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Molecular diversity analysis using Simple sequence repeats (SSRs) in 'A' and 'R' lines of *Ogura* CMS system in Indian mustard

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Abstract
Fifty-eight SSR primers were used for molecular diversity analysis among 45 'A' and 'R' lines (5 A lines and 40 restorers) of Indian mustard. Out of 58 primers 33 primers were found to be polymorphic. A total of 95 alleles were detected across all 45 genotypes. The PCR amplified products ranged from 70 bp to 350 bp in size. The number of alleles per locus varied from 2 to 5 with an average of 2.89. The PIC value varied from 0.206 to 0.749 with an average of 0.519. The highest PIC value was obtained for BG 132 (0.749) followed by BG 114 (0.744). The UPGMA cluster analysis suggested demarcation of 45 genotypes into six distinct groups in dendrogram wherein three groups had single genotype (restorer line) each. Cluster VI consisted of all five Ogura CMS lines only. In the remaining clusters, restorer lines were distributed. Based on similarity coefficient, cross combinations *viz*., RH 0749-OA & IOR-17, RH 0749-OA & IOR-20 and RH 119-OA & OR-17 were identified as diverse and might be suitable for using parent in heterosis exploitation in Indian mustard.

Keywords: Indian mustard, 'A' and 'R' lines, SSRs, molecular diversity

Introduction
Indian mustard [*Brassica juncea* (L.) Czern & Coss.], commonly known as raya, is one of the major oilseed crops cultivated in the Indian sub-continent. The predominant oilseed *Brassica* species plays a pivotal role in the vegetable oil economy. *B. juncea* (AABB; 2n=36) is a natural amphiploid arisen from hybridization between *B. rapa* (AA; 2n=20) and *B. nigra* (BB; 2n=16) followed by chromosome doubling which led to the evolution of it (U, 1935). The wild form of *B. rapa* and *B. nigra* are found in the Middle East hence, this region is the most probable place of origin of *B. juncea* (Prakash and Hinata, 1980) [11].

Indian mustard plays a crucial role in the Indian oilseed economy and shares about 23.5% area with 24.2% production of total oilseeds in the country. The oil content in Indian mustard varies from 38-46% (Tomar *et al*. 2015) [19]. Mustard oil is low in saturated fat and high in monounsaturated and polyunsaturated fat along with a significant amount of omega-3 fatty acids. The mustard oil-cake is a valuable protein source and recommended feed of cattle. The mustard seed contains some of the essential nutrients such as selenium, magnesium, dietary fibre, omega-3 fatty acids, vitamin B3, calcium, protein and zinc (Kaur *et al.*, 2019) [6].

In India during *rabi*, 2018-19 Indian mustard was grown in an area of 6.94 million hectares with an output of 7.20 million tons. Yet, India meets 57% of the domestic edible oil requirements through imports and ranked 7th largest importer of edible oils in the world. The further boom in human population and improved living standards has led to a rapid increase in the per capita oil consumption. Thus, there is an urgent need to increase the yield potential through genetic interventions to meet the present and future oil requirements. Heterosis breeding could be a potential alternative to substantially increase the productivity of Indian mustard (Ghosh *et al.*, 2002) [4]. Attempts to exploit hybrid vigour have been stimulated by the availability of a large number of CMS sources. These are *nap*, *polima*, *ogura*, *tournefortii*, *axyrrhina*, *siifolia*, *catholica*, *sinapis*, *trachystoma*, *moricandia* and *lyratus*. Among these, *Ogura* (*Ogu*) CMS shows very stable and complete sterility suitable for hybrid development.

Hybrid development depends upon the magnitude of heterosis. Heterosis in crosses involving diverse parents is generally higher than related parents. Hence, the selection of parents based

on diversity estimates is a vital criterion to initiate a hybrid development program (Banga *et al.,* 2015) [3]. Diversity analysis based on DNA markers is more reliable than conventional methods. DNA or molecular markers are unique DNA sequences that can be traced or identified using molecular biology techniques. These markers can be used for diversity analysis in different crops. Some of the currently used DNA molecular markers for diversity analysis include RFLP, RAPDs, AFLP, SSR etc. The SSR markers are more advantageous compared to other marker systems. They are reproducible, co-dominant in nature, abundant, widely distributed throughout the genome, highly polymorphic and easily scorable representing multi-allelic variation (Adhikari *et al.,* 2017) [1]. Numerous SSR markers have been used in different *Brassica* species such as in *B. juncea* (Vinu *et al.*, 2013; Pratap *et al.*, 2015) [21, 12], *B. napus* (Wang *et al.*, 2012; Li *et al.*, 2013) and *B. rapa* (Suwabe *et al.* 2002; Ramchiary *et al.* 2011) [18, 13]. Hence, we used SSR markers for diversity analysis among the CMS and restorer lines.

