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F_2 SCREENING OF SESAME (SESAMUM INDICUM L.) GENOTYPES BY USING MOLECULAR MARKERS

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Abstract: Bulk segregant analysis an approach to analyze the F_2 individuals of white seed coat colour IC-203871 X brown seed colour AKT-101 to screen and identify the markers linked to the seed coat colour trait in sesame. 2 DNA bulks namely white bulk and brown bulk were setup by pooling equal amount of DNA from ten randomly selected plants generated from a segregating population from a single cross. A total of 35 (15 RAPD; 20 SSR) primers were screened out of which 2 primers were polymorphic between parents and F_2 bulks were then used to screen 60 segregating F_2 progeny. RPI-7 (33.33% polymorphism) and Hs-1871 (100% polymorphism) showed linkage with the brown seed coat colour in sesame. Out of 60 individuals of the progeny, the polymorphic band was seen in 26 (RAPD) and 14 (SSR) samples showing it linkage with the brown seed coat colour. The inheritance for seed coat colour was under the control of two independent dominant genes and segregated in the ratio of 9:3:4.

***Index Terms* - Seed coat colour, bulk segregant analysis, RAPD, SSR, polymorphism**

I. INTRODUCTION

Sesame (Sesamum indicum L.), a member of the order Tubiflorae, family Pedaliaceae having chromosome number 2n=26. It is an important annual oilseed crop in the tropics and warm subtropics, where it is usually grown in small plots (Bedigian and Harlan, 1986). Sesame is mostly grown under moisture stress with low management input by small holders. Sesame is described as the "Queen of oilseeds" as it contains high oil (50-60%), protein (18-25%), calcium, phosphorous, oxalic acid and excellent qualities of seed oil (Prasad, 2002) and meal recovered from the extraction process is rich in tryptophan and methionine.

India is the largest producer of sesame in the world and ranks first in terms of sesame-growing area (24%). In India sesame is cultivated on an area of 1566.1 thousand hectare with a productivity 448 kg/ha and 743.9 thousand tonnes production in 2017-18 (Anonymous).

Two types of molecular markers have been used in the present study, random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR). RAPD markers provide a quick method for generating genetic maps and analyzing populations. SSRs or microsatellites are preferred for genetic analysis as they offer several advantage compared to other molecular markers such as high reproducibility, high polymorphism, being multi-allelic, co-dominant, higher relative abundance and extensive genome coverage (Zane *et al*, 2002). But the development of SSRs is costly and time consuming (Squirrell *et al.,* 2003).

Genetic variability in sesame has also been researched by molecular techniques *viz.,* Isozymes, RAPD, AFLP, SSR, and ISSR has been used as molecular markers to date. On the other hand efforts were also made for trait mapping in sesame by Uzun *et al.,* (2003) by using AFLP markers. A genetic linkage map in sesame was first reported by Li-Bin Wei *et al*., (2009) based on F_2 segregating population of an intraspecific cross.

Bulk segregant analysis (Michelmore *et al.* 1991) an approach to identify the marker in specific regions of the genome. Two pooled DNA samples of individuals from a segregating population originating from a single cross were done. Markers that are polymorphic between the pools are genetically linked to the loci decisive the trait accustomed construct the pools.

The present study was initiated with the main objective of, development of F_2 population and to screen and identify the molecular markers linked to seed coat colour trait in sesame. This is the unique work where a combination of bulk segregant analysis, RAPD and SSR primers were used to identify the markers in specific regions of the genome.

II. MATERIALS AND METHOD

The breeding work was carried out at the farm field of Department of Agricultural Botany, College of Agriculture, Latur, (Maharashtra). The molecular work was carried out at the Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, (Maharashtra).

2.1 Materials

The genotypes were obtained from Department of Agricultural Botany, College of Agriculture, Latur (Maharashtra).

2.2 Methodology

The selection of genotype was done on basis of contrasting morphological characters (Fig 1). The female parent IC-203871 with white seed coat colour was crossed with male parent AKT-101 having brown seed coat colour and the F_1 plants were selfed to generate a segregating population for the seed coat colour trait. An F_2 mapping population of 60 individuals was constructed from this cross. The F_2 individuals were phenotypically scored either as white or as brown and were included in the BSA scheme as two bulks contrasting for the trait of interest. DNA was extracted from 10 individual plants from each of the two bulks, from pooling the equal weights of fresh leaves.

