

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(4): 20-27
www.biochemjournal.com
Received: 08-01-2026
Accepted: 10-02-2026

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Assessment of molecular diversity among Indian mustard [*Brassica juncea* (L.) Czern and coss] genotypes using SSR marker

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i4Sa.8047>

Abstract

Genetic diversity can be assessed through molecular markers at the DNA level because of their abundance as compared to morphological characters and independence of environmental influences. The present investigation was carried out at the Biotechnology Laboratory of Department of Genetics and Plant Breeding at C. P. College of Agriculture, S. D. Agricultural University, Sardarkrushinagar during 2021. Molecular diversity of twenty-two mustard genotypes was analysed using simple sequence repeat (SSR) analysis. DNA extraction was carried out by CTAB method. Total 30 SSR primers were screened across all the genotypes of mustard. Twenty alleles were amplified and high polymorphism (100%) was found from 10 SSRs primers (BR-A01, BR-A03, BRA05, BRMS-08, BRMS-37, BRMS-240, SR-9222, SR-94102, BG-41 and BG-48). Number of alleles per locus was 2 for polymorphic primers and 1 for monomorphic primers, with an average of 1.45 alleles per locus. The Polymorphic Information Content (PIC) value ranged from 0.09 to 0.22, with an average of 0.06. Similarity indices derived from Jaccard's similarity co-efficient were estimated on the basis of twenty-two SSR primers ranged from 0.60 to 1.00. Genotypes, GMMo2108A and GMMo2102A showed minimum similarity indicating the presence of genetic diversity among the genotypes of mustard. A dendrogram based on the UPGMA analysis showed two main clusters and four sub-clusters. Results of similarity indices, dendrogram, SSR is useful in the exploration of genetic diversity in mustard. Overall results suggested that SSR can be a great tool to explore genetic diversity in mustard.

Keywords: SSR, Indian mustard, molecular diversity, molecular marker, primer

Introduction

Indian mustard [*Brassica juncea* (L.) Czern and Coss], commonly known as rai or raya, belongs to the family *Brassicaceae* and genus *Brassica*. Indian mustard is a natural amphidiploid ($2n = 36$) derived from interspecific cross of *B. campestris* ($2n = 20$) and *B. nigra* ($2n = 16$). It is largely a self-pollinated crop (85-90%). However, owing to insects, especially honeybees, the extent of cross-pollination varies from 4.0 to 16.6 per cent (Rambhajan *et al.*, 1991) [13]. India occupies first position in area and second in production after China. Genetic diversity of the germplasm is the major interest for the plant breeder. So, they are producing high yielding varieties which can tolerate environmental fluctuations for increasing the production and productivity of pulses. Genetic diversity can be assessed through molecular markers at the DNA level because of their abundance as compared to morphological characters and independence of environmental influences. It can give information about genetic diversity and structure of germplasms from various geographical origins (Akkale *et al.*, 2010) [2]. In addition, they allow more accurate characterization of the genetic variations and genetic relationships. Thus, molecular markers can successfully provide an alternative tool for genetic analysis.

Variations of the DNA sequences (base-pair additions, substitutions, deletions or sequence patterns) are the bases for molecular marker to detect polymorphisms. List of structural markers is long viz., RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), Cleaved Amplified Polymorphic Sequence (CAPS), Microsatellite or Simple Sequence Repeat (SSR), Inter-SSR (ISSR), etc. SSRs are short tandem repeats of 1 to 6 bases that occur randomly and frequently in the eukaryotic genome (Tautz and Renz, 1984) [17].

SSR markers are abundant, locus specific, reproducible, hyper variable, codominant in nature and uniformly present in the nuclear and organelle genome.

The SSRs markers are more advantageous compared to other marker systems. They are reproducible, co-dominant in nature, abundant, highly polymorphic and easily scorable representing multi-allelic variation (Adhikari *et al.*, 2017) [1]. Numerous SSRs markers have been used in different *Brassica* species such as in *B. juncea* (Vinu *et al.*, 2013; Pratap *et al.*, 2014) [18, 10], *B. napus* (Li *et al.*, 2013) [7] and *B. rapa* (Suwabe *et al.*, 2002) [16]. Hence, we used SSR markers for diversity analysis.

Materials and Methods

Total genomic DNA Isolation procedure

For the isolation of genomic DNA, twenty-two diverse mustard genotypes were grown in Agronomy Instructional Farm, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar. All the procedure start from the DNA isolation to PCR amplification was conducted at biotechnology laboratory of Department of Genetics and Plant Breeding, Chimanbhai Patel College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar. The isolation of genomic DNA of Indian mustard genotypes was carried out as per CTAB method of Doyle and Doyle (1987) [4]. Various stock solutions and buffers were prepared and used during isolation of genomic DNA and during gel electrophoresis. The primers used for present study (Table 1) was taken from Pratap *et al.* (2014) [10], Prajapati *et al.* (2014) [9], Sharma *et al.* (2018) [15], Rajpoot *et al.* (2020) [12], Kumar *et al.* (2020) [6], Panigrahi *et al.* (2018) [8].

