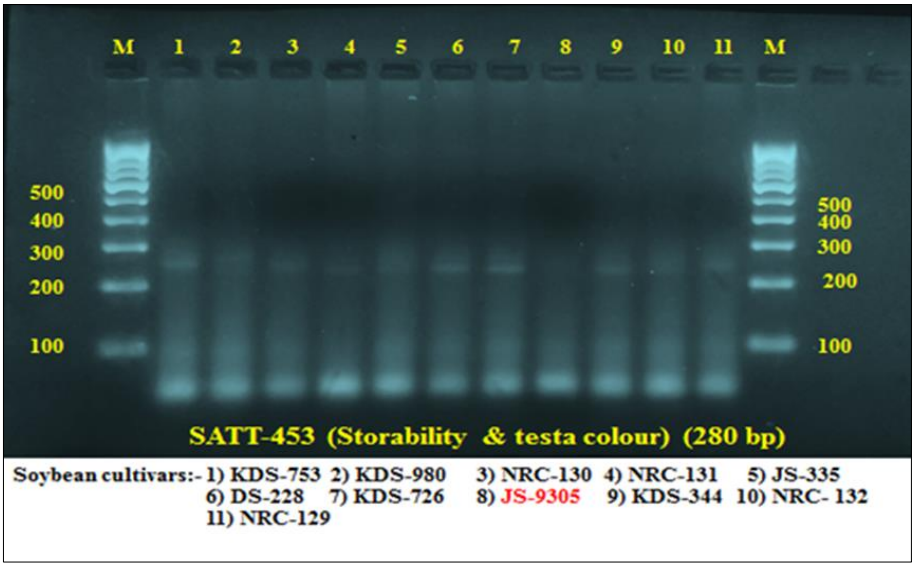

Plate 1: DNA amplification of SATT 453 SSR molecular markers

SATT 618

SATT 618 produced specific allelic bands with respect to storability and testa colour was observed. The band corresponding to an allelic size 117 bp. Soybean cultivars KDS-753, KDS-980, NRC-130, NRC-131, JS-335, DS-228, KDS-726 JS-9305, KDS-344 and NRC-129. While, no amplification were found in the NRC- 132 soybean cultivars (Plate 2) The black seeded soybean showed higher storability due to activity of some free fatty acids and phenolic compounds. SATT 618 produced specific allelic bands making them candidate markers for linkage with seed storability and testa colour (Hosamani *et al.*, 2013) [4].

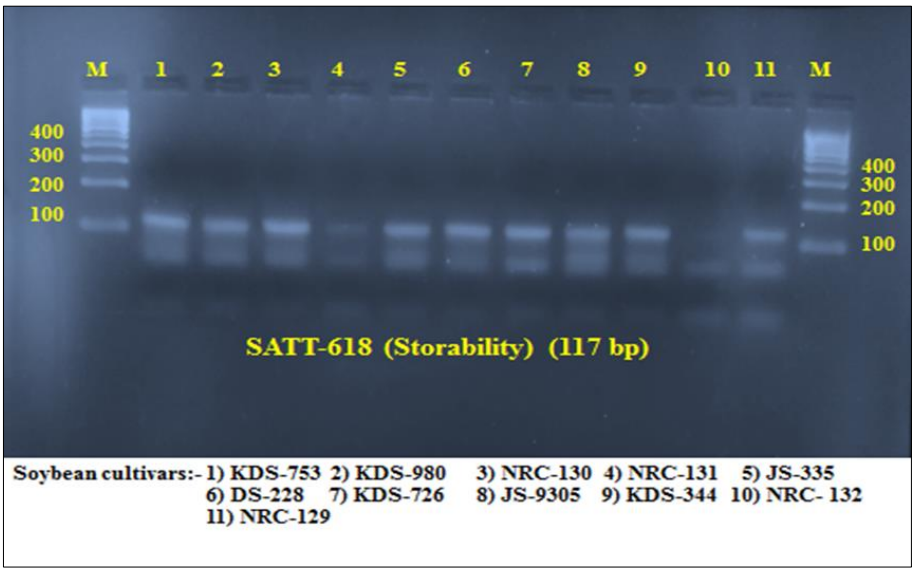

Plate 2: DNA amplification of SATT 618 SSR molecular markers

SATT 400

The high rate of monomorphic SSR loci (100%) detected in the present study. However, the lower allele number and PIC values indicated low allelic diversity in the present set of soybean cultivars. This SSR primer has been reported to produce specific allelic bands with respect of storability with band size of 205 bp were observed in soybean cultivars. The amplification observed in primer SATT 400 is illustrated in Plate 3. This primer were amplified in the all soybean cultivars *viz.*, JS-335, KDS-726, KDS-753, KDS-344, DS-