Materials and Methods

Plant Material

Forty-five 'A' and 'R' lines (Table. 1), including five cytoplasmic male sterile lines ('A' line) and forty restorer lines ('R' line), of Indian mustard were grown in paired rows of 5m at the Oilseeds Research Area, Department of Genetics and Plant Breeding, CCS HAU, Hisar during *Rabi*, 2016-17. The row-to-row spacing was kept at 30 cm and the plant-to-plant spacing was maintained at 10 cm.

Genomic DNA Isolation, Purification and Quantification

Total genomic DNA was extracted from the fresh leaves of the four-week-old plants according to the CTAB method suggested by Saghai-Maroof *et al*. (1984) [15] with a slight modification. The precipitated DNA pellets were washed with cold ethanol (70%) and allowed to air dry overnight. These pellets were re-suspended into 100 µL of 10X T.E. buffer and stored at -20 °C. The quality and quantity of DNA were checked by gel electrophoresis using 0.8% agarose gel along with standard lambda DNA (50 ng/µL).

SSR Markers

A total of 58 SSR primers (Table. 2) were used for PCR amplification to determine genetic diversity in 45 Indian mustard 'A' and 'R' lines. The primers showing monomorphic banding patterns were excluded from further analysis. Finally, data produced by polymorphic SSR primers were used to analyze diversity and collect other information.

PCR Amplification

PCR amplification was carried out in a PCR machine (Bio Rad, USA). The total volume of PCR reaction mixture was 20 µl per sample, containing template DNA (1.0 µl), 10X PCR buffer (2.0 µl), reverse and forward primer (1.0 µl), 10 mM dNTP mix (0.4 µl), 5U*Taq* DNA polymerase (0.5 µl) and nuclease-free water (13.1 µl). The PCR tubes were set on the wells of the thermal cycler. The machine was run as follows: Initial denaturation at 95.0 °C for 3 min; 40 cycles of denaturation at 94.0 °C for 1 min; annealing for 1 min at a particular temperature of primer; and extension at 72.0 °C for 1 min. Final extension was done at 72.0 °C for 7 min, and then the products were held at 4.0 °C. The amplified products were stored at -20 °C until further use. The PCR products were resolved by electrophoresis in TBE buffer through 3.5% horizontal electrophoresis containing ethidium bromide. A DNA ladder of 50 bp was run along with the PCR products. DNA bands were visualized in UV-trans-illuminator in the dark chamber of the Image Documentation System, which is linked to a computer that also runs the digital imaging system.

SSR Data Analysis

The size of the amplicons was measured by comparing the migration of amplified fragments with that of a known size DNA ladder (50 bp). All distinct bands (SSR markers) were given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each primer. The scores obtained from all polymorphic markers in the SSR analysis were pooled to create a single data matrix and construct a UPGMA dendrogram using NTSYS–pc software (Rohlf, 1998). Markers were classified as informative when PIC was ≥0.5. Polymorphic information content (PIC) values for each SSR were estimated by determining the frequency of alleles per locus using the following formula

