Journal of Oilseed Brassica, 13 (2) : 94-99, July 2022

Molecular and morphological diversity of the *mori* CMS lines and restores in Indian mustard (*Brassica juncea* L.)

Raju Ram Choudhary*, Ram Avtar, Pawan Kumar, Manjeet Singh and Mahavir Bishnoi

Department of Genetics & Plant Breeding, CCS Haryana Agricultural University, Hisar 125004, Haryana, India
**Corresponding author: rajuramchoudhary33@gmail.com*
(Received: 24 January 2022; Revised: 18 March 2022; Accepted: 18 March 2022)

Abstract

Indian mustard represents one of the most important oilseed crops in India, nevertheless, their genetic diversity is barely known. A better understanding on this topic is essential for the proper utilization of genotypes in different target breeding programmes. We evaluated the genetic diversity among 15 Indian mustard (*Brassica juncea* L.) genotypes including 10 *mori* CMS lines and five restorers of *mori* CMS. For this, we used A and B genome specific SSR markers and phenotypic data on yield and yield contributing traits. Out of the 35 primers tested, 15 reported polymorphic and a total of 38 alleles were amplified. The range of PIC value was 0.11-0.71 with mean value of 0.36 demonstrating moderate discriminating capability of SSR markers used for this study. Cluster analysis for SSR profile was performed using DARwin v 6.0 and for morphological traits was performed using WARD's method through Euclidean distance estimation by JMP v.14 software. Genotypes were grouped into four clusters based on genetic distances and into three clusters based on morphological dissimilarity. Three SSRs in our study have PIC values of more than 0.50 suggesting their effectiveness in distinguishing studied genotypes. The use of genotypes from different cluster in hybridization is assuring to produce feasible heterotic combinations, which will be helpful in development of superior cross combination.

Keywords: Genetic diversity, *mori* CMS, phenotypic diversity, SSR markers, principal coordinates

Introduction

Among the nine major oilseed crops widely grown in India, rapeseed-mustard occupies the second most important position after soybean because of its greater sustainability and adaptability under varied agro-ecological situations. India is ranked 3rd in world after Canada and China both in acreage (19.3 %) and production (11.3 %) of brassica oilseeds. (Singh *et al*., 2020). In India, Rapeseed-mustard is ranked 2nd in both acreage (23.33 %) and production (26.24 %) after Soybean. Among rapeseed-mustard species, four species viz. *Brassica juncea, B. napus, B. rapa* and *B. carinata* are cultivated in about 6.23 million hectares' area and produce 9.25 million tons in India (FAOSTAT, 2020). *Brassica juncea* accounts for about 75-80 % of the total rapeseed-mustard area and production. In India, the average productivity is around 1511 kg/ha *i.e.* very low as compared to Germany (3303 kg/ha), France (3182 kg/ha), Canada (2241 kg/ha) and China (2052 kg/ha) (FAOSTAT, 2020). Owing to this, India meets ~60 % of its interior oil requirements through import from other countries (Singh *et al.,* 2022). Increase in the productivity and oil content of brassica oilseed are the most important breeding aspect that can reduce the dependence and import of edible oils from foreign countries and save the millions of rupees. To meet out the present edible oil requirements, there is an urgent need to increase the yield potential of *B. juncea* through genetic interventions. The maximum utilization of any species for breeding and its adaptation to changing environments depend on the level of genetic diversity it holds (Singh *et al*., 2013). Genetic distance among parents may be attributed to their differences for number of genes and their functional relations in a given environment (Nei, 1976). Evaluation of genetic diversity among CMS lines has significant implications for the improvement of *B. juncea* through heterosis breeding. Heterosis breeding may be the one of the viable options to meet out the present requirements in future. Knowledge on genetic diversity in *B. juncea* could help plant breeders and geneticists to understand the structure of germplasm, predict which combinations would produce the best hybrids and facilitate to widen the genetic base of breeding material for selection (Qi, 2008).