228, KDS-980, NRC-130, NRC-131, NRC-129, JS-9305 and NRC- 132 varying in storability (good and poor) and seed coat colour (black and yellow). The increase in longevity was associated with the expression of genes encoding protective chaperones such as heat shock proteins and the repression of nuclear and chloroplast genes involved in a range of chloroplast activities (Lima *et al.*, 2017) [10].

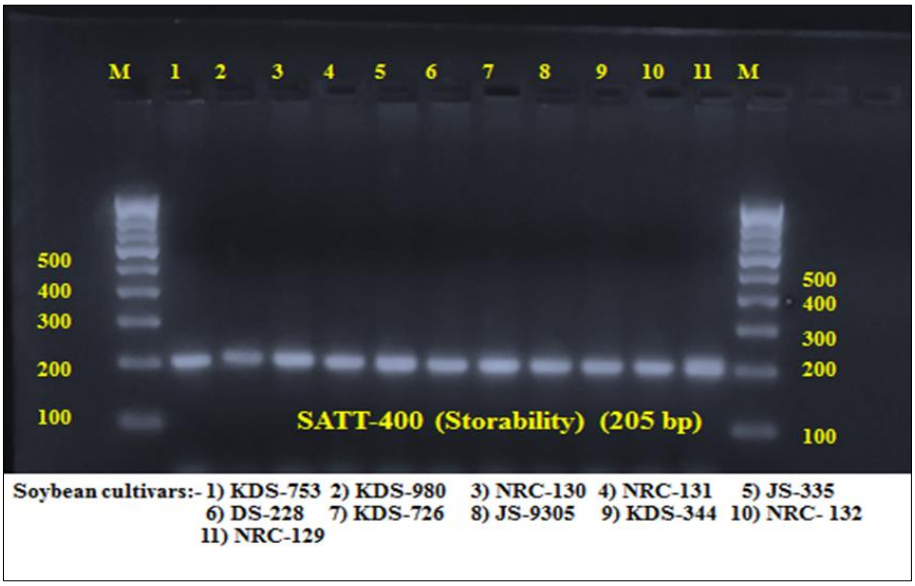

Plate 3: DNA amplification of SATT 400 SSR molecular markers

SATT 143

The SSR primer has been related to storability and it was observed that a band corresponding to an allelic size 251 bp. Among the soybean cultivars DNA amplification were found in KDS-753, KDS-980, NRC-131, DS-228, KDS-344 NRC-129 and NRC- 132 cultivars while, in the NRC-130, JS-335, KDS-726 and JS-9305 no amplification was found (plate 4). The seed coat color and leachate conductivity of soybean has been correlated with seed longevity and black seed coat color has been reported to be positively correlated with better seed longevity (Pawar *et al.*, 2017) [13].