Genomic DNA Isolation, Purification and Quantification

The fresh leaves were taken from 20-25 days old seedlings and were made to grind to a fine powder using liquid nitrogen in a pestle and mortar and were filled into 2 ml centrifuge tube. 1 ml of CTAB extraction buffer and 10µl β-mercaptoethanol was added into the tubes and kept in a

water bath for 45 minutes to 1 hour, where tubes are given a jerk for every 10-15 minutes interval. After incubation the tubes were centrifuged at 12000 rpm for 12 minutes at 24°C. The supernatant was carefully transferred to a fresh tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added and again centrifuged for 12 minutes at 12000 rpm. The supernatant was transferred to a fresh tube and given a RNase A treatment and kept in water bath at 37°C for 45 minutes. Equal volume of chloroform: isoamyl alcohol (24:1) was added after the RNase A treatment and centrifuged at 12000rpm for 12 minutes. The supernatant was taken and equal volume of chloroform: isoamyl alcohol (24:1) was added and again centrifuged at 12000 rpm for 12 minutes. After centrifugation the supernatant was transferred to 1.5 ml centrifuge tube and 0.6 volume of chilled isopropanol was added and stored overnight at -20°C for better precipitation of DNA. Next day morning, the fine pellets of DNA will be obtained after it has been given a centrifugation at 4°C for 10 minutes at 10000 rpm. The pellets obtained was washed with 70% ethanol, twice and kept in the oven for the evaporation of ethanol. Later, the pellets were made to dissolve in 100 µl of 1X TE buffer by tapping on the tubes and stored at -20°C.

The quantification of nucleic acid was performed using UV spectrophotometer. The good quality DNA will have the O.D. in the range of 1.8-2.0. The O.D. below 1.8 or 1.7 indicates protein contamination and O.D. above 2.0 will be indicating contamination of RNA. The 0.8% agarose gel run will show bands with less shearing indicates good quality and amount of DNA.

PCR Amplification

For the PCR based amplification total thirty number of SSR primers were used for the validation. PCR reactions were performed in the reaction volume of 15 µl containing 1.5µl of Taq buffer (10X) with MgCl₂, 0.5 µl dNTP mix (10 mM), 1.0 µl of each primer (5 p mol), 0.1 µl of Taq DNA polymerase and 1.0 µl of genomic template DNA (50ng/ml).

Table 1: List of SSR primers used for PCR amplification in the present study

Sr. No.	Primer		Sequence	No. of nucleo tides	Tm value (°C)	% GC
1.	BR-A01	F	CCGTTTTTATGTCACAAATCT	21	52.01	33.33
		R	AAACAAAACGAACCTTTGTCAG	21	52.01	33.33
2.	BR-A03	F	ATCGTCTCTTCGTCTTGCT	21	55.92	42.86
		R	CGTAAACTGAAACCATTCAC	21	53.97	38.10
3.	BR-A04-9627	F	ATGGAATCTGCTCATCTCAC	20	55.25	45.00
		R	TAAGCTGCAATGATCAAAGAT	21	52.01	33.33
4.	BR-A04-154	F	TTTGAACGATACACAACAACA	21	52.01	33.33
		R	GTTGGTCCACGAGTAAAAGAT	21	55.92	42.86
5.	BR-A05	F	ATAAAGATTGATGGGAGGAG	21	53.97	38.10
		R	GGTGGAGGAGGATAGTTGTAG	21	59.82	52.38
6.	BRMS-01	F	GGTGGCTCTAATTCCTCTGA	20	57.30	50.00
		R	ATCTTTCTCTCACCAACCCC	20	57.30	50.00
7.	BRMS-05	F	ACCTCCTGCAGATTCGTGTC	20	59.35	55.00
		R	GCTGACCTTTCTTACCGCTC	20	59.35	55.00
8.	BRMS-07	F	AAATTGTTTCTCTTCCCAT	20	51.15	35.00
		R	GTGTTAGGGAGCTGGAGAAT	20	57.30	50.00
9.	BRMS-08	F	AGGACACCAGGCACCATATA	20	57.30	50.00
		R	CATTGTTGTCTTGGGAGAGC	20	57.30	50.00
10.	BRMS-14	F	CCGTAAGGAATATTGAGGCA	20	55.25	45.00
		R	TTCCCAATTCTCAAACGGTA	20	53.20	40.00
11.	BRMS-27	F	GTGCTTGATGAGTTTCACATTG	22	56.53	40.91
		R	GCAGGCGTTGCCTTTATGTA	20	57.30	50.00
12.	BRMS-30	F	TCAGCCTACCAACGAGTCATAA	22	58.39	45.45