Genetic diversity among CMS lines and restorer lines can be determined using morphological, biochemical and molecular methods. Molecular markers are important tools for the analysis of genetic diversity and characterization of plant genotypes as they are highly reproducible and

reliable. In addition, the genetically divergent parents can also be utilized to obtain the desirable recombinants in segregating generations. Keeping above fact into consideration, the main objective of present study was the molecular diversity analysis by SSR markers.

Materials and Methods

The present study was carried out using 10 diverse *mori* based cytoplasmic male sterile lines and 5 restores of Indian mustard. All the genotypes were raised in randomized block design with three replications in two rows of 4 m length maintaining spacing of 45 cm × 15 cm (row × plant) at Research area of Oilseeds Section, GPB, CCS HAU, Hisar during *Rabi* 2018–19. The morphological data was recorded for 12 traits *viz*; days to 50 % flowering, days to maturity, plant height, number of primary branches per plant, number of secondary branches per plant, main shoot length, numbers of siliquae on main shoot, siliquae length, number of seeds per siliquae, oil content (%), seed yield per plant and 1000-seed weight. The oil content was determined through Soxhlet method of oil extraction (Danlami *et al.,* 2015).

Plant material and DNA extraction

Genomic DNA of all 15 genotypes was isolated from the fresh leaf tissue using 2 % CTAB extraction protocol (Murray and Thompson, 1980). Quantity and quality of genomic DNA was checked on 0.8% agarose gel and diluted to a concentration of 50 ng/ìL after quantification.

Molecular marker evaluation

A total of 35 SSR primers were used in present study

Table 1: List of 35 SSR primer sequences depicting annealing temperature and amplification profile