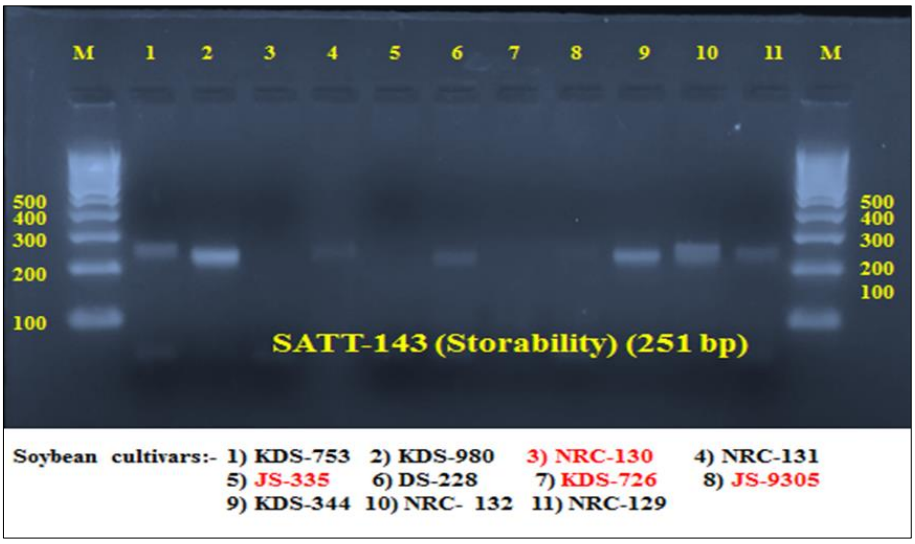

Plate 4: DNA amplification of SATT 143 SSR molecular markers

SATT 162

Eleven soybean cultivars known to amplify SATT 162 SSR markers corresponding to an allelic size 200bp to NRC-131, KDS-726, JS-9305 and NRC-132 soybean cultivars. Whereas, KDS-753, KDS-980, NRC-130, JS-335, DS-228, KDS-344, and NRC-131 soybean cultivars exhibited a band correspond to 180 bp (plate 5). Black seed coat colour may contribute towards better seed longevity. Good storer black-seeded varieties had lesser gap between the seed coat and cotyledon than the poor storer yellow-seeded varieties (Kuchlan *et al.*, 2010) [9].

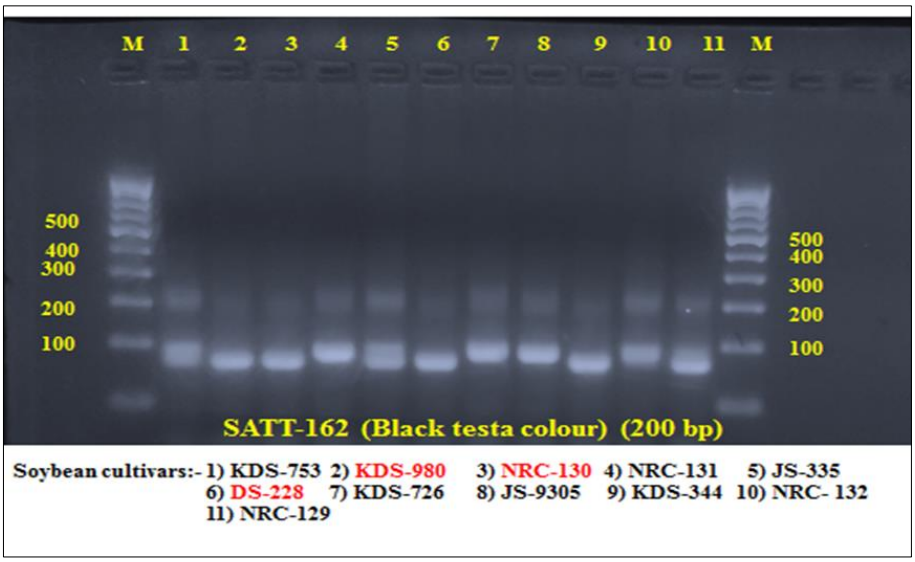

Plate 5: DNA amplification of SATT 162 SSR molecular markers

SATT 286

This SSR primer has been reported to produce specific allelic bands with respect to seed storability. The distinct band corresponding to 217 bp was amplified in all soybean cultivars *viz.*, JS-335, KDS-753, KDS-344, DS-228, KDS-980, NRC-130, NRC-129, NRC-132, JS-9305, KDS-726 and NRC-131 (plate 6).

