Journal of Oilseed Brassica, 16 (1) : 110-116, January, 2025

Assessment of genetic diversity for salinity tolerance in Indian mustard using SSR markers

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(Received: 25 August 2024; Revised: 20 September 2024; Accepted: 12 November2024)

https://doi.org/10.56093/JOB.v16i1.16

Abstract

Salinity presents a major challenge for Indian mustard cultivation, especially during the seedling stage in salt-affected soils. Developing salt-tolerant mustard lines through targeted breeding, supported by germplasm screening and genetic variability assessment, can enable cultivation in these areas. This study aimed to evaluate salinity tolerance and assess genetic diversity among 48 Indian mustard genotypes under greenhouse conditions at the seedling stage, exposed to control, 8 dSm^{-1}, and 12 dSm^{-1} salinity stress levels. Molecular diversity was analysed using 59 SSR primers, of which 24 polymorphic primers produced 48 distinct alleles. The mean polymorphism information content (PIC) was 0.40, with a gene diversity of 0.49. Based on Jaccard's dissimilarity coefficients, the genotypes clustered into two main groups. These SSR markers hold potential for marker-assisted selection, and the genetically diverse genotypes identified can serve as valuable parents in breeding programs aimed at enhancing salt tolerance and yield in Indian mustard. Overall, the identified genetic diversity and promising salt-tolerant germplasm provide a foundation for developing salt-tolerant cultivars in Indian mustard breeding efforts.

Keywords : *Germplasm screening, genetic diversity, Indian mustard, salinity tolerance, SSR markers*

Introduction

Brassica oilseeds rank as the world's third most important source of edible oil, following soybean and palm, cultivated across more than 50 countries. In India, rapeseed-mustard is the second most essential oilseed crop after soybean, contributing approximately 25% of the country's total oilseed production (Yadav *et al*., 2019). Brassica oilseeds account for 23.4% of the total oilseed cultivation area and 24.2% of overall oilseed output (Jat *et al*., 2019). Haryana is a leading mustard-producing state, with 0.647 million hectares under cultivation, a production of 1.31 million tonnes, and a productivity rate of 20.28 q/ha, the highest in the country (Anonymous, 2020-2021).

Despite the crop's significance, mustard production in India remains insufficient to meet the growing demand, primarily due to various biotic and abiotic stresses, with salt stress being a critical constraint. Globally, around 95 million hectares of land are affected by high salinity (Szabolcs, 1994), and in India, approximately 6.74 million hectares are classified as salt-affected (Kumar and Sharma, 2020). In Haryana alone, about 0.23 million hectares out of the total 4.42 million hectares are impacted by salinity, which substantially affects mustard productivity (Mandal and Sharma, 2010).

Molecular markers, especially microsatellite markers (SSRs), have proven valuable in genetic analysis and the development of salt-tolerant plant varieties (Tanksley, 1989). SSR markers facilitate the selection of suitable parental lines, diversity and pedigree analyses, and are particularly advantageous as they remain unaffected by environmental conditions and the plant's developmental stage (Parida, 2010). Currently, molecular markers are widely used for analyzing genetic diversity within and among populations. SSR markers are especially favored due to their high reproducibility, co-dominance, abundance, genome-wide distribution, easy scoring, and multi-allelic variability (Powell *et.al*., 1996). Although a substantial number of SSR markers have been developed for *B. rapa* (Suwabe *et al.*, 2002; Ramchiary *et al*., 2011) and *B. napus* (Wang *et al*., 2012; Li *et al*., 2013), relatively few are characterized in *B. juncea* (Hopkins *et al.*, 2007; Vinu *et al*., 2013; Pratap *et al*., 2015; Avtar *et al*., 2016). Understanding genetic diversity is fundamental to any breeding program, as it supports the sustainable development of resilient plant populations.

Therefore, the present study focuses on assessing genetic diversity and identifying salt-tolerant genotypes in Indian mustard (*B. juncea*) using SSR markers, aiming to provide a genetic foundation for breeding programs

directed at developing resilient, salt-tolerant mustard cultivars.