		R	AAGGTCTCATACGATGGGAGTG	22	60.25	50.00
13.	BRMS-31	F	TGCCACCAATGACAATGACACTAC	25	61.34	44.00
		R	GATGCACTGGGACCACCTTACATTTT	25	61.34	44.00
14.	BRMS-37	F	CTGCTCGCATTTTTTATCATAC	22	54.66	36.36
		R	TACGCTTGGGAGAGAAAACTAT	22	56.53	40.91
15.	BRMS-240	F	CAAGAGTATTTGTGTGGGTTGACTC	25	61.34	44.00
		R	AAATAACGAACGGAGAGAGAGAGAG	25	61.34	44.00
16.	BRMS-324	F	AACTTAACCGAAACCGAGSTAGGTG	25	62.98	48.00
		R	AATCTCGAAATTCATCGACTTCCTC	25	59.70	40.00
17.	SSR Na10-D09	F	AAGAACGTCAGATCCTCTGC	21	57.87	47.62
		R	ACCACCACGGTAGTAGAGCG	20	61.40	60.00
18.	SSR Na10-D11	F	GAGACATAGATGAGTGAATCTGGC	24	61.01	45.83
		R	CATTAGTTGTGGACGGTCGG	20	59.35	55.00
19.	SR-7223	F	AGGACCCGACTTTCCTTGTT	20	57.30	50.00
		R	ACCAAACCTCGGCGTACAAAT	20	55.25	45.00
20.	SR-9222	F	CACCGAACAAAACCTGAGGGT	20	57.30	50.00
		R	CGTTTCACTGCGTTCTACCA	20	57.30	50.00
21.	SR-94102	F	ATCCCCAACTACCCTCACC	20	59.35	55.00
		R	AGGATGAGCAAAGGAAAGCA	20	55.25	45.00
22.	BG-41	F	TCCTCCGACAACAACAACTCAA	22	58.39	45.45
		R	ATCTAACCCGTCTGCGAATCTG	20	60.25	50.00
23.	BG-48	F	CACGAAAGCTGTAGAGGCATGA	22	60.25	50.00
		R	TCTTTTCTGTCCATGAGATTCAA	24	57.59	37.50
24.	BG-100	F	TGGTAAGTGTACTTCCATATGTTT	27	57.37	29.63
		R	TGTGATGGATTGAATCGAGGTC	22	58.39	45.45
25.	BG114	F	GCAAAATCCATTGGTAATCAGGA	23	57.08	39.13
		R	TGGGCAAGTCACACTCACTCA	21	59.82	52.38
26.	BG132	F	AATGCCCATCCCTCCTTGAT	20	57.30	50.00
		R	TTTGAACACTCATTGTGGTGATGA	24	57.59	37.50
27.	BRMS-017	F	GGAAAGGGAAGCTTCATATC	20	55.25	45.00
		R	CTGGAAAGCATACACTTTGG	20	55.25	45.00
28.	BRMS-033	F	GCGGAAACGAACACTCCTCCCATGT	25	66.26	56.00
		R	CCTCCTTGTGCTTTCCCTGGAGACG	25	67.90	60.00
29.	BRMS-011	F	GAACGCGCAACAACAAATAGTG	22	58.39	45.45
		R	CGCGTCACAATCGTAGAGAATC	22	60.25	50.00
30.	BrgMS339	F	CTACCTGAAGATGACCCAGACG	22	62.12	54.55
		R	GCATACAAACCTCGTCTAAGC	22	60.25	50.00

Results and Discussion

Total 30 SSR primers were used for screening of twenty-two genotypes for the assessment of molecular diversity. Out of 30 SSR primers, 10 primer pairs were found to be polymorphic, 12 primers found monomorphic while rest of the primers not amplified properly among the mustard genotypes. The results obtained using 10 polymorphic primers and 12 monomorphic primers out of total 30 have been presented in Table 2. The SSR amplification profiles obtained in the present study with primers BR-A01, BR-A03, BR-A05, BRMS-08, BRMS-37, BRMS-240, SR-9222, SR-94102, BG-41 and BG-48 were found polymorphic. The results obtained using 10 polymorphic primers have been presented in Table 2 and result is shown in Plate-1 To 10. Number of alleles per locus was found to be 2 alleles. Previous studies by Rajpoot *et al.* (2020) [12] found an average of 3.86 alleles per locus which was higher than present study. The band size of primers BG-41 and BG-48 used in present genetic diversity study in mustard was in accordance with molecular size of 160-260 bp as reported by Neeraj *et al.* (2020) [6], the band size of BRMS-37 was in accordance with 137-176 bp as reported by Prajapati *et al.* (2014) [9] whereas, the band size of BRMS-017 was in accordance with 200-350 bp as reported by Prajapati *et al.* (2014) [9].