2.3 DNA isolation

DNA was extracted according to the Doyle and Doyle (1987) method with minor modifications. Young leaf tissue was crushed with 100 ml extraction buffer (20 mM ethylene diamine tetra acetic acid (EDTA), 2.5 NaCl, 100 mM Tris-HCl: pH 8 and 10 mM b-mercapto-ethanol). The sample was incubated at 65^0C for 45 min and then emulsified with an equal volume of chloroform–isoamyl alcohol (24: 1) and centrifuged at 12,000 rpm 10 min. The aqueous phase was removed and subjected to a second chloroform–isoamyl alcohol extraction. The aqueous phase was again removed and incubated at 20^0C for 30 min. After incubation, the solution was mixed with 500µl of ethanol to precipitate DNA. The nucleic acid precipitate was washed twice with 70% ethanol and dried at room temperature. The pellet was dissolved with TE buffer (10 mM Tris-HCl: 1 mM EDTA: pH 8) and stored in 20^0C. The inhibitors are probably phenolics and polysaccharides that co precipitate with the DNA in alcohol.

2.4 Molecular marker analysis:

A total of 35 (15 RAPD; 20 SSR) primers were used in the present analysis (Table No. 1). The primers used in this investigation were ordered from the Eurofins Genomics, India. PCR reaction mixtures for RAPD were prepared with the volumes of 25 mL containing, 2.00 mL of the extracted DNA, (25 ng/mL), 3.50 mL (10X) assay buffer, 2.00 mL (10 mM) primer, 0.33 mL (0.2 mM) dNTPs, 0.40 mL (1 units) *Taq* polymerase and 16.77 mL sterile distilled H_2O. The PCR reaction was carried out in a DNA thermal cycler (BIORAD) programmed to run the following temperature profile: 94^0C for 5 min initial denaturation then 35 cycles consisting each of a denaturation step for 30 seconds at 94^0C, an annealing step for 30 seconds at 37^0C: an extension step for 30 seconds at 72^0C and the final extension for 10 min at 72^0C. PCR reaction mixtures for SSR were prepared with the volumes of 20 mL containing, 1.00 mL of the extracted DNA (25 ng/mL), 3.50 mL (10X) assay buffer, 1.00 mL (10 pmol) forward and 1.00mL of reverse primer, 0.2 mL (0.2 mM) dNTPs, 0.2 mL (1 units) *Taq* polymerase and 13.10 mL sterile distilled H_2O. The PCR reaction was carried out in a DNA thermal cycler (BIORAD) programmed to run the following temperature profile: 94^0C for 10 min initial denaturation then 35 cycles consisting each of a denaturation step for 30 seconds at 94^0C, an annealing temperature was optimized for each primers: an extension step for 1.5 minutes at 72^0C and the final extension for 10 min at 72^0C. Agarose gel (1.2%) electrophoresis was performed to separate the amplified products. 7 mL of PCR amplified product was loaded with 2 mL of loading dye. The voltage was maintained at 70 V for 1.0 hour and the bands were visualized under gel documentation system (Alpha Imager TM1200, Alpha Innotech Corp., CA, USA).

Table No. 1 List of primers used in F_2 analysis

Sr. N	Primer ID	Sequence (5'- 3')
1	OPA-1	CAGGCCCTTC
2	OPA-2	TGCCGAGCTG
3	OPA-3	AGTCAGCCAC
4	OPA-4	AATCGGGCTG
5	OPA-5	AGGGGTCTTG
6	OPA-6	GGTCCCTGAC
7	OPA-7	GAAACGGGTG
8	OPA-8	GTGACGTAGG
9	OPA-8	GGGTAACGCC
10	OPA-10	GTGATCGCAG
11	RPI-1	AAAGCTGCGG
12	RPI-2	AACGCGTCGG