$$PIC = 1 - \sum x_i^2$$

Where, x^i is the relative frequency of the i^{th} allele of the SSR loci

Results and Discussion

SSR Marker-Based Divergence and Allelic Diversity Analysis

Out of 58 primers used in the study, three primers were not amplified, 22 were monomorphic and 33 primers generated polymorphic bands (Table 3 & 4). The level of genetic diversity among 45 lines was evaluated by calculating allele amplified and PIC value for each polymorphic marker. A total of 95 alleles were detected with 33 polymorphic SSR markers across 45 lines. The overall size of the PCR amplified products ranged from 70 bp (BG93) to 350 bp (BG100, BG132, and BG133). The molecular size difference between the smallest and largest allele at a locus varied from 10 bp (BG126) to 220 bp (BG93). The number of alleles per locus varied from 2 to 5 with an average of 2.89 (Table 5). This was consistent with some earlier reports of 2.79, 2.9 and 3 average alleles per locus (Prajapat *et al*. 2014; Avtar *et al*. 2016; Singh *et al*. 2016) [10, 2, 17]. The highest number of alleles (5) was recorded with primers BG41 and BG132, followed by 4 alleles detected with primers BG48, BG100, BG111, BG114, BG129, and BG157. The lowest number of alleles (2) was detected at several loci. The average number of alleles per locus observed in this study was 2.89; in comparison, Sudan *et al*. (2016) [17] reported slightly lower value of 2.37 alleles per locus. The variability in the number of alleles detected per locus might be due to the use of diverse lines and different SSR primers. The calculated PIC value of each marker varied widely from 0.206 to 0.749 with an average of 0.519. The highest PIC value was obtained for BG132 (0.749), followed by BG114 (0.744), BG100 (0.733) and BG41 (0.729). However, BG119 (0.206), BG116 (0.234) and BG135 (0.278) had low PIC values and could be considered as the least powerful markers. The average PIC value (0.519) observed in our study was consistent with previous estimates of SSR marker analysis in Indian mustard by Avtar *et al*. (2016) [2] and Patel *et al*. (2018) who reported average PIC values of 0.529 and 0.555, respectively. On the other hand, the observed value was higher than the values of 0.46, 0.28 and 0.31 reported by Turi *et al*. (2012) [20], Gupta *et al*. (2014) [5], and Sudan *et al*. (2016) [17], respectively.

Similarity Coefficients and UPGMA Cluster Analysis

In this study, the dendrogram is constructed according to the UPGMA cluster analysis based on the similarity coefficient by using SAHN subprogram of NTSYS-pc. The range of similarity coefficients was found to be 0.49 to 0.91. The cluster dendrogram analysis revealed six clusters demarcated at a cut-off value of 72% of similarities, below which the similarity values narrowed conspicuously (Fig 1). Clusters I, II and III consisted of a single line, i.e., OR-11, OR-9 and IOR-11 respectively. Cluster IV consists of the maximum number of lines, i.e., 27 'R' lines. Cluster V is comprised of 10 'R' lines only. Interestingly, Cluster VI consisted of five 'A' lines only. The details about the clustering pattern of genotypes are given in Table 6. Based on this cluster analysis, we conclude that all A-lines were derivatives of a single parent that must be different from the R line. So, there may be a chance for heterosis exploitation. Among the different combinations of 'A' and 'R' lines, the minimum similarity coefficient value of 0.53 was found between RH 8812-OA & IOR-2 followed by 0.54 between RH 8812-OA & IOR-3 and RH 8812-OA & IOR-14. There were also other combinations having low similarity coefficient value such as 0.57 between RH 0749-OA & IOR-17, RH0749-OA & IOR-20, and RH 119-OA & OR-17. Among all CMS-lines, RH 8812-OA was the most diverse. Thus, it can be concluded that the above parental combinations, based on similarity coefficient values can be utilized successfully for the hybrids development in Indian mustard.

Table 1: List of genotypes used for molecular diversity analysis

Sr. No.	Genotype	Sr. No.	Genotype
'A' lines			
1.	RH 8812-OA	18.	IOR-18
2.	RH 0749-OA	19.	IOR-19
3.	RH 0406-OA	20.	IOR-20
4.	RH 0119-OA	21.	IOR-21
5.	RH 30-OA	22.	OR-1
'R' Lines		23.	OR-2
1.	IOR-1	24.	OR-3
2.	IOR-2	25.	OR-5
3.	IOR-3	26.	OR-6
4.	IOR-4	27.	OR-7
5.	IOR-5	28.	OR-9
6.	IOR-6	29.	OR-11
7.	IOR-7	30.	OR-12
8.	IOR-8	31.	OR-13
9.	IOR-9	32.	OR-14
10.	IOR-10	33.	OR-15
11.	IOR-11	34.	OR-16
12.	IOR-12	35.	OR-17
13.	IOR-13	36.	OR-18
14.	IOR-14	37.	OR-21
15.	IOR-15	38.	OR-22
16.	IOR-16	39.	OR-23
17.	IOR-17	40.	OR-24

Table 2: A brief description of SSR primers used during the present investigation