Based on the simple sequence repeat data, cluster analysis was performed by using genetic similarity values and a dendrogram (Fig. 1) was generated showing the genetic

relationships among twenty-two genotypes of mustard. The dendrogram was constructing based on UPGMA (un weighted pair-group method with arithmetic mean) by using Jaccard's similarity coefficient through NTSYSpc (version 2.0) software. Jaccard's similarity coefficient values for 22 genotypes ranged from 0.60 to 1.00 with average of 0.88 indicating moderate level of genetic variation existing among the mustard genotypes used in this study. Kumar *et al.* (2020) [6] also reported Similarity coefficient average of 0.72 among 30 cultivars. Clustering pattern of dendrogram generated by pooled SSR data of twenty-two genotypes by using 22 primer pairs constructed using UPGMA showed two major clusters A and B formed at a similarity coefficient of 0.93 (Fig : 1). The similarity coefficient was ranged from 0.86-1.00 which is an indicative of presence of moderate variation among genotypes. Relationships between the mustard cultivars were graphically represented in the form of dendrogram. These results indicated that this genotypes GMMo2122A, GMMo2130A, GMMo2128A, GMMo2102A were found to be the most diverse type that the genetically divergent parents could be chosen which indicate adequate polymorphism.

The knowledge of genetic relationships among the genotypes provides useful information to mark breeding programs and germplasm resource management (Roldan-Ruiz *et al.*, 2001) [14]. The present investigation suggested that SSR markers are suitable to evaluate genetic diversity in mustard and suitable to individually distinguish all the

twenty-two genotypes of mustard. Molecular data must be useful for exposing DNA patterns especial for a given genotype or set of genotypes.

Overall, it was concluded that the genetic diversity derived in this investigation might be useful in future mustard improvement programmes and selection in mustard crop should be based on the phenotypic characters as well as based on the molecular markers analysis. Therefore, studies on morphological and molecular markers are completely

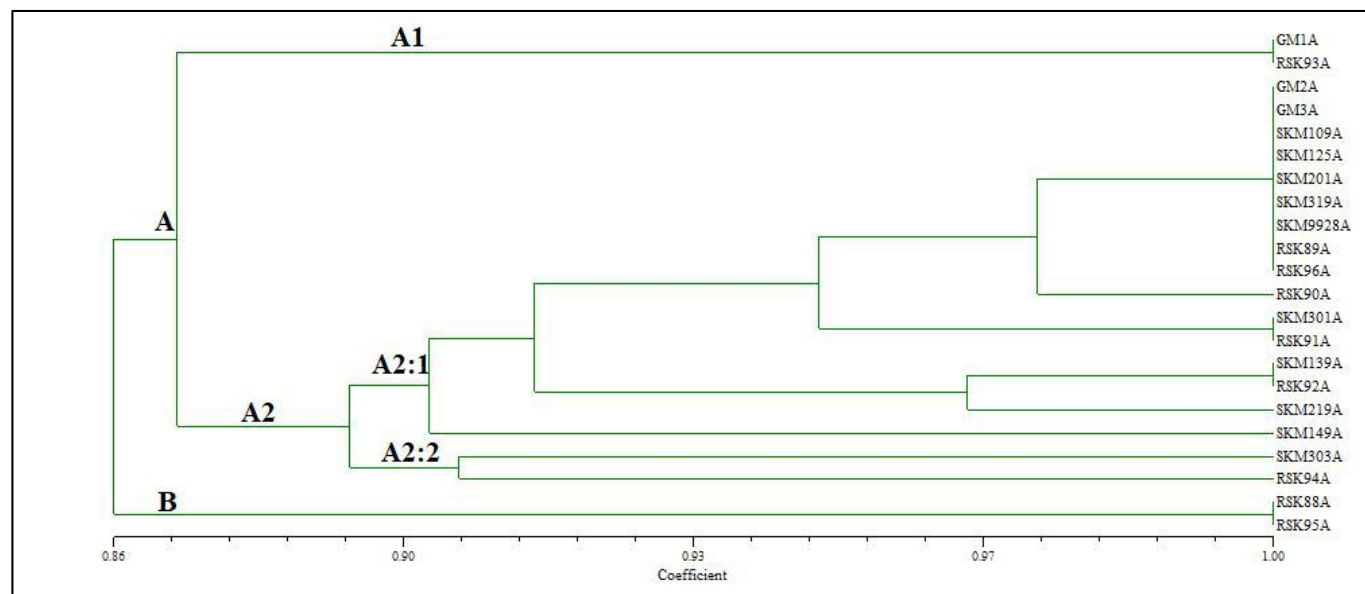
useful in analysis the genetic diversity in mustard. Normally, it can be observed that the combination of microsatellites and morphological data can be highly informative so that they could be effective and accurate tools for recognizing genetic diversity in mustard germplasm. The assessment of genetic divergence and relatedness among breeding materials has significant implications for the improvement of crop plants (Chandra *et al.*, 2013) [3].