S. No.	Primer Name	Forward Primer	Reverse Primer	Amplification	Annealing Temp.
1.	BG1	GCTGGCTGCACAATAACAGA	GTACCACTGGAGGAGCTTCG	M	57
2.	BG 2	GGCCTTTGGAGGTGACTGTA	CAGGGATATGCGGTCTTTCT	P	56
3.	BG3	AGGAACGTTGCAGAGCAGTT	GTCTGTGACGGAATCAGCAA	M	56
4.	BG4	CTGGTTTGGTTCGGTTTGAT	CCTGACAAATAGCAAGAAGTCG	P	54
5	BG6	TGAGGGTGAGGATGGTGATG	GCACAGTACACCGACGCCTA	P	58
6.	BG7	GGGATATTGAAGACCCGCAAA	TCTCCCGGTGGCTTAAAGAA	P	56
7.	BG 8	TTGGATCAATCAAACTAAACCCTGA	CCAAAATGCCAACAAAAGCA	M	54
8.	BG10	GGGAAAGAAGAAAGAGGGAGAGG	TTTCAAAAATTTTAATTGCACAGA	M	53
9.	BG12	TCGATTGAAACACTGAACATTGA	GCGTTTTCTGTTTTCCCAATAA	P	53
10.	BG15	CATAACCACACGGCCTCCTC	AAGTCATGCCCATTCGCCTA	M	57
11.	BG16	CGAATCCGACGTGAATTTGA	ATTGAGAAGGTCCGCCATGA	P	55
12.	BG18	CCCTCGACTGTCGACGGTAT	ACACCCCTTCCTGATTGT	P	56
13.	BG22	TCTCCTTTTTATCCCGCAAACA	TTGCTAGCCAGTTTGCTCAAGA	M	56
14.	BG23	GAGGCAAAAGCGAAGGTGAA	AGCACCCAAACACTCCCAAA	M	57
15.	BG25	CTCCTCCCCCTTCTTTACCG	CACGAAACCAAGCATGTCGT	M	57
16.	BG30	GGATGTTCACGCCGTATGTG	CCATAAACTGCATTGTTTGAATTG	P	54
17.	BG41	TCCTCCGACAACAACAACTCAA	ATCTAACCCGTCTGCGAATCTG	M	57
18.	BG48	CACGAAAGCTGTAGAGGCATGA	TCTTTTCCTGTCCATGAGATTCAA	M	54
19.	BG89	TGCCAACAAATCAAGGATGC	CCGAAGTTCACTTGTTATTCCAAC	P	54
20.	BG91	ACGTGGCATTCATTAAACGG	GAAAGAGAGATCCTTCAGCCAA	M	54
21.	BG93	TGTAAGTCACGTTCGGTTTGCT	AGGCATGTATGGAGATGTAGAGTGA	M	56
22.	BG108	TTTGGGCATCACGGATCTCT	CAAAAATAAGAAAGCGACAGCTGAA	M	55
23.	BG 109	AAGCCGGTTCTGCAAGTGTT	CATGGCATCCTACGTGGACA	P	56
24.	BG110	GCATACTTCAATTCTTTGAGGACCA	GCAGCATTCCCCTATGTTGG	P	55
25.	BG121	CAACCACATGAGATTGGTTTAGTT	GAAATGGTTTTGGAGCGGTA	P	53
26.	BG126	AGAACGAGTCGCGAGGATT	AGTGGGTGGAAGTTCGGTTA	P	56
27.	BG127	GCGCCATCTAAACCGATATT	TACCGCGCCATTGATACATA	P	53
28.	BG128	CCCTAGTCCGTTTGGGTTAGGT	CCTAATCGCTCTTTTGATTTTGGA	M	56
29.	BG129	CGGAGATAACCGGAATGGAA	GGATGCTCTGAGACACCCAAA	M	55
30.	BG135	TGATGAAGAATGGTGCATGG	TTCGAATCTCATCAGCTGCAC	M	54
31.	BG136	TTGGAGAGGTCTGGGCTTTG	TCTCGCCTTGTTGTGAATCAA	M	55
32.	BG158	TGTGAGAATGCAGTCCAAAACT	TCTGGTCATGATGGTGGAAA	M	54
33.	BG160	TTGGGAAGGTTCTGTCCAAC	CGCGTCACAATCGTAGAGAA	M	55
34.	BG 162	TGCCACTGAGACTTCCTCCT	GAGGTTTGGGAGATGCAGAG	P	57
35.	BG164	GCTCGTTTCGATTTGGTCTC	GGCCATGGAGAGAGAGAGAG	M	55

(Table 1). PCR reactions were standardized in a 10 µL reaction volume having 50 ng genomic DNA, 1 mM PCR buffer, 1.0 U Taq DNA polymerase (Thermo Fisher Scientific), 0.2 mM dNTP mix, 1.5 mM MgCl2 and 0.2 µM of each primer. DNA amplifications were performed in the BIO-RAD T100™ Thermal cycler. The configured PCR framework was as follows: an initial 2-minute denaturation at 94ºC followed by 40 cycles of 1 min at 94ºC, annealing temperature according to primer sequences for 1-minute, 2 min extension at 72ºC and a final extension of 7 min at 72ºC. The amplified fragments were resolved on 2 % agarose gel along with DNA ladder (Thermo Fisher Scientific SM0371, 50 bp). Gel was imagined under UV-light and recorded in the program for gel documentation.

SSR scoring and analysis

An amplified band at each position was scored as 1 for presence and 0 for absence. The size (in nucleotide base pairs) of the amplified bands was further determined based on its migration related to the standard 50-bp DNA ladder. Genetic cluster analysis and was performed using DARwin v 6.0. Cluster analysis performed by DARwin v 6.0 was supported by PCoA (Principal Coordinated Analysis) assessed using GenAlex version 6.5 (Peakall and Smouse, 2006). Further analysis of molecular variance of sub-populations identified using genetic cluster analysis was performed by GenAlex version 6.5 (Peakall and Smouse, 2006). The parameters computed in sub-populations for genetic diversity were the number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), observed gene diversity (h), and unbiased gene diversity (uh) following the protocol given by Nei and Li, 1979. The polymorphism information content (PIC) and expected heterozygosity/ gene diversity (He) values of each primer were determined according to Liu (1998) and Botstein *et al*. (1980), respectively.