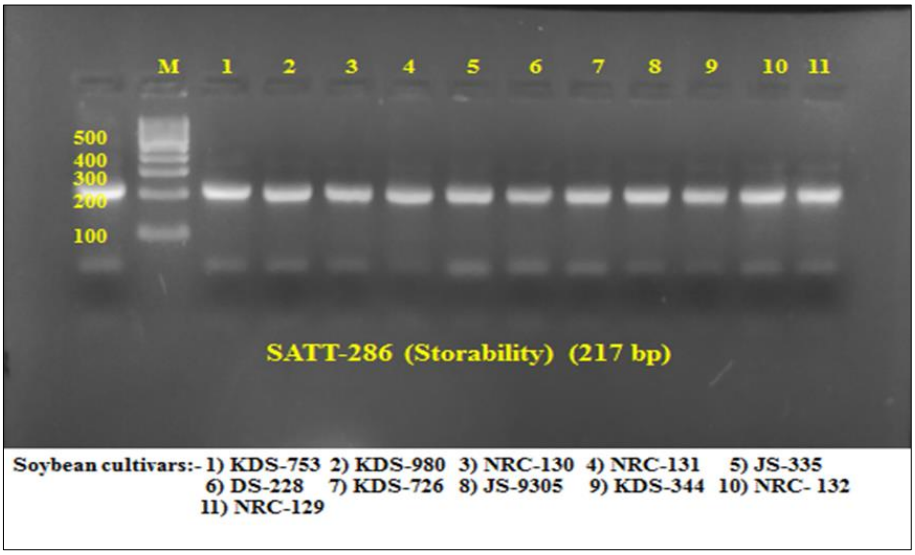

Plate 6: DNA amplification of SATT 286 SSR molecular markers

SATT 523

SATT 523 SSR has been related to black testa colour and it was observed that a band corresponding to an allelic size 180 to 190 bp. This SSR marker were amplified in NRC-130, KDS-980, JS-335, JS-9305 and NRC-129 soybean cultivars with corresponding allelic size of 190 bp. However, KDS-753, NRC-131, DS-228, KDS-726, KDS-344 and NRC- 132 soybean cultivars did exhibit a band correspond to 180 bp (plate 7). Vita-E, lignin, calcium content and activity of antioxidative enzymes appeared to be positively correlated with soybean seed longevity and levels were higher in black and brown seed coat color progenies (Pawar *et al.*, 2017) [13].

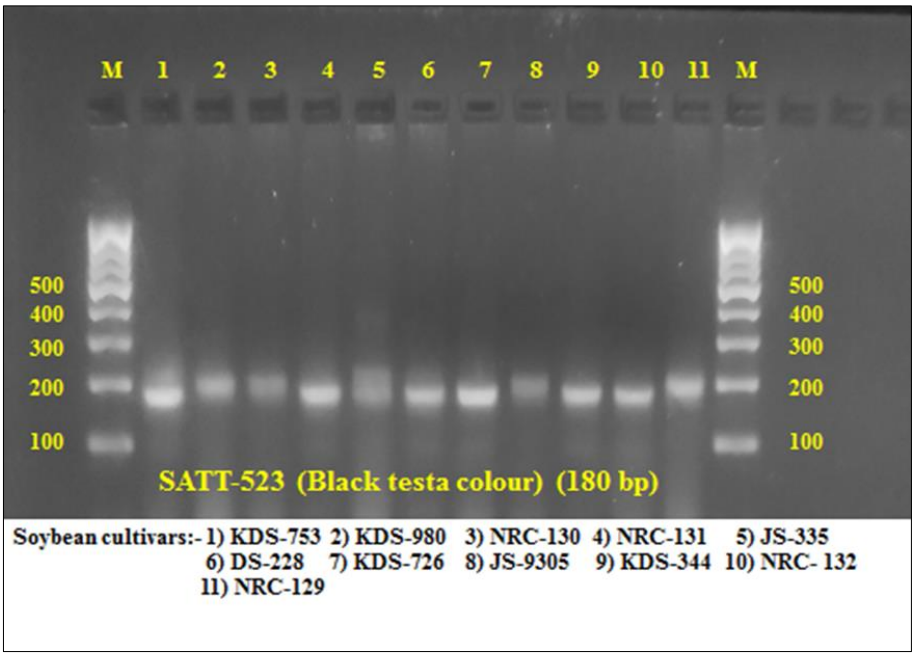

Plate 7: DNA amplification of SATT 523 SSR molecular markers

SATT 184

The distinct band corresponding to 168 bp was observed in seed hardiness soybean cultivars *i.e.* JS-335, KDS-753, DS-228, KDS-980, KDS-726, KDS-344, NRC-130, NRC-129, NRC-132 and JS-9305. However, in the NRC-131cultivars no amplification was found for the respective primers (plate 8). This SSR primer has been reported to produce specific allelic bands with respect to seed hardiness. Hard seededness in soybean quantitative traits that affect the germination rate, viability and storability of soybean seed (Keim *et al.*, 1990) [8]. Seed calcium content and hardness constitute determining characteristics of soybean (Orazaly *et al.*, 2018) [12].