13	RPI-3	AAGCGACCTG
14	RPI-4	AATCGCGCTG
15	RPI-7	ACATCGCCCA
16	Y1966	F-ACAGCACTTACCCCAAAGGA R-TGGGAGCCAACTTTCATTCT
17	Y751	F-ATGCCACGGAGTACAATTT R-TGCAAGAGTGGAAGTTGAAA
18	Y1248	F-AATGTCAGCTGCCTATTTCC R-AAGACAGGCGATGTCATCTT
19	Y1623	F-TCAACTATCAGTCCAAT R-AAAGAGACCCACAAG
20	Y817	F-CTTACTTTCCAACCTCTTTC R-TCGCTTAATTCTTTGGATGC
21	Y1082	F-CCTACCAGCCAAAAAGAAA R-TCAAGGGTTTCAAGTTCCAT
22	Y1223	F-CGGGCGATAAAATTCAGA RTTGGCTTCAACAGCTACAAA
23	Y2029	F-TGGTTCTCTACGGTTTCTCC R-GTTGGCTACGGTTACAATCC
24	Y2128	F-TAGGTCGAGGGGATCGATAG R-CTAACAGGGGTGACATAGGG
25	Hs1125	F-TTCAGCCCACTTCACTTCAG R-ACGCTCGCACTTCTTTTTCT
26	Hs1153	F-AAGGAGCTGAAGAAGCCAA R-GTTGCATCATTTCATGGAGG
27	Hs1282	F-CGTAAACGTCGTCGTCTTGT R-CCCAGAAAATCCAGAGAAG
28	Hs1385	F-GAGAAAGTTCAGGTGTGCGA R-TTGTCCAGAAGCCCTTTCTT
29	Hs1514	F-CCATGAGTGTTGGCTTTCTC R-CTGCTGTAAATGACGGGATG
30	Hs1871	F-GCATTTCAGGTGGACAAATG R-TAATGAGCAGAGGCACAAC
31	Hs1972	F-TTCGGTGGCATTAGCTGTAG R-TTGTGGGCATAGGTGGTAGA
32	Hs1007	F-TCCATCACCTCCTCATCAAA R-CCGAGGACACAAGGAATCTT
33	Hs1011	F-GGATAGGGTGAGGGCTGATA R-CGTGGCAATAGACCAAATCA
34	Hs1026	F-TGCAGTCTTTGCTTTTGTCC R-TCCATCTCTCAATCCACAGC
35	Hs1977	F-ACAGTATTTGGGACTTGCCC R-GCCGTTTCCTTGTCTGATTT

III. Results and Discussion

Sesame is an unexplored crop which needs a focused research for its genetic improvement in several fronts. Major sesame traits that require attention to achieve higher yields are harvest index, seed retention, uniform maturity, and resistance to abiotic and biotic stresses. Foremost requirement for this is the assessment of genetic diversity available in the crop. RAPD and SSR markers have been effectively used for the assessment of molecular diversity, and the data were integrated with the other markers for construction of genetic map in several crop plants.

Several approaches have been suggested to saturate genomic regions of interest with molecular markers. Bulked segregant analysis provides a rapid, technically simple alternative tool for identifying markers linked to specific regions of the genome. The only prerequisite is the existence of a segregating population resulting from a single cross that segregates for the gene of interest. The success of the approach will depend on the genetic divergence between the parents in the target region.

The morphological observation for the qualitative trait seed coat colour (Baydar *et al.,* 2000) was observed after 10 days of harvesting. The trait was evaluated for all the 60 segregating population.

Bulk segregant analysis and molecular markers were used to analyze the F_2 individuals of white seed coat colour IC-203871 X brown seed coat colour AKT-101 to screen and identify the molecular marker linked to seed coat colour in sesame. For carrying out of bulk segregant analysis equal amounts of DNA from pooled samples of individuals from a segregating population originating from a single cross were done.

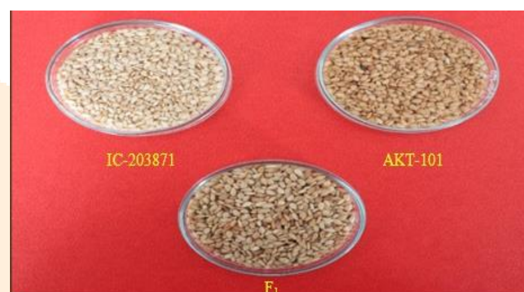

Fig 1: Parents IC-203871(white), AKT-101 (dark brown) and their F_1 (light brown) differing in seed coat

Bulk segregant analysis uses two DNA bulks namely white bulk and brown bulk were setup by pooling equal amount of DNA (Gemenet *et al.,* 2010) from ten randomly selected plants from segregating individuals. A total of 35 (15 RAPD; 20 SSR) primers were used in the present analysis. In F_2 screening, markers that are polymorphic between the pools and are genetically linked to loci determining the trait were used to construct the pools. Here out of the total 35 primers screened, we found a single RAPD RPI-7 (33.33% polymorphism) primer and a single SSR primer Hs1871 (100% polymorphism) which distinctly shows the difference between the bulks.

