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SSR marker and morphological trait-based genetic diversity assessment in *mori* cms based lines and restorers in Indian mustard [*Brassica juncea* (L.) Czern & Coss]

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Abstract

Indian mustard is a vital oilseed crop with significant economic and industrial importance. This study aimed to evaluate the genetic diversity among 15 genotypes of Indian mustard (*Brassica juncea* L.), consisting of 10 Mori CMS lines and 5 restorer lines, using SSR markers and agro-morphological traits. Out of 87 SSR primers screened, 40 showed successful amplification. A total of ninety-five alleles were amplified, with an average of 2.38 alleles per locus. The major allele frequency ranged from 0.30 to 0.93, with an average of 0.63 per SSR locus. The polymorphism information content (PIC) values ranged from 0.12 to 0.67, averaging 0.40, indicating moderate discriminative power. Gene diversity per locus ranged from 0.12 to 0.72, with an average of 0.45. Notably, six SSR markers exhibited PIC values greater than 0.50, demonstrating their effectiveness in differentiating the genotypes. Genetic divergence based on morphological traits was analyzed using Mahalanobis's D^2 statistics. Cluster analysis of SSR profiles, performed with NTSYS software version 2.0, grouped the 15 genotypes into four clusters, while morphological traits analyzed using Torchers method grouped the genotypes into four clusters. Utilizing genotypes from different clusters for hybridization could potentially yield feasible heterotic combinations, contributing to the development of superior cross combinations.

Keywords: *Genetic diversity, Mori CMS, SSR marker, morphological traits, Mahalanobis's D^2 statistics*

Introduction

Indian mustard (*Brassica juncea* L. Czern & Coss.) is an economically important oilseed crop of rapeseed-mustard group, belonging to family Brassicaceae. It is an amphidiploid crop (AABB, 2n = 36) with a physical genome size of 922 Mb (Kumar *et al.,* 2021) which evolved by natural hybridization between two primary diploids *B. rapa* (AA, 2n = 20) and *B. nigra* (BB, 2n = 16), followed by subsequent chromosomal duplication in nature. 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.

To date, several type of DNA-based marker systems were used for evaluation of genetic diversity in Indian mustard germplasm accessions including random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers. Among these molecular markers, SSR markers are the most preferred markers for molecular characterization and genetic diversity evaluation due to their co-dominant and multi-allelic inheritance, reproducibility and abundance (Viera *et al* . 2016). Scattered efforts had been made for genetic diversity evaluation in Indian mustard (Vinu *et al*., 2013; Nanjundan *et al.,* 2015; Pratap *et al*., 2015 and Singh *et al*., 2018)

Given the significance of genetic diversity for breeding programs, this study aimed to assess the genetic diversity among 15 genotypes of Indian mustard, including 10 Mori CMS lines and 5 restorer lines, using SSR markers and agro-morphological traits. This research seeks to identify the most diverse genotypes to support breeding programs by facilitating the development of superior cross combinations through effective heterotic breeding.

Materials and Methods

Plant material

The present study was carried out using 15 genotypes including 10 *Mori* CMS based lines and 5 restores of Indian mustard obtained from ICAR-DRMR, Bharatpur. SSR-genotyping work had been carried out in Molecular Biology Laboratory of ICAR-DRMR, Bharatpur, Rajasthan, India.

Experimental design

The genotypes were grown in a randomized block design (RBD) with three replications during the *rabi* season of 2023-24 at the Research Area of ICAR-Indian Institute of Rapeseed-Mustard Research, Sewar, Bharatpur. Each genotype was sown in two rows of 3 meters in length, maintaining a spacing of 45 cm between rows and 15 cm between plants.

Morphological data collection

Morphological data were recorded for 18 traits to evaluate the phenotypic variation among the Indian mustard genotypes. The traits included days to 50% flowering, days to maturity, main shoot length, plant height, number of primary branches,number of secondary branches per plant, number of siliquae on the main shoot, number of siliquae per plant, siliqua length, number of seeds per siliqua, biological yield, seed yield per plant, 1000-seed weight, oil content, canopy temperature, leaf area index and membrane stability index. These measurements provided a detailed phenotypic profile of each genotype, contributing to the understanding of genetic diversity and aiding in the selection of superior genotypes for breeding programs.