Statistical Analysis

Cluster analysis for morphological traits was performed using WARD's method through Euclidean distance estimation by JMP v.14 software. Constellation plot was drawn for graphical representation of morphological clustering. Analysis of variance performed by using the principles of Fisher (1930).

Results and Discussion

Analysis of variance

The analysis of variance for 12 yield and its attributing traits showed highly significant differences that revealed the presence of considerable amount of variation among different genotypes taken for present investigation (Table

Table 2: Analysis of variance for yield and component traits

Source of variation	DF	PH	NPB	NSB	MSL	NSMS	SL	NSS	DF	DM	TW	SY	OC
Replication	2	9.9	0.2	0.3	2.7	93.6	0.0	0.2	0.4	2.7	0.1	1.5	4.7
Genotypes	14	879.8**	0.5**	14.3**	134.4**	128.8**	0.8**	9.9**	58.0**	19.0**	1.8**	125.0**	1.1**
Error	28	2.1	0.1	0.2	7.7	48.2	0.0	0.1	0.4	0.6	0.1	0.2	0.4

*Significant at *P = 0.05* and **Significant at *P = 0.01*; DF – Degree of freedom; PH-Plant height (cm); NPB-No. of primary branches; NSB-No. of secondary branches; MSL-Main shoot length (cm), NSMS-No. of siliquae on main shoot; SL-Siliqua length (cm); NSS-No. of seeds/siliqua; DF-Days to flowering (50 %); DM-Days to maturity; TW-1000-seed weight (g); SYP-Seed yield/plant (g)

2). Significant differences among the 43 genotypes for all the traits investigated have been reported earlier by Verma *et al*. (2021) and Kaur *et al.* (2022).

Genetic divergence and cluster analysis

Based on the phenotypic divergence analysis, all 15 CMS and restore lines of mustard were grouped into three clusters (Fig. 1). A total of four *mori* CMS lines viz., MA-1-30, MA-9518, MA-8701 and MA-9705 grouped into cluster – A. Cluster-B comprised of five CMS lines (MA-023, MA-8812, MA-9301, MA-9811and MA-9702) and one restore, MR-31. While, Cluster-C grouped five genotypes including four restores (MR-9, MR-38, MR-43 and MR-44) and only one CMS line (MA-270). When we crossed the *mori* CMS lines with restores during 2017-18 and evaluated during 2018-19, found that two hybrids namely MA-9301 x MR-44 (12.8 %) and MA-8701 x MR-38 (12.4 %) from diverse parents showed significant positive economic heterosis over standard check DMH-1 (Choudhary *et al*., 2020a) while on the basis of per se performance and specific combining ability, crosses namely MA-9301 x MR-44, MA-8701 x MR-38 and MA-9705 x MR-31 were observed as superior cross combinations for seed yield in desirable direction (Choudhary *et al*., 2020b). These specific crosses also result from crossing of studied diverse parents and that proves that selection of genotypes based on genetic as well as phenotypic diversity is rewarding for heterosis breeding. Our findings are consistent with preceding studies where Singh *et al.* (2017) grouped 15 Indian mustard genotypes into three major clusters and Singh *et al*. (2021) also obtained 3 main clusters while evaluating 16 Indian mustard genotypes for selection of sclerotinia stem rot resistance.