Table 2: Particulars of polymorphic SSR primers used in the study

Sr. No.	Primers	Range of Molecular weight (bp)	No. of Total alleles	No. of amplified bands	Polymorphic Information Content (PIC) value	Polymorphism
1.	BR-A01	50-100	2	16	0.22	Yes
2.	BR-A03	100-200	2	20	0.09	Yes
3.	BR-A05	100-200	2	19	0.09	Yes
4.	BRMS-08	300-400	2	15	0.21	Yes
5.	BRMS-37	120-200	2	16	0.22	Yes
6.	BRMS-240	150-210	2	14	0.14	Yes
7.	SR-9222	400-500	2	20	0.09	Yes
8.	SR-94102	150-250	2	18	0.09	Yes
9.	BG-41	120-180	2	18	0.16	Yes
10.	BG-48	150-200	2	20	0.09	Yes
11.	BR A04-9627	300-350	1	16	0.00	No
12.	BR A04-154	50-250	1	19	0.00	No
13.	SR-7223	400-500	1	21	0.00	No
14.	BRMS-01	250-300	1	20	0.00	No
15.	BRMS-05	100-200	1	10	0.00	No
16.	BRMS-017	230-250	1	21	0.00	No
17.	BRMS-30	320-350	1	22	0.00	No
18.	BRMS-324	250-300	1	16	0.00	No
19.	SSR Na10-D09	100-300	1	17	0.00	No
20.	BG-114	100-200	1	19	0.00	No
21.	SSR Na10-D11	100-300	1	09	0.00	No
22.	BRMS-033	100-200	1	15	0.00	No
Total :		-	32	381	1.40	-
Mean :		-	1.45	17.32	0.06	-

Table 3: Distribution of genotypes based on the clusters from UPGMA dendrogram from SSR primer

Cluster	Sub-cluster	Genotypes	No. of genotypes
	A1	GMMo2102A, GMMo2128A	02
	A2:1	GMMo2103A, GMMo2105A, GMMo2106A, GMMo2108A, GMMo2109A, GMMo2111A,	
A	A2	GMMo2113A, GMMo2114A, GMMo2116A, GMMo2119A, GMMo2121A, GMMo2123A, GMMo2124A, GMMo2125A, GMMo2127A,	16
		GMMo2131A	
	A2:2	GMMo2118A, GMMo2129A	02
B		GMMo2122A, GMMo2130A	02



GM1A-GMMo2102A, GM2A-GMMo2103A, GM3A-GMMo2105A, SKM109A-GMMo2106A, SKM 125A-GMMo2108A, SKM 139A-GMMo2109A, SKM 149A-GMMo2111A, SKM 201A-GMMo2113A, SKM 214A-GMMo2114A, SKM 301A-GMMo2116A, SKM 303A-GMMo2118A, SKM 319A-GMMo2119A, SKM 9928A-GMMo2121A, RSK 88A-GMMo2122A, RSK 89A-GMMo2123A, RSK 90A-GMMo2124A, RSK 91A-GMMo2125A, RSK 92A-GMMo2127A, RSK 93A-GMMo2128A, RSK 94A-GMMo2129A, RSK 95A-GMMo2130A, RSK 96A-GMMo2131A

Fig 1: UPGMA dendrogram of twenty-two genotypes of Indian mustard based on SSR primers

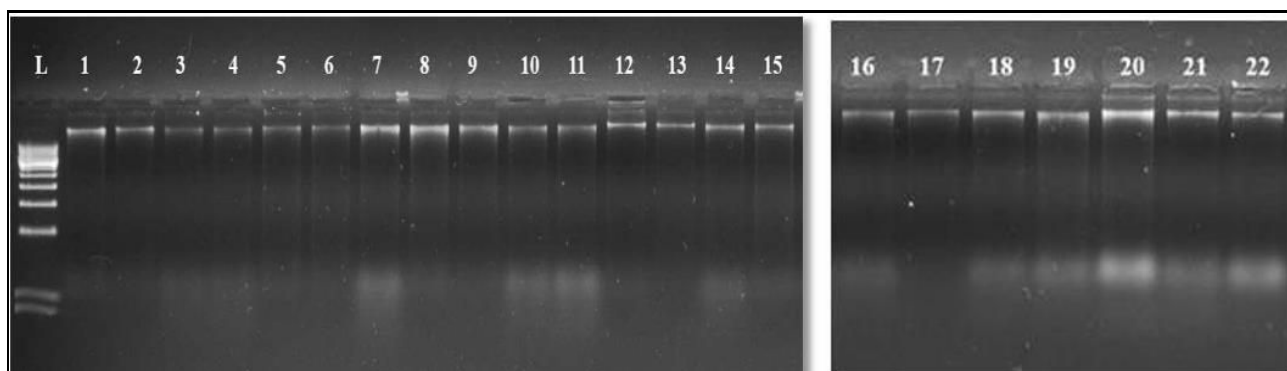


Plate 1: Separation of genomic DNA of mustard on 0.8% agarose gel electrophoresis