DNA extraction and purification

Genomic DNA of all 15 genotypes was isolated from the fresh leaf tissue using CTAB extraction protocol of Thakur *et al*., (2013). The quality and quantity of genomic DNA was examined by running on 0.8% agarose gel electrophoresis along with a lambda DNA ladder and diluted to a concentration of 50ng/µl after quantification.

SSR primers and polymerase chain reaction (PCR) amplification

A set of 87 genomic-SSRs were chosen for genotyping of Indian mustard varieties.The mastermix was prepared in a 2.0 ml centrifuge tube and the PCR reactions were standardized in a 25 µl reaction volume having 50 ng genomic DNA, 1X PCR buffer, 1.0 U Taq DNA polymerase (GCC Biotech, India), 0.2 mM of each dNTP, 2.0 mM $MgCl_2$ and 400 nM of each primer. DNA amplifications were performed in the BIO-RAD T100TM Thermal cycler. In thermal cycler, initial denaturation cycle comprised of 94 ^{0}C temperature for 5 min which was followed by 45 cycles at 94 ^{0}C for 30s, 50-60 ^{0}C (depending on the annealing temperature of the SSR primers) for 30 s, 45 s of extension at 72 ^{0}C and in the last, primer extension step at 72 ^{0}C for 7 min. PCR amplicons containing 5 µl loading dye were resolved in a 3.5% Super Fine Resolution (SFR) agarose (Amresco, USA). Further, gel pictures were taken in a gel documentation unit (Syngene Gel Doc, UK).

SSR scoring and analysis

The SSR marker data was scored in a binary format assigning '1' for the presence and '0 for the absence of a particular allele. The resulting binary matrix was subjected to Power Marker software v 3.25 (Liu and Muse, 2005) for computation of major allele frequency, PIC value and gene diversity for each individual SSR marker. 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. Clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on unweighted pair group arithmetic mean

(UPGMA) using SHAN module of NTSYS software version 2.0 (Rohlf, 1998).

Statistical analysis

Analysis of variance performed by using the principles of Fisher (1930). Mahalanobis' D^2 statistics (1936) were used to assess the morphological divergence. Cluster analysis for morphological traits was performed using Torcher's method.

Heterozygosity of a marker was calculated following Nei, (1978) as below:

$$\text{Heterozygosity (He)} = 1 - \sum_{i=1}^{k} P_i^2$$

Polymorphism information content (PIC) of each primer was calculated following Botstein *et al.* (1980) as below:

$$\text{PIC} = 1 - \sum_{i=1}^{k} P_i^2 - \left(\sum_{i=1}^{k} P_i^2\right)^2 - \sum_{i=1}^{k} P_i^4$$

Where,

Pi= the frequency of the i^{th} allele.

K= the total number of different alleles at the specific locus.

Results and Discussion

Genetic divergence and cluster analysis

Based on the phenotypic divergence analysis, all 15 mustard genotypes were grouped into four clusters (Table 1). Out of the 4 clusters, Cluster 1 was the largest, comprising 12 genotypes (MJA 10, MJA 27, MJR 2, MJA 38, MJA 3, MJA 35, M 78, MJR 5, MJR 6, MJA 9, MJR 7 and MJR 4), indicating a considerable level of similarity among these genotypes (Fig. 1). Cluster 2, Cluster 3, and Cluster genotype (MJA 1, MJA 36, MJA 37 respectively). Cluster 1 had the highest intra-cluster distance (122.41). The highest inter-cluster distance was between Clusters 2 and 4 (525.79), and the lowest between Clusters 2 and 3 (221.71), (Table 2). Our findings are consistent with preceding studies where Singh *et al* . (2017) and Singh *et al* . (2021).