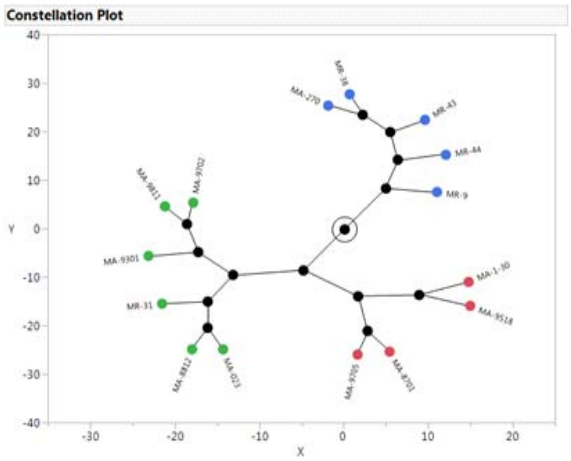

Fig. 1: Constellation plot of morphological data based on WARD's method of clustering

According to different phenotypic cluster mean values, highest cluster mean value for seed yield was recorded in case of cluster-B and minimum in cluster – A (Table 3). Cluster-A grouped the genotypes with maximum value for plant height, days to maturity, test weight and oil content. Cluster-B grouped genotypes with maximum value for primary branches, secondary branches, main shoot length, number of siliquae on main shoot, siliquae length, number of seeds per siliquae, days to maturity and seed yield. These results indicated that seed yield directly affected by these traits and selection based on these traits rewarding for increment in seed yield of Indian mustard. Cluster-C does not have maximum values for any trait and have minimum value for most of the traits indicated that this cluster is diverse from other clusters.

Table 3: Phenotypic cluster mean for twelve characters in Indian mustard

Cluster	No. of Genotypes	PH	PB	SB	MSL	NSMS	SL	NSS	DF	DM	TW	SY	OC
A	4	203.3	4.6	7.5	73.2	45.70	3.8	9.29	48.5	143.3	5.7	14.1	39.2
B	6	201.7	5.0	11.0	74.1	50.79	4.3	12.22	47.9	144.3	5.6	26.3	38.6
C	5	176.7	4.6	10.8	66.2	39.36	3.6	11.94	41.8	139.1	4.7	20.5	38.9

Out of 35 SSR primers, 15 amplified polymorphic band patterns. Fifteen polymorphic primers amplified overall 38 alleles that displaying clear band size differences. A total of 38 polymorphic amplified bands ranged between 70-900bp. The PIC value and gene diversity (expected heterozygosity) both are good indicator for genetic divergence measures among the germplasm (Singh *et al*., 2021). The range of PIC value was 0.11-0.71 with mean value of 0.36 demonstrating moderate discriminating capability of SSR markers used for this study (Table 4). This result is in agreement with previous study of Singh *et al.* (2021) and Avtar *et al.* (2016). Three SSRs in our study have PIC values of more than 0.50 suggesting their effectiveness in distinguishing studied genotypes. The gene diversity (H-index) provides an estimate of genetic distance among genotypes in germplasm set. The average expected heterozygosity/gene diversity (H- index) was recorded to be 0.42 with maximum value (0.75) for BG4

Table 4: List of polymorphic markers with PIC value and He index

S.No.	Primer	Number of alleles	Gene diversity (He)	PIC value
1.	BG2	2	0.12	0.11
2.	BG4	5	0.76	0.72
3.	BG6	2	0.49	0.37
4.	BG7	2	0.14	0.13
5.	BG12	3	0.50	0.45
6.	BG16	2	0.50	0.37
7.	BG18	4	0.70	0.65
8.	BG30	2	0.50	0.38
9.	BG89	2	0.24	0.21
10.	BG109	3	0.48	0.42
11.	BG110	2	0.48	0.36
12.	BG121	2	0.50	0.37
13.	BG126	2	0.18	0.16
14.	BG127	2	0.12	0.11
15.	BG162	3	0.66	0.59

Table 5: Different parameters computed in sub-populations for genetic diversity

Parameters of Sub-population	Na	Ne	I	h	Uh
Mean	1.52	1.41	0.35	0.23	0.35
SE	0.07	0.04	0.03	0.02	0.04

Here, number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), observed gene diversity (h), and unbiased gene diversity (uh)

and minimum value (0.11) for BG2 and BG 127. Nei and Li's (1979) method of genetic diversity divide the total population into two sub-populations. The mean for both sub-population for different parameters mentioned in Table 5.