Sr. No.	Primer Name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Ann. Temp
1.	BG41	TCCTCCGACAACAACAACTCAA	ATCTAACCCGTCTGCGAATCTG	67.5°C
2.	BG48	CACGAAAGCTGTAGAGGCATGA	TCTTTTCCTGTCCATGAGATTCAA	64.5°C
3.	BG89	TGCCAACAAATCAAGGATGC	CCGAAGTTCACTTGTTATTCCAAC	65.0°C
4.	BG90	TGGCTAAAGTCTATGTTACCCTTA	AATATTTACATCAAGATAACAACACAA	59.5°C
5.	BG91	ACGTGGCATTCATTAAACGG	GAAAGAGAGATCCTTCAGCCAA	64.5°C
6.	BG92	GACACGTGGCATTCATTAAACG	TCCTTCAGCCAAACCCAGAA	65.5°C
7.	BG93	TGTAAGTCACGTTCGGTTTGCT	AGGCATGTATGGAGATGTAGAGTGA	68.0°C
8.	BG94	CCTTCAAAAGAAAGGAGGGGAA	GAGAGAGAGAGAGGGCATAATAAAAGC	66.0°C
9.	BG95	AGCTGCAAGAAAGCGCAAAA	ATTGCCGAACCTCACTTCCAT	67.5°C
10.	BG96	TCTTGCGAATGTTCCGGTTT	CCAAAATTAAGAGAATAAATGATTGG	59.5°C
11.	BG97	AATTCGTCTTGCGTCGGTAAAA	GCCTAGAGATAAACGTGGACCG	66.5°C
12.	BG98	CTGTGATCCGAGGGAAGAAGGT	GATAGTAACGCCTCCAGGTCCC	70.5°C
13.	BG99	CAGAGCGAGCTGCAAGACAG	CATTGCCGAACCTCACTTCC	67.5°C
14.	BG100	TGGTAAGTGTACTTCCATATAATGTTT	TGTGATGGATTGAATCGAGGTC	62.5°C
15.	BG102	GGACCGACTTTAGCAAGTCCA	GGGTAGCTTAGAAGATCATCTCTTTGG	67.0°C
16.	BG104	CATGCGGAAACCCGTTAAAA	AAAGCAACCACCCCATTCAA	65.5°C
17.	BG105	TCGATCTTTTTGCGGTGGAT	TTGCAATGGGCATTACATCCT	65.5°C
18.	BG106	TTTTCCCTTTAAATTTCAATTGCTT	GGAAGTGAAGGTGAAGAGGAGTG	60.0°C
19.	BG108	TTTGGGCATCACGGATCTCT	CAAAAATAAGAAAGCGACAGCTGAA	65.0°C
20.	BG109	AAGCCGGTTCTGCAAGTGTT	CATGGCATCCTACGTGGACA	68.5°C
21.	BG110	GCATACTTCAATTCTTTGAGGACCA	GCAGCATTCCCCTATGTTGG	66.0°C
22.	BG111	ACCCGAAAAGAGAATATGGCCT	ACAGTGGCGTTAGGTGGGG	67.5°C
23.	BG112	TTTCAGCCATGGAGGACGTT	AGCATTGCACCAGTCTCAAAA	66.0°C
24.	BG113	ATTGCTTCCGGAACTTGTCG	GCGTCACAGAGGCGGTTATT	67.0°C
25.	BG114	GCAAAATCCATTGGTAATCAGGA	TGGGCAAGTCACACTCACTCA	64.5°C
26.	BG115	GAGGAGGAGGAGAAGGAGGA	CCATCTTTGAAAAACCCCAAT	62.0°C
27.	BG116	ATGGGGGTTCTAGCGAAAAG	CTTTCAATTGAGTTTCTCGTAGTTCTT	64.5°C
28.	BG117	CTGGAAAGCATACACTTTGGTG	CAAAGGATTTCCCTCGATCA	63.0°C
29.	BG118	TGTGCTTGCGTTTTAAAGGA	GCAAAACCCACAGGTCAGAT	63.5°C
30.	BG119	TTGTGAAATGGTGTCGAAGC	GCACGAGAATGCAAAGTTGA	64.5°C
31.	BG120	TTTGAAACGACGATCAACACA	GCTTCATCTGCTTACTATGGTTTTT	63.5°C
32.	BG121	CAACCACATGAGATTGGTTTAGTT	GAAATGGTTTTGGAGCGGTA	64.5°C
33.	BG122	CCATTATTTGAAAATACCATTTGTTG	AAATATCAATGATGGATTGGTGA	60.5°C
34.	BG123	CCAACGGAAGGGATGTTAAA	TTACCCTGCACACACACACA	63.0°C
35.	BG124	TAGATCATTTCACACGGTGGAT	TCATAGCGACAAAAGTGACAGG	64.0°C
36.	BG125	CGAACCGCGAACATAGTGTA	TAAGTGCCAGTCCATTGCAT	65.0°C
37.	BG126	AGAACGAGTCGCGAGGATT	AGTGGGTGGAAGTTCGGTTA	67.8°C