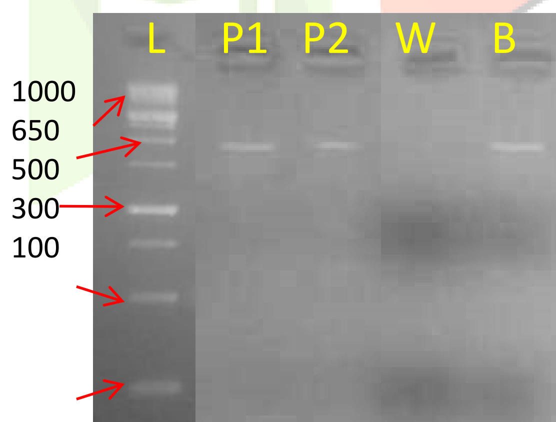

Fig 2: RAPD marker detecting polymorphisms between bulks made for seed coat colour. The four lanes result from PCR amplification with a l0-mer oligonucleotide primer: Rpi-7. The first and second lanes contain parental DNA from IC-203871 and AKT-101. The third lane contains bulked DNA from the homozygous white seeded individuals (W), and the fourth lane contains bulked DNA from the homozygous brown seeded individuals (B): L-1kb ladder

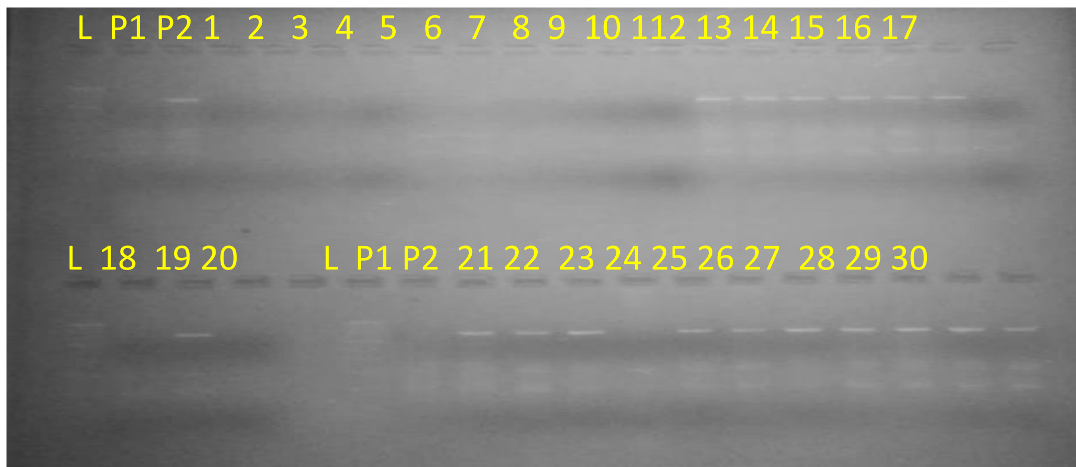

Fig 3(RAPD profile, RPI-7): L- ladder; P1-IC-203871; P2-AKT-101; 1-10- F2 individuals used for developing white bulk; 11-20- F_2 individuals used for developing brown bulk; 21-30- individual F_2 progeny of F_2 population