Materials and Methods

Plant materials

This study was conducted in the greenhouse of the Department of Genetics and Plant Breeding at CCS Haryana Agricultural University, Hisar, India (latitude 29°10' N, longitude 75°46' E, elevation 215.2 m). The plant material consisted of 48 Indian mustard genotypes, obtained from the Oilseeds Section, Department of Genetics and Plant Breeding at CCS HAU, Hisar, and the Central Soil Salinity Research Institute, Karnal (Table 1).

Table 1: List of genotypes used in this study

SNo.	Genotypes	Source
1.	BPR 543-2	DRMR, Bharatpur/ India
2.	CS 52	CSSRI, Karnal/India
3.	CS 58	CSSRI, Karnal/India
4.	RGN 486	ARS, Sri Ganganagar/ India
5.	NPJ 244	IARI, New Delhi/ India
6.	DRMRIJ 18-62	DRMR, Bharatpur/ India
7.	DRMRCI 128	DRMR, Bharatpur/ India
8.	TM 264	BARC, Mumbai/ India
9.	RB 110	RRS Bawal, CCS HAU, Hisar/ India
10.	NRCHB 101	DRMR, Bharatpur/ India
11.	PM 28	IARI, New Delhi/ India
12.	RB 50	RRS Bawal, CCS HAU, Hisar/ India
13.	RH(0E) 1701	CCS HAU, Hisar/ India
14.	RH(0E) 1801	CCS HAU, Hisar/ India
15.	RH(0E) 1806	CCS HAU, Hisar/ India
16.	RH(0E) 1808	CCS HAU, Hisar/ India
17.	RH 1424	CCS HAU, Hisar/ India
18.	RH 1906	CCS HAU, Hisar/ India
19.	RH 1921	CCS HAU, Hisar/ India
20.	RH 1923	CCS HAU, Hisar/ India
21.	RH 1926	CCS HAU, Hisar/ India
22.	RH 1936	CCS HAU, Hisar/ India
23.	RH 1974	CCS HAU, Hisar/ India
24.	RH 1975	CCS HAU, Hisar/ India
25.	RH 1999-15	CCS HAU, Hisar/ India
26.	RH 1999-19	CCS HAU, Hisar/ India
27.	RH 1999-21	CCS HAU, Hisar/ India
28.	RH 1999-22	CCS HAU, Hisar/ India
29.	RH 1999-23	CCS HAU, Hisar/ India
30.	RH 1999-24	CCS HAU, Hisar/ India
31.	RH 2046	CCS HAU, Hisar/ India
32.	RH 2049	CCS HAU, Hisar/ India
33.	RH 2050	CCS HAU, Hisar/ India
34.	RH 2051	CCS HAU, Hisar/ India
35.	RH 2054	CCS HAU, Hisar/ India
36.	RH 2055	CCS HAU, Hisar/ India
37.	RH 2065	CCS HAU, Hisar/ India
38.	RH 2066	CCS HAU, Hisar/ India
39.	RH 2069	CCS HAU, Hisar/ India
40.	RH 2070	CCS HAU, Hisar/ India
41.	RH 30	CCS HAU, Hisar/ India
42.	RH 0406	CCS HAU, Hisar/ India
43.	RH 725	CCS HAU, Hisar/ India
44.	RH 0749	CCS HAU, Hisar/ India
45.	RH 761	CCS HAU, Hisar/ India
46.	RH 8812	CCS HAU, Hisar/ India
47.	RVM 2	RVSKVV, Gwalior, MP/ India
48.	Varuna	CSAUA&T, Kanpur/ India

SSR Primers

A total of 59 random SSR primers were initially used, from which 24 polymorphic primers were selected for detailed analysis (Table 2). Primer sequences are available at http://mustard.pw.usda.gov. These primers were sourced from Imperial Life Sciences Pvt. Ltd, India.