38.	BG127	GCGCCATCTAAACCGATATT	TACCGCGCCATTGATACATA	63.5°C
39.	BG128	CCCTAGTCCGTTTGGGTTAGGT	CCTAATCGCTCTTTTGATTTTGGA	64.5°C
40.	BG129	CGGAGATAACCGGAATGGAA	GGATGCTCTGAGACACCCAAA	65.5°C
41.	BG131	TTCGACAGATTATTTCCGTTGG	AAAGAGGGAGGAAGAAGAAGGAG	63.5°C
42.	BG132	AATGCCCATCCCTCCTTGAT	TTTGAACACTCATTGTGGTGATGA	65.5°C
43.	BG133	CGAACCAAACCGAACCAAAC	GCCGAACAAAAATCAAAAACC	62.5°C
44.	BG134	CAGATTTTCGAAAGGTGGTTGG	CCATCACCCGAAAATCCAAA	64.5°C
45.	BG135	TGATGAAGAATGGTGCATGG	TTCGAATCTCATCAGCTGCAC	64.5°C
46.	BG136	TTGGAGAGGTCTGGGCTTTG	TCTCGCCTTGTTGTGAATCAA	65.5°C
47.	BG156	CATTGATGAGGCAAGACTTTGA	CACCAAAGCTTCTCAACTTTCTAA	64.5°C
48.	BG157	GGAGAGGTGTTTCTCGAACCT	TTCAGTGTGCTATGCAGATCG	66.5°C
49.	BG158	TGTGAGAATGCAGTCCAAAACT	TCTGGTCATGATGGTGGAAA	64.0°C
50.	BG159	CAAAAATTCAAAAACCCGTGA	CAAGCACCAAGAAGTTGCAG	60.5°C
51.	BG160	TTGGGAAGGTTCTGTCCAAC	CGCGTCACAATCGTAGAGAA	66.5°C
52.	BG161	ATGCTCGTGCCCACAAAA	AAACGTTTTATTTGCTTTACCTATTT	60.5°C
53.	BG162	TGCCACTGAGACTTCCTCCT	GAGGTTTGGGAGATGCAGAG	66.5°C
54.	BG163	TGTGAAACAACAACAACCACCT	GCGCTAGCTAAACCCACTCC	66.0°C
55.	BG164	GCTCGTTTCGATTTGGTCTC	GGCCATGGAGAGAGAGAGAG	65.5°C
56.	BG165	TTCATTCTCGGCACAAAACA	ATGGCCCAAGAAACATCAAT	63.0°C
57.	BG166	GCGTCATACTGTTGTTAGTTTATGG	GACATATTGCACATGCAAAGACT	64.0°C
58.	BG167	CAAGTTAAGGAATTAAGTTTCTCCTTC	CCAAAATTAAACAGCTCAAGAACA	62.5°C

Table 3: Allelic variation and PIC values for 33 polymorphic SSR loci identified among 45 Indian mustard genotypes