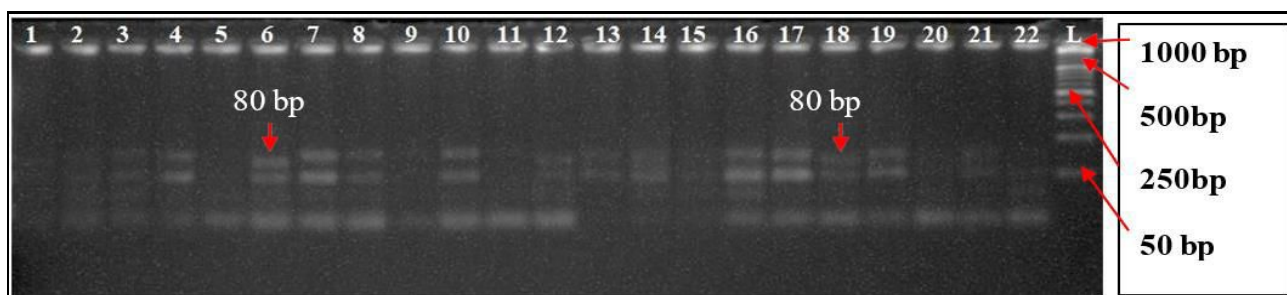


Plate 2: SSR profile with BR-A01

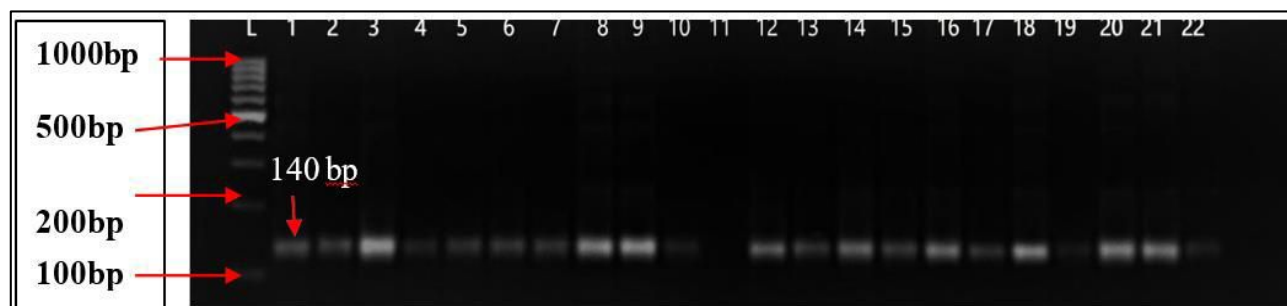
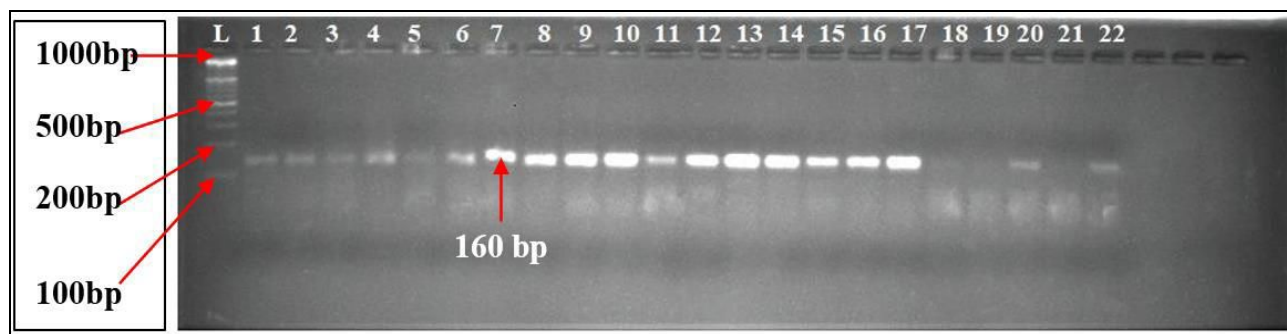