Table 2 : A brief description of polymorphic SSR primers used in present investigation

Primer	Forward Primer	Reverse Primer
BG 1	GCTGGCTGCACAATAACAGA	GTACCACTGGAGGAGCTTCG
BG 4	CTGGTTTGGTTCGGTTTGAT	CCTGACAAATAGCAAGAAGTCG
BG 9	GGTGACCACCTCCGTCTTCTT	CTGTATGGAGCCCCAAGCTC
BG 15	CATAACCACACGGCCTCCTC	AAGTCATGCCCATTCGCCTA
BG 19	CGTTGTGTGAAATCGCTCAAAT	TCCGAACTAGAAACCGAAAATATCC
BG 23	GAGGCAAAAGCGAAGGTGAA	AGCACCCAAACACTCCCAAA
BG 79	CGTTTCCTCAGCCTCCTTCA	TGCCTACATCCACCGGAGTT
BG 102	GGACCGACTTTAGCAAGTCCA	GGGTAGCTTAGAAGATCATCTCTTTGG
BG 105	TCGATCTTTTTGCGGTGGAT	TTGCAATGGGCATTACATCCT
BG 109	AAGCCGGTTCTGCAAGTGTT	CATGGCATCCTACGTGGACA
BG 113	ATTGCTTCCGGAACTTGTCG	GCGTCACAGAGGCGGTTATT
BG 114	GCAAAATCCATTGGTAATCAGGA	TGGGCAAGTCACACTCACTCA
BG 115	GAGGAGGAGGAGAAGGAGGA	CCATCTTTGAAAAACCCCAAT
BG 124	TAGATCATTTCACACGGTGGAT	TCATAGCGACAAAAGTGACAGG

BG125	CGAACCGCGAACATAGTGTA	TAAGTGCCAGTCCATTGCAT
BG126	AGAACGAGTCGCGAGGATT	AGTGGGTGGAAGTTCGGTTA
BG127	GCGCCATCTAAACCGATATT	TACCGCGCCATTGATACATA
BG129	CGGAGATAACCGGAATGGAA	GGATGCTCTGAGACACCCAAA
BG130	TTGTCAGGGCGGATTTAGGA	TGAACCAGGAGACTTCCACAA
BG142	TCGATGATTAGTTTAGTTATTTCACG	CCTCAAACCAAGGAAGATTTCA
BG144	TTGAATAACAACAATAACATTGGATAA	GAAAAGTTGTTGGACCCTTTCA
BG149	TATGTGAACCTTGCCCTGGT	ATTCCGCAATCAAATTCCAG
BG150	TCCAAAGATTTCAACGGTCA	CATGCATGAGGTTTGGTCGT
BG153	CCGTACAAAGATACGCACGA	ACAGAACTCGGGGCTACCTC

DNA amplification

Polymerase chain reaction (PCR) was conducted in 20 ìL reaction volumes, containing: 12.35 µL of nuclease-free water, 2 µL genomic DNA, 2 µL of 10X PCR buffer with $MgCl_2$, 0.40 µM of each forward and reverse primer (Integrated DNA Technology, India), 0.4 µL dNTP mix (Thermo Scientific) and 0.25 µL Taq DNA polymerase (Thermo Scientific).

PCR amplification protocol

PCR reactions were conducted in a T100™ Thermal Cycler (Bio-Rad) using 96-well plates. The PCR conditions were as follows:

1. Initial denaturation at 94°C for 2 minutes
2. Denaturation at 94°C for 1 minute
3. Annealing at 50–60°C (primer-specific) for 1 minute, for a total of 38 cycles
4. Extension at 72°C for 2 minutes
5. Final extension at 72°C for 7 minutes

Agarosegel electrophoresis

PCR-amplified DNA fragments were separated by electrophoresis in 2.0% (w/v) agarose gels. Agarose was melted in 0.5X TBE buffer, and ethidium bromide (1 µL/mL) was added once the gel was warm. The gel solution was poured into a casting plate with a comb to create wells, then allowed to set. After setting, the gel was placed in an electrophoresis chamber and submerged in 0.5X TBE buffer. Samples, mixed with 6X loading dye, were loaded into the wells, and electrophoresis was conducted at a constant voltage of 3 V/cm until the dye front reached the end of the gel. DNA bands were visualized using a UV transilluminator (Gel Imaging and Analysis System, Benchtop).

Allele scoring

SSR bands were scored based on their presence (1) or absence (0). The sizes of the amplified bands were estimated by comparing their migration with a 100 bp DNA ladder (Thermo Scientific) loaded alongside the samples to determine the approximate band size.