Sr. No	Primer	Amp. Range(bp)	Total no. of alleles	PIC
1.	BG41	160-250	5	0.729
2.	BG48	230-260	4	0.658
3.	BG89	130-150	2	0.499
4.	BG91	200-250	2	0.473
5.	BG93	70-290	3	0.298
6.	BG94	230-300	3	0.549
7.	BG95	80-100	2	0.316
8.	BG96	280-300	2	0.485
9.	BG99	120-270	3	0.690
10.	BG100	220-350	4	0.733
11.	BG105	190-240	3	0.430
12.	BG109	130-200	3	0.540
13.	BG111	120-320	4	0.727
14.	BG114	160-300	4	0.744
15.	BG116	140-160	2	0.234
16.	BG 119	200-300	2	0.206
17.	BG121	200-250	3	0.509
18.	BG123	220-200	2	0.480
19.	BG125	140-190	3	0.654
20.	BG126	140-150	2	0.483
21.	BG129	200-300	4	0.464
22.	BG132	180-350	5	0.749
23.	BG133	250-350	3	0.657
24.	BG135	270-300	2	0.278
25.	BG136	220-240	2	0.278
26.	BG156	180-220	3	0.486
27.	BG157	150-250	4	0.643
28.	BG158	250-300	2	0.497
29.	BG160	150-200	2	0.413
30.	BG161	160-250	3	0.606
31.	BG162	140-300	3	0.665
32.	BG 164	250-330	3	0.617
33.	BG 167	130-150	2	0.363

Table 4: Description of primers based upon amplification

Nature of Primer	Number of primers	Primer
Polymorphic primer	33	BG41, BG48, BG89, BG91, BG93, BG94, BG95, BG96, BG99, BG100, BG105, BG109, BG111, BG114, BG116, BG119, BG121, BG123, BG125, BG126, BG129, BG132, BG133, BG135, BG136, BG156, BG157, BG158, BG160, BG161, BG162, BG164 and BG167
Monomorphic primer	22	BG90, BG92, BG97, BG98, BG102, BG104, BG108, BG110, BG112, BG113, BG115, BG117, BG118, BG120, BG122, BG124, BG127, BG128, BG134, BG159, BG165 and BG166
Non-amplified	3	BG106, BG131 and BG163

Table 5: Summary of molecular analysis

Total number of genotype	45
Total number of primer tested	58
Total Number of polymorphic primer	33
Total number of monomorphic primer	22
Total number of polymorphic bands	83
Total number of monomorphic bands	12
Total number of bands	95
Size of amplified product (bp)	70 bp to 350bp
Per cent polymorphism	87.37 %
Number of alleles per locus	2-5
Average alleles per locus	1.64
PIC value range	0.206 to 0.749
Average PIC value	0.519

Table 6: Distribution of 45 Indian mustard genotypes in different clusters based on SSR markers analysis

Cluster No.	Number of genotypes	Genotypic details
I	1	OR-11
II	1	OR-9
III	1	IOR-11
IV	27	IOR-1, IOR-2, IOR-6, IOR-7, IOR-3, IOR-4, IOR-5, IOR-13, IOR-14, IOR-16, IOR-8, IOR-17, IOR-10, IOR-12, OR-1, OR-3, IOR-20, IOR-21, IOR-9, OR-7, IOR-15, IOR-18, OR-12, IOR-19, OR-2, OR-5 and OR-6
V	10	OR-13, OR-14, OR-15, OR-16, OR-23, OR-18, OR-21, OR-22, OR-24 and OR-17
VI	5	RH 8812-OA, RH 0749-OA, RH 30-OA, RH 0406-OA and RH 0119-OA