Table 1: Distribution of genotypes into different clusters based on morpo-physiological traits

Cluster	Genotypes
Cluster 1	MJA 10, MJA 27, MJR 2, MJA 38, MJA 3, MJA 35, M 78, MJR 5, MJR 6, MJA 9, MJR 7, MJR 4
Cluster 2	MJA 1
Cluster 3	MJA 36
Cluster 4	MJA 37

Table 2: Intra and inter cluster distance in genotypes

Cluster	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1	122.4119	264.7506	261.0543	230.374
Cluster 2	264.7506	0	221.7162	525.7948
Cluster 3	261.0543	221.7162	0	328.0608
Cluster 4	230.374	525.7948	328.0608	0

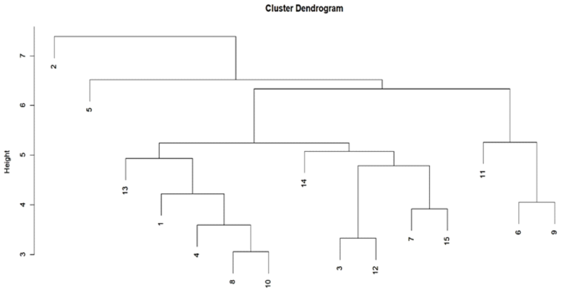

Fig.1 : Dendrogram Depicting genetic diversity among parental lines

Allelic diversity

Out of 87 SSR primers screened, 40 showed successful amplification (Fig. 3). A total of ninety-five alleles were amplified, with an average of 2.38 alleles per locus. The major allele frequency ranged from 0.30 to 0.93, with an average of 0.63 per SSR locus. The polymorphism information content (PIC) values ranged from 0.12 to 0.67, averaging 0.40, indicating moderate discriminative power. Gene diversity per locus ranged from 0.12 to 0.72, with an average of 0.45. Notably, six SSR markers exhibited PIC values greater than 0.50, demonstrating their effectiveness in differentiating the genotypes (Table 3). This result is in agreement with previous study of Singh *et al.*, (2021) and Avtar *et al.*, (2016).

A total of 12 SSRs produced 18 unique alleles for 12 varieties (Table 4). This result is in agreement with previous study of Singh *et al.* (2021) and Avtar *et al* . (2016).

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 2. A total nine genotypes viz., MJR6, MJR 5, MJA 37, MJA 3, MJA 10, MJR 4, MJR 2, MJA 36 and MJR 7 fells into cluster 2. Cluster 1 comprises three genotypes viz., MJA 27, MJA 9, and MJA 38, while only two CMS lines, M78 NA and MJA 35 fell into cluster-3. Most diverse CMS line viz.,

Table 3: Genetic diversity parameters of 40 polymorphic SSR markers for 15 genotypes

Marker ID	Total Amplified Bands	Polymorphism Percentage	Major Allele Frequency	Gene Diversity	PIC value
BrgMS388	2	100.00	0.55	0.50	0.37
SJ4933	3	100.00	0.62	0.50	0.42
SA0306	3	100.00	0.42	0.64	0.57
SB1935a	2	100.00	0.64	0.46	0.35
SJ8033	2	100.00	0.82	0.29	0.25
SJ13133	2	100.00	0.88	0.23	0.21
BrgMS383	3	33.33	0.35	0.67	0.59
BrgMS465	4	75.00	0.45	0.66	0.60
BrgMS4539	3	66.67	0.50	0.60	0.52
BrgMS216	3	33.33	0.47	0.56	0.46
BrgMS66	2	100.00	0.54	0.50	0.37
BrgMS361	3	66.67	0.50	0.56	0.46
BrgMS10	3	66.67	0.50	0.58	0.49
BrgMS841	3	100.00	0.40	0.66	0.58
BrgMS397	2	100.00	0.87	0.24	0.23
BrgMS787	2	50.00	0.52	0.50	0.37
BrgMS190	2	100.00	0.64	0.46	0.36
SB0372	2	50.00	0.88	0.21	0.19
BrgMS1474	2	100.00	0.55	0.50	0.37
BrgMS639	2	100.00	0.76	0.36	0.30
BrgMS639	2	100.00	0.50	0.50	0.38
SJ6842	2	100.00	0.78	0.35	0.29
BrgMS638	3	100.00	0.61	0.55	0.49
SJ3302RI	2	100.00	0.68	0.43	0.34
BrgMS233	3	100.00	0.48	0.56	0.46
BrgMS713	2	100.00	0.64	0.46	0.35
D09	2	100.00	0.65	0.46	0.35
C09	2	100.00	0.72	0.40	0.32
BRMS 006	4	25.00	0.30	0.72	0.67
cnu_m585a	2	50.00	0.56	0.49	0.37
C12	2	100.00	0.75	0.38	0.30
ENA3	3	66.67	0.60	0.56	0.50
cnu_m626a	2	100.00	0.93	0.13	0.12
nia_m053a	2	100.00	0.52	0.50	0.37
nia_m042a	2	100.00	0.93	0.12	0.12
cnu_m583a	2	100.00	0.93	0.12	0.12
Ra2-F09	2	50.00	0.56	0.49	0.37
Ra2-H04	2	100.00	0.50	0.50	0.38
F02	2	50.00	0.88	0.21	0.19
E12	2	50.00	0.75	0.38	0.30