Statistical analysis

Data were analysed using NTSYS software**.**

Results and Discussion

SSR polymorphism

To assess genetic diversity and relationships among 48 Indian mustard genotypes, 59 SSR primers were employed. Out of these, 24 primers were polymorphic, 27 were monomorphic, and 8 failed to amplify. The 24 polymorphic primers generated a total of 58 alleles, of which 48 were polymorphic, exhibiting distinct band sizes. Each primer produced an average of 2.5 alleles, ranging from 1 to 4 alleles per primer. Among the polymorphic markers, 13 primers generated 2 alleles each, 10 generated 3 alleles, while primer BG129 yielded a maximum of 4 alleles. The amplified fragment sizes ranged from 100 bp (BG124) to 900 bp (BG144), with allele size variations spanning from 35 bp (BG1, BG124) to 680 bp (BG153). Figure 1 shows the PCR amplification results of 48 Indian mustard genotypes using SSR primer BG150 on a 2% agarose gel. The primer generated polymorphic bands across the genotypes, indicating genetic variability. The amplified bands are of varying sizes, demonstrating allelic diversity at the SSR loci targeted by BG150. The presence of multiple alleles among the genotypes confirms the effectiveness

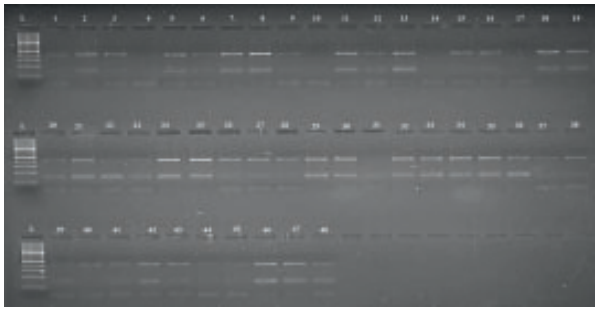

Fig. 1: PCR amplification profile with primer BG150 on 2 % agarose gel: L-100 bp DNA ladder, 1-48: genotypes

of BG150 for assessing genetic diversity in Indian mustard.

The polymorphism information content (PIC) values for these markers ranged from 0.09 to 0.66, with an average PIC of 0.40, indicating a moderate discriminatory power of the SSR markers. Primer BG129 exhibited the highest PIC value of 0.66, while BG130 had the lowest at 0.09, marking BG129 as the most informative and BG130 as the least informative primer. The polymorphic range among the primers varied between 33.3% and 100%. Specifically, primer BG115 exhibited 33.3% polymorphism, five primers (BG1, BG15, BG126, BG127, BG150) showed 50% polymorphism, two primers (BG113, BG149) exhibited 66.6% polymorphism, one primer showed 75%, and 15 primers achieved 100% polymorphism. The mean expected heterozygosity (He) was 0.49, with BG129 having the maximum value of 0.72 and BG130 the minimum at 0.09. Overall, 82.97% polymorphism was observed across the primers used in this study (Table 3).

Table 3: Polymorphic primers depicting number of alleles, per cent polymorphism, band size (bp) range and polymorphism information content (PIC) and heterozygosity/gene diversity (H_e)

Primer	Total no. of bands/ alleles	Monomorphic	Polymorphic	% Polymorphism	Band size (bp)	PIC	H value
BG1	2	1	1	50	115-150	0.33	0.42
BG4	2	0	2	100	200-280	0.37	0.50
BG9	2	0	2	100	200-250	0.31	0.38
BG15	2	1	1	50	270-700	0.37	0.49
BG19	2	0	2	100	270-400	0.37	0.49
BG23	2	1	1	100	110-290	0.26	0.30
BG79	3	0	3	100	110-400	0.58	0.66
BG102	2	0	2	100	110-190	0.37	0.50
BG105	3	0	3	100	320-600	0.53	0.59
BG109	3	0	3	100	195-250	0.50	0.59
BG113	3	1	2	66.6	300-500	0.14	0.14
BG114	2	0	2	100	250-300	0.35	0.45
BG115	3	2	1	33.3	115-600	0.59	0.66
BG124	2	0	2	100	100-135	0.37	0.50
BG125	3	0	3	100	140-200	0.49	0.57
BG126	2	1	1	50	110-160	0.37	0.49
BG127	2	1	1	50	215-600	0.37	0.50
BG129	4	1	3	75	180-600	0.66	0.72
BG130	2	0	2	100	200-400	0.09	0.09
BG142	3	0	3	100	160-280	0.50	0.58
BG144	3	0	3	100	380-900	0.59	0.66
BG149	3	1	2	66.6	160-400	0.34	0.41
BG150	2	1	1	50	150-400	0.37	0.49
BG153	3	0	3	100	120-800	0.48	0.57