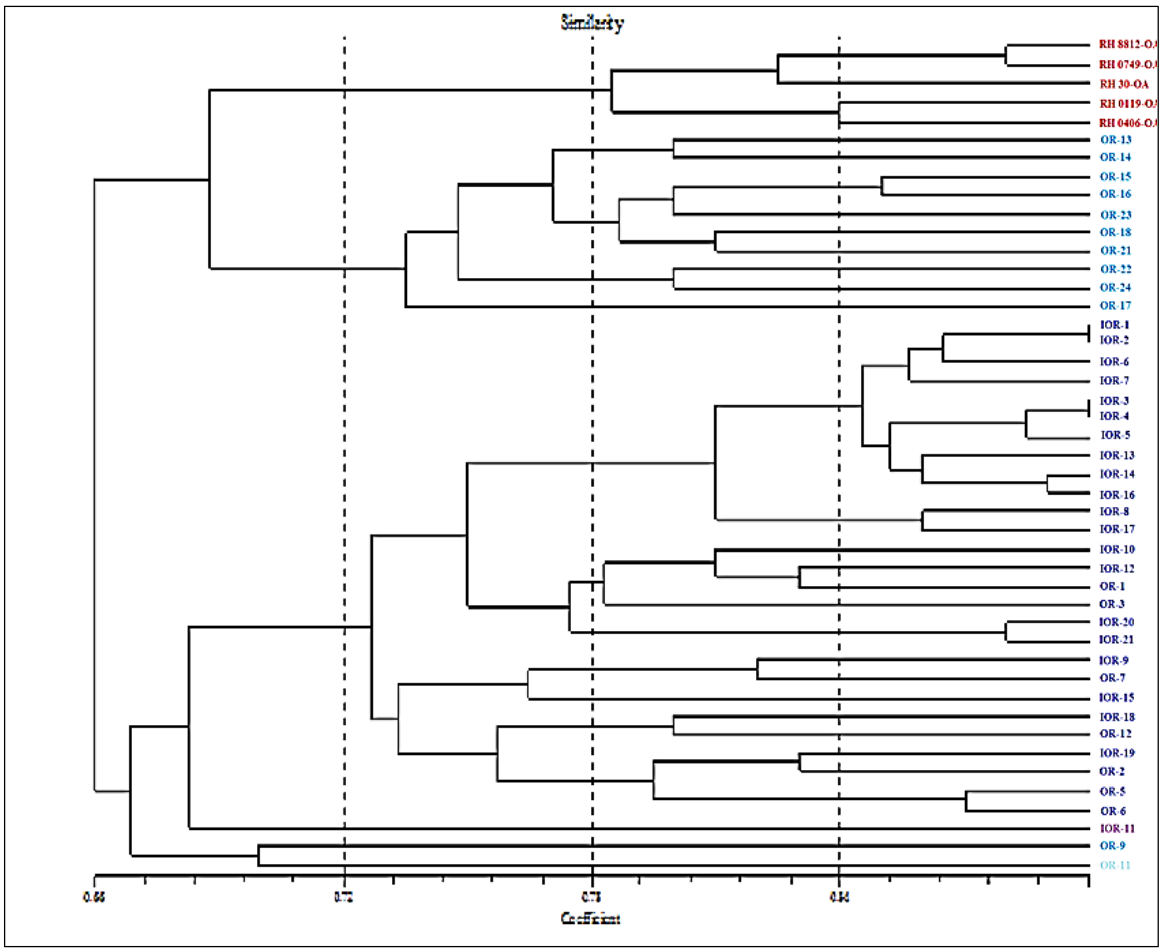

Fig 1: Dendrogram showing the clustering pattern of 45 Indian mustard genotypes based on 58 SSR markers