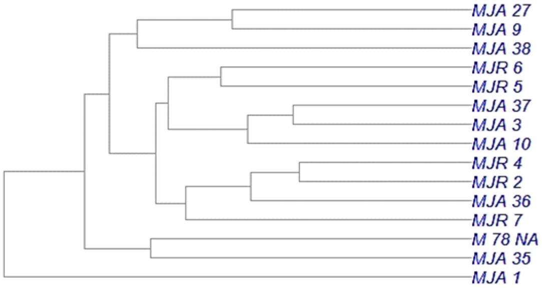

Fig. 2: UPGMA dendrogram showing genetic diversity in 15 genotypes based on 40 SSRs

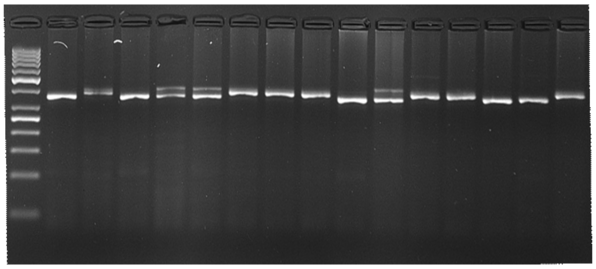

Fig. 3: A representative gel picture depicting the amplification profile of SSR marker SA0306 in 15 Indian mustard germplasm accessions

Table 4: Unique alleles for selected Indian mustard genotypes

Marker	Genotype	Unique allele (bp)
SJ4933	MJR 2	360
SJ13133	M 78	320
BrgMS216	MJA 38	310
	M 78	310
BrgMS361	MJA 1	270
	M 78	280
BrgMS397	MJA 35	360
	M 78	380
SB0372	MJA 37	240
	M 78	240
SJ6842	MJA 9	290
	MJA 27	290
BrgMS233	MJA 10	220
cnu_m626a	MJA 37	220
nia_m042a	MJR 6	360
cnu_m583a	MJA 1	220
F02	MJA 10	250
	MJR 5	250

Table 5: Distribution of genotypes into different clusters based on SSR marker analysis

Cluster	Genotypes
Cluster 1	MJA 24, MJA 9, MJA 38
Cluster 2	MJR6, MJR 5, MJA 37, MJA 3, MJA 10, MJR 4, MJR 2, MJA 36, MJR 7
Cluster 3	M78 NA, MJA 35
Cluster 4	MJA 1

MJA 1 fell into cluster – 4 (Table 5). These CMS lines gives best cross combinations with restorers of cluster – 1 and cluster – 2.

Conclusion

This study revealed considerable phenotypic and genotypic variation among the Indian mustard genotypes examined. Although, morphological characterization show different clusters than the molecular characterization. Cluster analysis revealed relatively more diversity at molecular level as compared to morphological level. So, there was no relation between morphological and molecular diversity. The significant presence of private alleles suggests that these accessions can be valuable sources of novel genes for mustard breeding programs. Cluster analysis identified distinct genetic groupings, indicating that hybridization between genotypes from different clusters could produce promising heterotic combinations. These insights are crucial for developing effective breeding programs for Indian mustard, facilitating the identification and use of genetically diverse genotypes to improve the crop's genetic makeup.

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