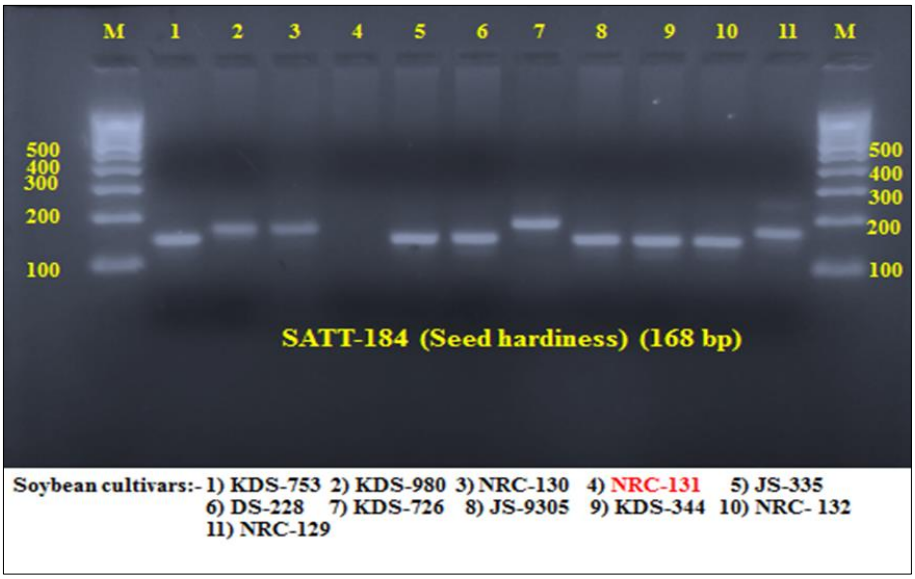

Plate 8: DNA amplification of SATT 184 SSR molecular markers

SAT_170

The role of seed hardness is crucial in quality attributes for soybeans because it affects water absorption, seed coat permeability, and overall texture. This SSR primer has been reported to produce specific allelic bands with respect to seed hardiness. The distinct band corresponding to 180 bp was observed in soybean cultivars *viz.*, JS-335, KDS-753, DS-228, KDS-980, NRC-130, NRC-129, NRC-132 and JS-9305. However, NRC-131 cultivar no amplification were found for the respective primers (plate 9). These results demonstrated that genes underlying seed hardness were actively expressed in the interval between stage 2 and stage 3, which was in parallel with the mass filling period in the development of soybean seeds (Yuanpeng Bu, 2018) [19]. Research is under way to confirm the identified QTLs for soybean seed hardness in multiple populations with different genetic backgrounds (Zhang *et al.*, 2008) [20].

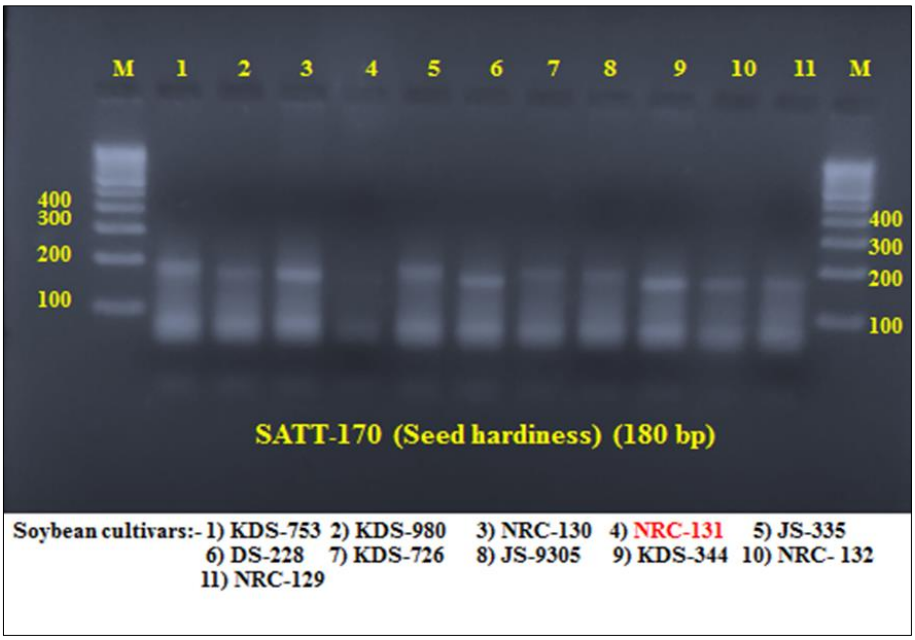

Plate 9: DNA amplification of SATT_ 170 SSR molecular markers

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