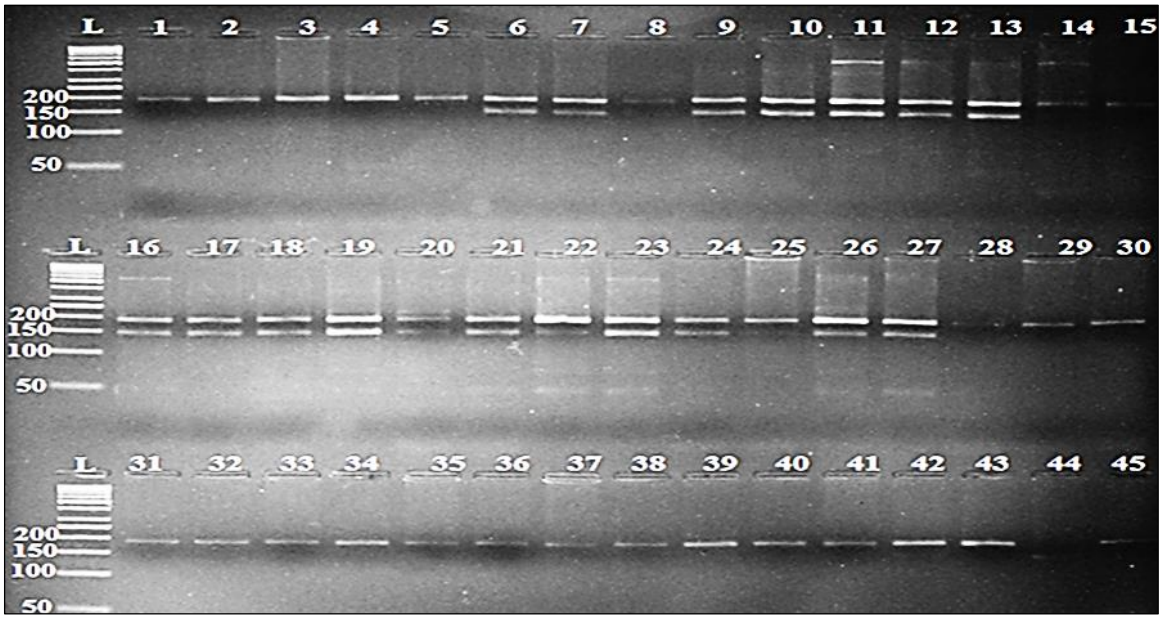

Fig 2: Agarose gel showing allelic polymorphism among 45 Indian mustard genotypes using SSR primer BG 160

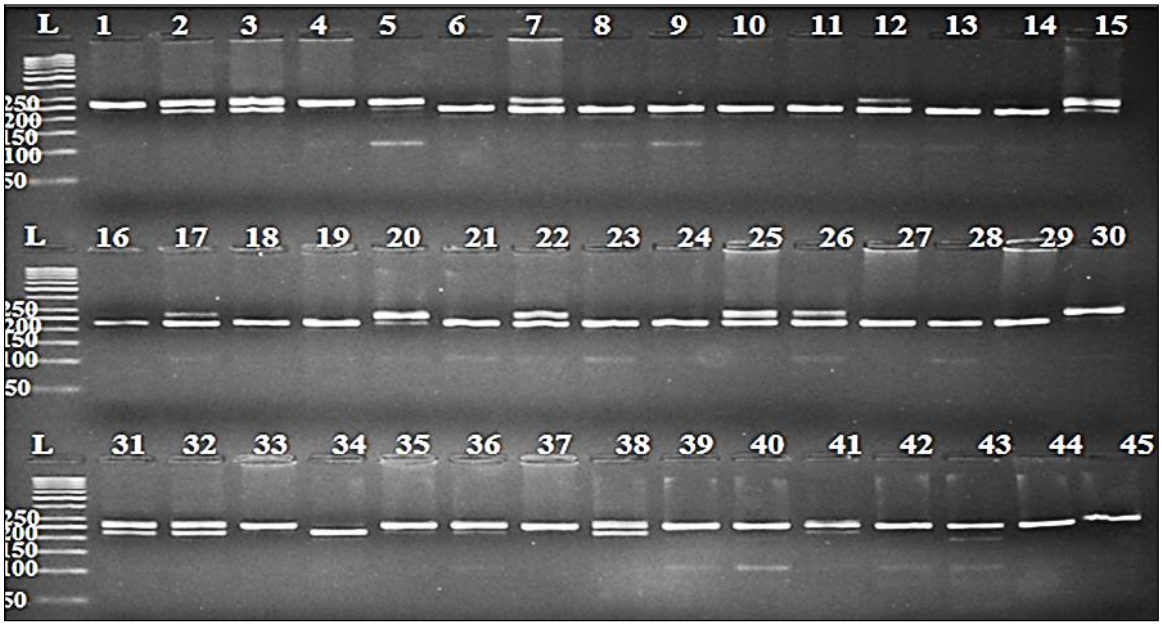

Fig 3: Agarose gel showing allelic polymorphism among 45 Indian mustard genotypes using SSR primer BG 99

1- RH 8812-OA, **2**- RH 0749-OA, **3**- RH 406-OA, **4**- RH 119-OA, **5**- RH 30-OA, **6**- IOR-1,**7**- IOR-2, **8**- IOR-3, **9**- IOR-4, **10**- IOR-5, **11**- IOR-6, **12**- IOR-7, **13**- OR-8, **14**- IOR-9,**15**- IOR-10, **16**- IOR-11, **17**- IOR-12, **18**- IOR-13, **19**- IOR-14, **20**- IOR-15, **21**- IOR-16, **22**- OR-17, **23**- IOR-18, **24**- IOR-19, **25**- IOR-20, **26**- IOR-21, **27**- OR-1, **28**- OR-2, **29**- OR-3, **30**- OR-5, **31**- OR-6, **32**- OR-7, **33**- OR-9, **34**- OR-11, **35**- OR-12, **36**- OR-13, **37**- OR-14, **38**- OR-15, **39**- OR-16, **40**- OR-17, **41**-OR-18, **42**-OR-21, **43**-OR-22, **44**-OR-23 and **45**-OR-24

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