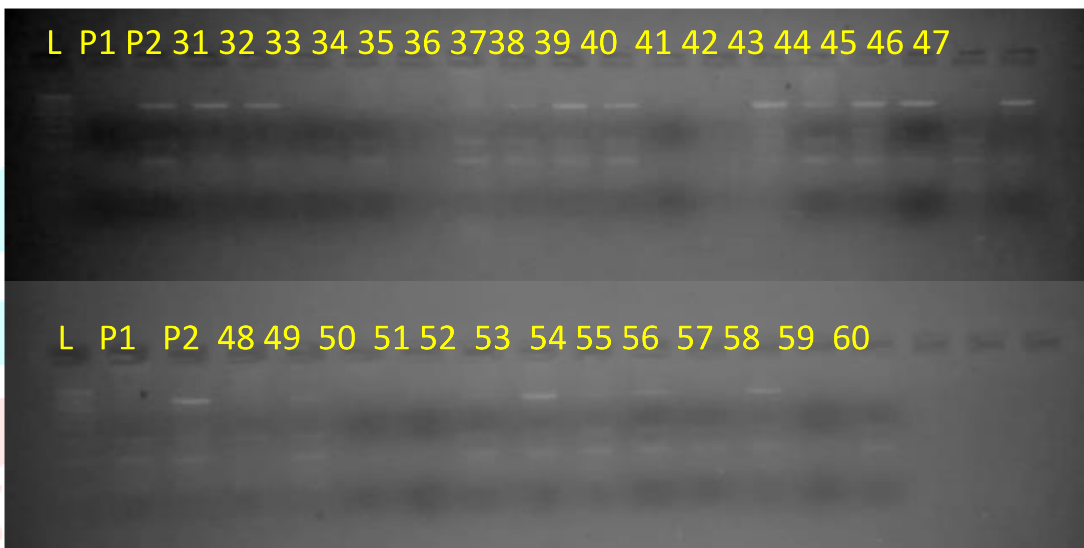

Fig 4(RAPD profile, RPI-7): L- ladder; P1-IC-203871; P2-AKT-101; 31-60- individual F_2 population

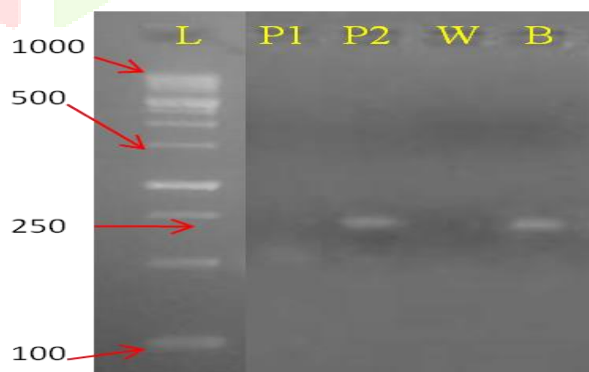

Fig 5: SSR marker detecting polymorphisms between bulks made for seed coat colour. The four lanes result from PCR amplification with a primer: Hs-1871. The first and second lanes contain parental DNA from IC-203871 and AKT-101. The third lane contains bulked DNA from the homozygous white seeded individuals (W), and the fourth lane contains bulked DNA from the homozygous brown seeded individuals (B): L- 1kb ladder

Fig 6 (SSR profile): L- ladder; P1-IC-203871; P2-AKT-101; 1-10- F2 individuals used for developing white bulk; 11-20- F_2 individuals used for developing brown bulk; 21-29- individual F_2 progeny