Genetic distance and cluster analysis

The genetic dissimilarity coefficients ranged from 0.079 to 0.94. The highest dissimilarity (0.94) was recorded between genotypes RGN 486 and RH 1424, while the lowest dissimilarity (0.079) was observed between RH 2054 and RH 2055. Notably, RH 1424 was identified as the most diverse genotype, showing high dissimilarity with several other genotypes, including 0.94 with RGN 486, 0.93 with PM 28, 0.92 with RB 110, and 0.88 with NRCHB 101. Similarly, RH (OE) 1801 and PM 28 displayed a high level of genetic dissimilarity, with RH (OE) 1801 having coefficients of 0.92 with RGN 486 and 0.91 with PM 28 and RB 110. PM 28 also exhibited a dissimilarity coefficient of 0.92 with RGN 486 and 0.90 with RB 110.

A dendrogram was constructed using the UPGMA method based on Jaccard's dissimilarity matrix, which categorized the 48 genotypes into two primary clusters (Fig. 2). Group A comprised 44 genotypes, further divided into subgroups A1 and A2. Group B included four genotypes, forming two subgroups, B1 and B2 (Fig. 3). Potential cross combinations between genotypes were proposed based on their dissimilarity coefficients (Table 4).

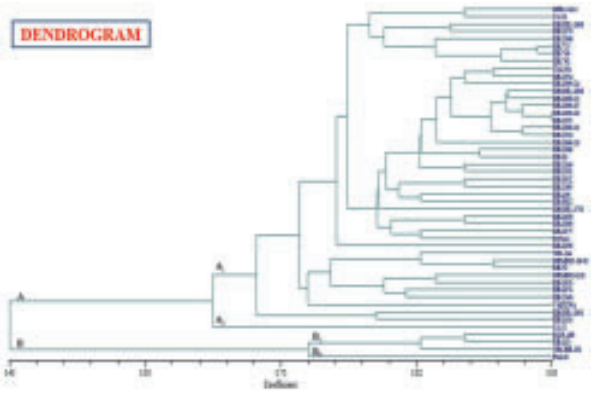

Fig. 2: Genetic relationship among 48 genotypes of Indian mustard revealed by UPGMA based dendrogram constructed using genotyping data of 59 SSRs primers

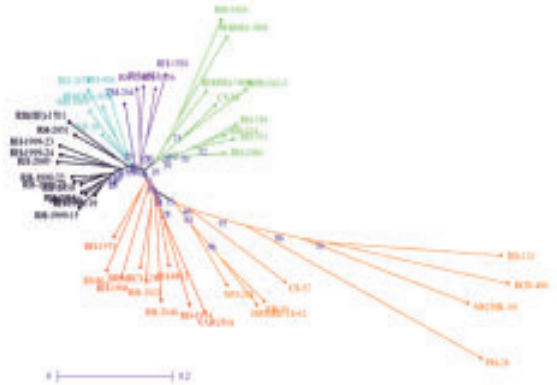

Fig. 3: Genetic relationship among 48 genotypes of Indian mustard revealed by NJ tree constructed using genotyping data of 24 polymorphic SSRs

Table 4: Possible cross combinations between genotypes based on dissimilarity coefficient

Dissimilarity coefficient	Genotypes
0.94	RGN 486 and RH 1424
0.93	PM 28 and RH 1424
0.92	RGN 486 and PM 28
0.92	RGN 486 and RH(0E) 1801
0.92	RB 110 and RH 1424
0.91	PM 28 and RH(0E) 1801
0.90	RB 110 and PM 28
0.90	RB 110 and RH(0E) 1801

While morphological and agronomic traits have traditionally been used to assess genetic diversity, they cover only a limited part of the genome, are influenced by environmental factors, and vary across developmental stages. In contrast, molecular markers provide a reliable means for evaluating genetic diversity, as they are not affected by environmental conditions and are independent of the plant's developmental stage (Gomez *et al*., 2004; Marvaldi *et al*., 2002). Studies indicate that molecular marker data can more accurately classify genotypes in terms of genetic diversity and phylogenetic relationships than phenotypic variables alone (Franco *et al*., 2001). Among molecular markers, SSRs are frequently used in genetic diversity studies due to their high reproducibility and co-dominance (Ali *et al*., 2008).