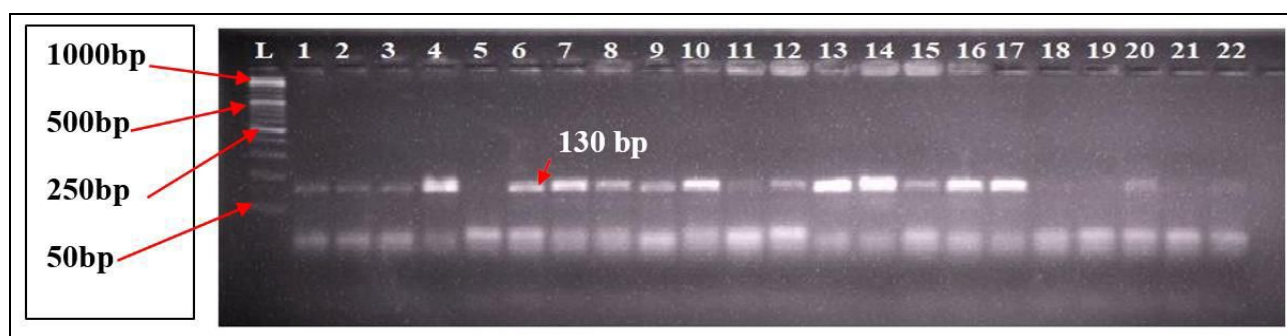
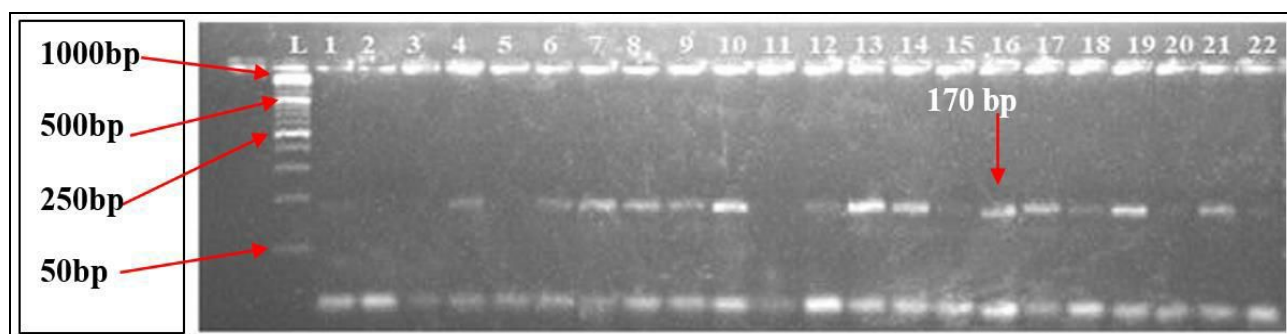
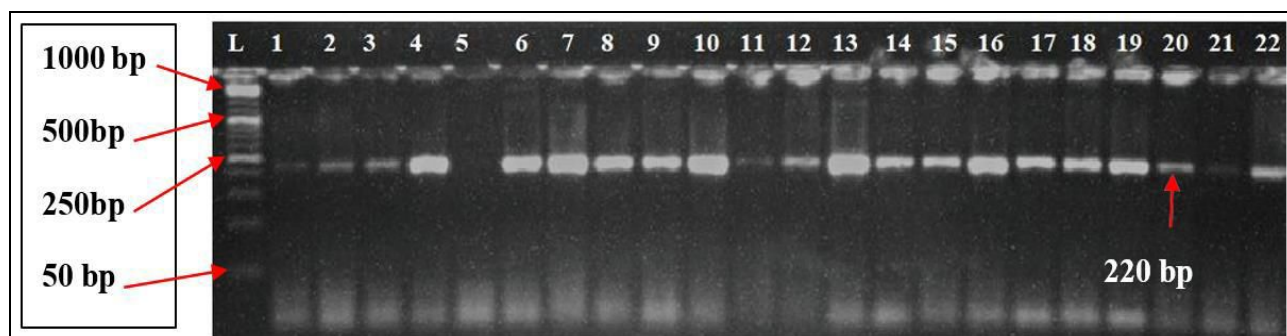