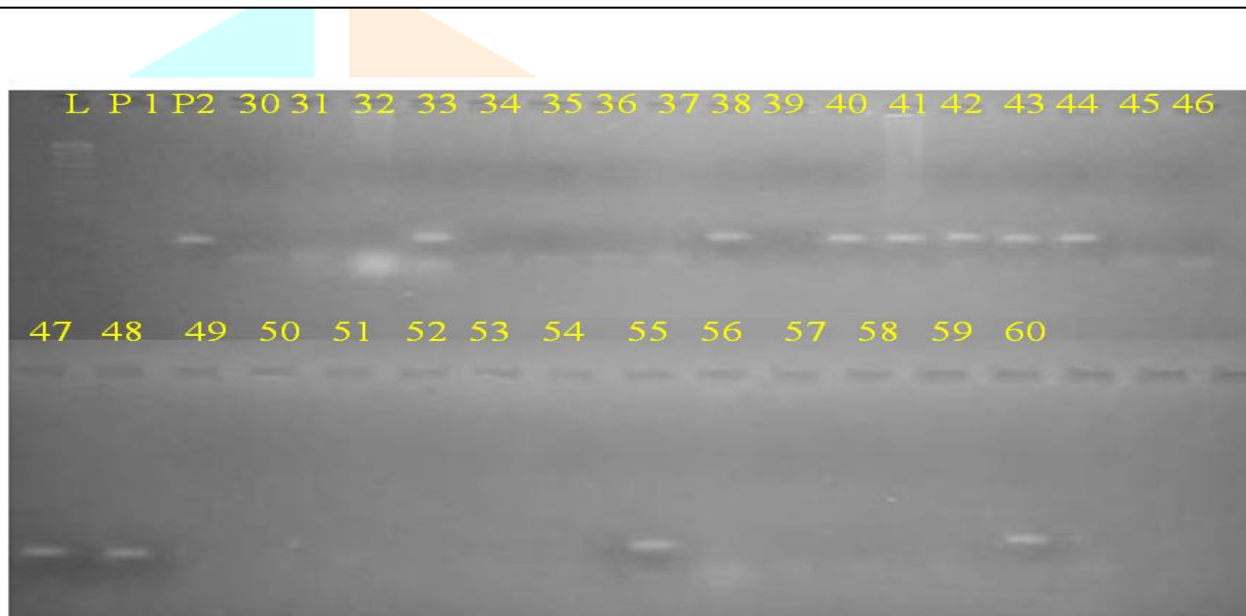

Fig 7 (SSR profile): L- ladder; P1-IC-203871; P2-AKT-101; 01-60- individual F_2 progeny

This work was supported by Ummu (2010) in accordance with genetic characterization; showed only five polymorphic markers among 318 EST based SSR markers of Turkish sesame accessions. Genetic analysis showed that Turkish sesame accessions have fairly low genetic diversity. Marri *et al*., (2005) used 210 microsatellite markers in rice to screen the parents (*O. rufipogon and O. sativa*) for identifying polymorphic markers. Eight markers (38%) detected polymorphism. The lower percentage of polymorphism may be due to higher degree of genetic similarity between *O. rufipogon and O. sativa* used in this study.

Out of sixty F_2 individuals of bulk the polymorphic band was seen in 26 (RAPD RPI-7; Fig 3 and 4) and 14 (SSR Hs1871; Fig 6 and 7) samples shows it linkage with brown seed coat colour. Respective primers generated a fragment in brown parent and their bulks but not in the white parent and their bulks (Fig 2 and 5). It confirmed that above mentioned primers were tightly linked to brown seed coat colour in sesame. The inheritance for seed coat colour was under the control of two independent dominant genes and has segregated in the ration of 9:3:4 (Chi-square is satisfactory). Table value of χ^2 (Table No. 2) at 0.05 level of probability at 2 df (degrees of freedom) is 5.99. Hence the data observed is in good fit of expected ratio of 9:3:4. Sesame is self-pollinated crop and it is very difficult to maintain F_2 populations. Hence results are produced on the basis of available population of F_2.

Table No. 2 Mode of inheritance of seed coat colour in the F_2 progeny in the cross between IC-203871×AKT-101 of sesame

Generations	No of plants	Observed frequency			Expected frequency			Ratio	Chi square
		W	LB	BB	W	LB	BB		
F2	60	26	12	22	33.75	11.25	15	9:3:4	4.99

W-white seed coat: LB-light brown: DB-dark brown

This finding is in confirmation with the earlier report by Baydar (2000) who reported that segregation ratios for seed coat colour was not only 3:1 but also segregate epistatically in the ratio of 9:3:4.

IV. CONCLUSION

A total of 60 f2 bulk segregant population was generated from the cross IC-203871 X AKT-101. The parents selected for the crossing were phenotypically and genotypically diverse. For the standardization of DNA extraction protocol and PCR procedure, varied concentrations of DNA isolation components were used. Particularly, in sesame which requires higher concentration of sodium chloride (2.5M up to 4M).

In F_2 screening, the primers RPI-7 (Fig 2) and Hs-1871 (Fig 5) produces a polymorphic band male parent AKT-101 and same band was observed in “B” bulk showing it linkage with seed coat colour *i.e.* brown seed coat colour in sesame. A total of 124 amplicons were generated by RAPD primers, out of which 104 were polymorphic giving total 84.27% polymorphism among 02 sesame genotypes. The primers OPA-5, OPA-8, OPA-10, RPI-2, RPI-3 and RPI-4 show 100% polymorphism. A total of 13 amplicons were generated by SSR primers, out of which 6 were polymorphic giving total 36.36% polymorphism among 02 sesame genotypes. The primers Hs1385, Hs1514, Hs1153 and Hs1871 show 100% polymorphism. However, the present study showed that, even though the primer shows 100% polymorphism among parents but fail to generate polymorphic band in respective F_2 bulk of progeny and *vice versa*.

Bulked segregant analysis could be extended to the analysis of' genetically complex traits by screening bulks of informative individuals. From the studies done so far it is clear that, several such crosses and BSA studies need to be done to confirm the markers for QTLs and characterize the sesame species.

V. ACKNOWLEDGMENT

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