In this study, we analyzed molecular diversity among 48 genotypes of Indian mustard using SSR markers. Indian mustard, a natural amphiploid, exhibits limited intra-specific diversity, reflecting its narrow genetic base (Thakur *et al.*, 2018). The development of PCR-based molecular marker techniques, such as SSRs, has enabled detailed molecular diversity studies in Indian mustard, facilitating the identification of genotypes with favorable agronomic traits. The 24 polymorphic SSR primers used in this study generated a total of 48 alleles, with an average of 2.5 alleles per primer, aligning with findings by Sudan *et al*. (2016), Nanjundan *et al*. (2015) and Avtar *et al*. (2016), who reported similar allele counts.

The polymorphism information content (PIC) and gene diversity (H_e) values are effective indicators of genetic variation within crop germplasm. Our study yielded an average PIC value of 0.40, with a range from 0.09 to 0.66, which is consistent with previous studies by Singh et al. (2017), Avtar *et al.* (2016) and Prajapat *et al.* (2014). Minor variations in PIC values are likely due to differences in genotypes and sample sizes. According to Botstein *et al.* (1980), SSR markers with PIC values between 0.25 and 0.50 are moderately informative, while those above 0.50 are highly informative. In our study, five SSR markers had PIC values exceeding 0.50, indicating their effectiveness in distinguishing between genotypes and detecting polymorphism at specific loci. The average gene diversity (H_e) was 0.49, slightly higher than the mean PIC value, as expected, since gene diversity tends to be greater than PIC.

The overall polymorphic percentage of 82.97% demonstrated considerable genetic variation among the markers, though slightly lower than the 100% polymorphism reported by Nanjundan *et al*. (2015) for a different set of Indian mustard genotypes. This difference may be attributed to the distinct genotypes and markers used in each study. The Jaccard's dissimilarity coefficients among the 48 genotypes ranged from 0.079 to 0.94, indicating higher genetic diversity than previously reported by Abbas *et al*. (2009), who found a range of 0.05 to 0.61. Our findings support the efficacy of SSR markers in estimating genetic distances within Indian

mustard genotypes.

The dendrogram constructed from Jaccard's dissimilarity matrix grouped the 48 genotypes into two main clusters. Group A included 44 genotypes, further divided into two subgroups (A1 and A2), while Group B contained four genotypes, also split into two subgroups (B1 and B2). These results demonstrate that SSR marker data effectively differentiate genotypes based on genetic diversity. The clustering pattern observed in the dendrogram was consistent with the structure analysis, affirming the accuracy of the grouping and supporting the utility of SSR markers in Indian mustard genetic diversity studies.

Conclusion

This study utilized 59 SSR primers to assess genetic diversity among 48 Indian mustard genotypes for salinity tolerance, with 24 primers identified as polymorphic, yielding a total of 48 alleles across genotypes with an average of 2.5 alleles per primer. The PIC value of 0.40 and mean gene diversity (H_e) of 0.49 indicate a moderate level of genetic variation among the genotypes, valuable for breeding programs targeting salinity tolerance. Cluster analysis based on Jaccard's dissimilarity coefficients grouped the genotypes into two major clusters: Cluster A, comprising 44 genotypes including the checks CS 58 and CS 52, and Cluster B, which included four genotypes. The high similarity coefficient (0.95) between genotypes RH 2055 and RH 1999-19 suggests a close genetic relationship. Overall, these findings provide a solid foundation for the use of SSR markers in selecting diverse and salt-tolerant Indian mustard genotypes, facilitating the development of resilient cultivars through targeted breeding efforts.

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