Based on SSR diversity, all the 15 genotypes of mustard were grouped into four clusters (Fig. 2). The maximum number of genotypes comes under cluster 1. A total nine

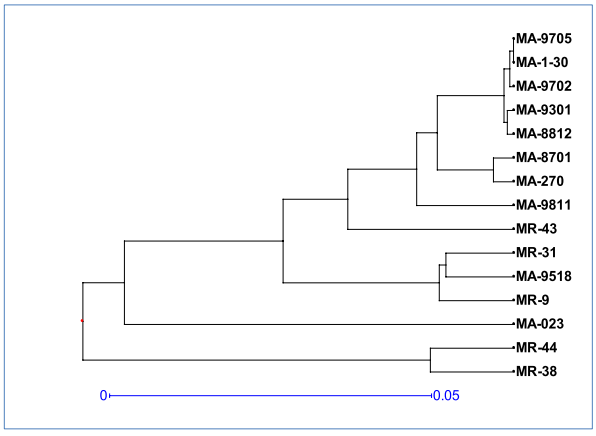

Fig. 2: Dendrogram derived from UPGMA cluster analysis using SSR profile

genotypes viz., MA-1-30, MA-9705, MA-9702, MA-9301, MA-8812, MA-8701, MA-270, MA-9811 and MR-43 fells into cluster 1.

Cluster 2 comprises three genotypes *viz*., MR-31, MA-9518 and MR-9 while only one CMS line, MA-023 fell into cluster-3. Most diverse two restores viz., MR-38 and MR-44 fell into cluster – 4. These restores gives best cross combinations with CMS lines of cluster – 1 and cluster – 2 (Choudhary *et al*., 2020a, Choudhary *et al.,* 2020b).

To support the molecular cluster analysis, Principal Coordinate Analysis (PCoA) was performed using DARwin 6.0 programme (Fig. 3). The PCoA analysis further confirmed the positions and grouping of genotypes. PCoA based on SSR marker showed the scattering of all restores (MR-9, MR-31, MR-38, MR-43 and MR-44) in two right hand side quadrants. The Six *mori* CMS lines viz., MA-270, MA-1-30, MA-9301, MA-8812, MA-9702 and MA-9705 were all together grouped in left sided quadrant while three restores viz., MR-38, MR-43 and MR-44 were together grouped in single quarter. The results of PCoA are accordance with previous study of Singh *et al*., 2013. The genotypes for hybridization may be chosen from widely separated quadrants.

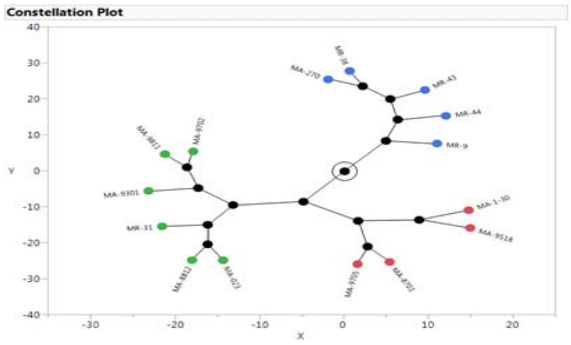

Fig. 3: Principal coordinates analysis using SSR marker-based similarity coefficient matrix of 10 *mori* CMS lines and 5 restorers of *B. juncea*

Conclusion

Panel of genotypes used in the study is having significant variation at phenotypic as well genotypic level. The presence of significant number of private alleles suggested that these accessions can be utilized as sources of novel genes in mustard breeding programmes. The use of genotypes from different cluster in hybridization is assuring to produce feasible heterotic combinations, which has been reported in the current programme.