Plate 3: SSR profile with BR-A03

**Plate 4:** SSR profile with BR-A05**Plate 5:** SSR profile with BRMS-08**Plate 6:** SSR profile with BRMS-37**Plate 7:** SSR profile with BRMS-240**Plate 8:** SSR profile with SR-9222

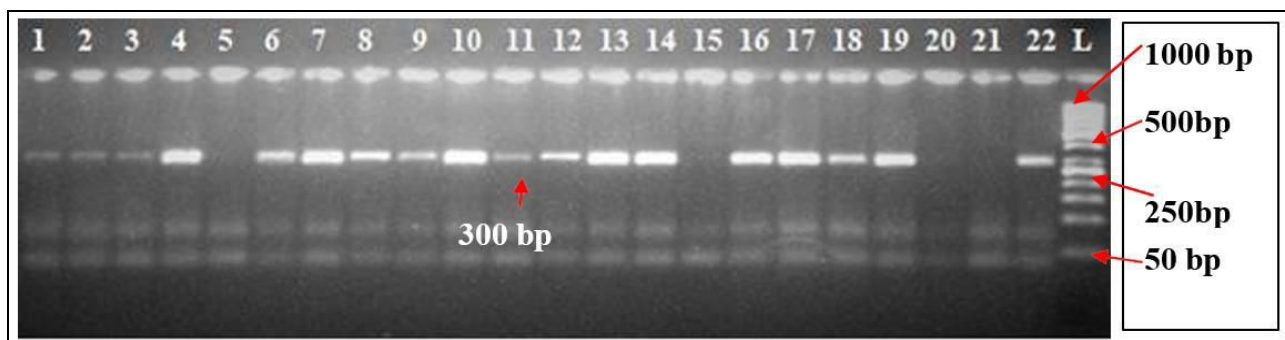


Plate 9: SSR profile with SR-94102

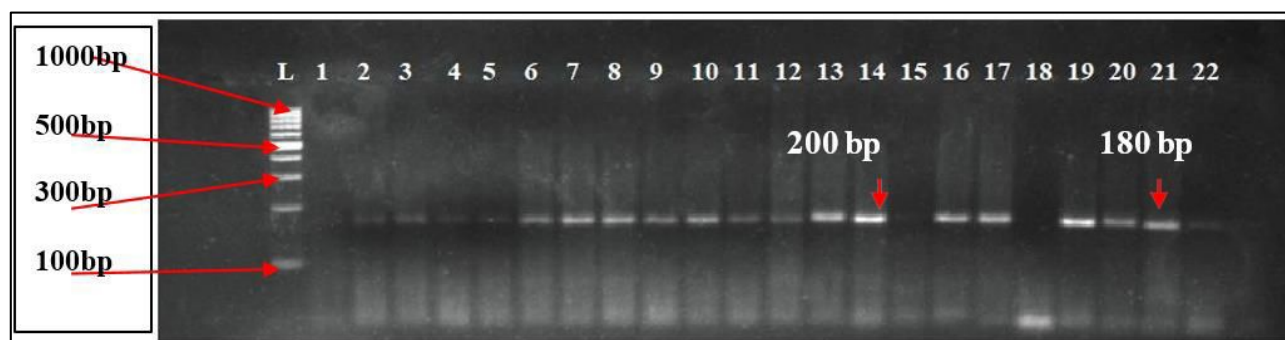


Plate 10: SSR profile with BG-41

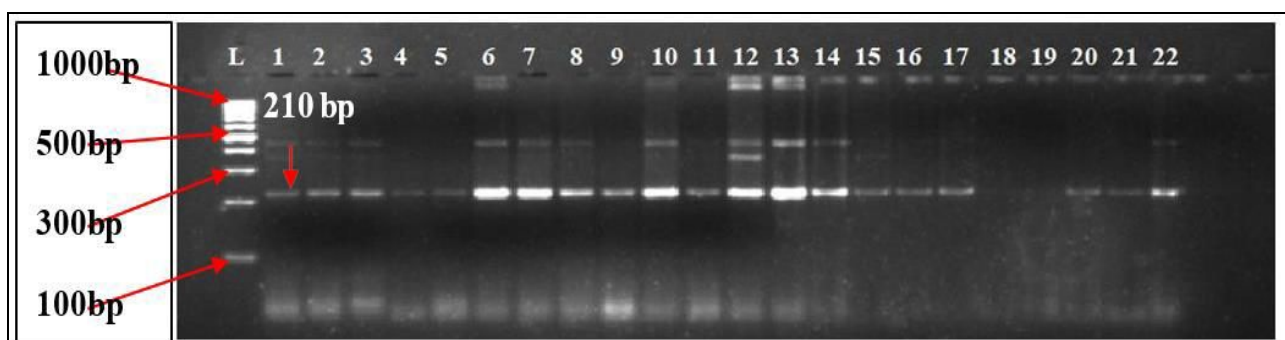


Plate 11: SSR profile with BR A04-9627

L = 100 bp and 50 bp DNA Ladder					
1	GMMo2102A	9	GMMo2114A	17	GMMo2125A
2	GMMo2103A	10	GMMo2116A	18	GMMo2127A
3	GMMo2105A	11	GMMo2118A	19	GMMo2128A
4	GMMo2106A	12	GMMo2119A	20	GMMo2129A
5	GMMo2108A	13	GMMo2121A	21	GMMo2130A
6	GMMo2109A	14	GMMo2122A	22	GMMo2131A
7	GMMo2111A	15	GMMo2123A		
8	GMMo2113A	16	GMMo2124A		

Conclusion

Jaccards's similarity coefficient values for 22 genotypes ranged from 0.60 to 1.00 with average of 0.88 indicating moderate level of genetic variation existing among the mustard genotypes used in this study. The similarity coefficient was ranged from 0.86-1.00 which is an indicative of presence of moderate variation among genotypes. Cluster A was sub-divided into two sub-clusters A1 and A2. Twenty genotypes were grouped in one major cluster A. From the molecular analysis it is envisaged that GMMo2130A and GMMo2122A were the most diverse genotypes, which should be given more emphasis during selection programme for the improvement of seed yield in Indian mustard.

Acknowledgement

Special thanks to Oilseeds Research, Sardarkrushinagar for giving material and C. P. College of agriculture along with Directorate of research, S.D.A.U. for giving research facility.

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