References

Avtar R, Rani B, Jattan M, Manmohan, Kumari N and Rani A. 2016. Genetic diversity analysis among elite gene pool of Indian mustard using SSR markers and phenotypic variations. *Bioscan* **11**: 3035–41.

Botstein D, White RL, Skolnick M and Davis RW. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Amer J Hum Genet* **32**: 314.

Choudhary RR, Avtar R, Sheoran R and Kumar D. 2020a. Heterosis studies based upon Mori CMS system in *B. juncea*. *J Oilseed Brassica* **11**: 116-120.

Choudhary RR, Avtar R, Sheoran RK, Samita and Kumar D. 2020b. Combining Ability Studies Based on Mori CMS System in Indian mustard (*B. juncea*). *Curr Appl Sci Technol* **39**: 58-66.

Danlami JM, Arsad A and Zaini MAA. 2015. Characterization and process optimization of castor oil (*R. communis*) extracted by the soxhlet method using polar and non-polar solvents. *J Taiwan Inst Chem Eng* **47**: 99-104.

FAO Statistics 2020. FAOSTAT Statistics Division. Food and Agriculture Organization of the United Nations (https://www.fao.org/faostat/en/#country).

Fisher RA. 1930. The Genetical Theory of Natural Selection. Clarendon, Oxford.

Kaur S, Singh RKV and Gupta S. 2022. Genetic variability analysis in different F_1 combinations in Indian mustard (*B. juncea*). *J Oilseed Brassica* **13**: 59-63.

Liu BH. 1998. Statistical Genomics: Linkage, Mapping and QTL Analysis. CRC Press, Boca Raton, Florida, USA.

Murray MG and Thompson WF. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8**: 4321–4325.

Nei M. 1976. Analysis of gene diversity in subdivided populations. *Proc Natl Acad Sci USA* **70**: 3321-3323.

Nei M and Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**: 5269–5273.

Peakall ROD and Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* **6**: 288–295.

Qi X, Yang J and Zhng M. 2008. AFLP-based genetic diversity assessment among Chinese vegetable mustards (*B. juncea*). *Genet Resour Crop Evol* **55**: 705-711.

Singh M, Avtar R, Lakra N, Pal A, Singh VK, Punia R, Kumar N, Bishnoi M, Kumari N, Khedwal RS and Choudhary RR. 2022. Early oxidative burst and anthocyanin-mediated antioxidant defence mechanism impart resistance against *S. sclerotiorum* in Indian mustard. *Physiol Mol Plant Pathol* **120**: 101847.

Singh M, Avtar R, Pal A, Punia R, Singh VK, Bishnoi M, Singh A, Choudhary RR and Mandhania S. 2020. Genotype-specific antioxidant responses and assessment of resistance against *S. sclerotiorum* causing Sclerotinia rot in Indian mustard. *Pathogens* **9**: 892.

Singh S, Singh VV, Ambawat S, Dubey M and Singh D. 2017. Screening and estimation of allelic differentiation in Indian mustard using SSR markers for background selection. *Int J Curr Microbiol Appl Sci* **6**: 2506–16.

Singh M, Avtar R, Singh VK, Mahavir, Kumari N, Anu and Rathore V 2021. Genetic diversity in Indian mustard (*B. juncea*) genotypes for stem rot (*S. sclerotiorum*) using SSR markers. *Indian J Agric Sci* **91**: 914-919.

Singh N, Vasudev S, Yadava, DK, Kumar S, Naresh S, Bhat RS and Prabhu KV. 2013. Assessment of genetic diversity in *B. juncea* Brassicaceae genotypes using phenotypic differences and SSR markers. *Revista de biologia tropical* **61**: 1919-1934.

Verma K, Tripathi MK, Tiwari S and Tripathi N 2021. Analysis of genetic diversity among *B. juncea* genotypes using morphophysiological and SSR markers. *Int J Curr Microbiol App Sci* **10**: 1108-1117.