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COMPARATIVE ASSESSMENT OF GENETIC DIVERSITY AT MORPHOLOGICAL AND MOLECULAR LEVELS IN INDIAN MUSTARD GERMPLASM

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ABSTRACT

The comparison of morphological and molecular methods of estimating the genetic diversity was done using seventy-three Indian mustard genotypes. Data were recorded on twelve quantitative traits to assess the morphological diversity. Thirty-four SSR primers were used to study the genetic diversity among genotypes. ANOVA revealed that the mean sum of squares of genotypes were highly significant for all the characters under investigation suggesting presence of transmissible variation. Mahalanobis D^2 statistics-based prediction of morphological diversity classified the genotypes into four clusters whereas UPGMA based clustering at molecular level divided the genotypes into five groups at 63 per cent similarity coefficient. The highest inter-cluster distance was recorded between the cluster II and cluster IV in morphological diversity while the polymorphism information content varied from 0.39 to 0.66 with average of 0.57. Only 47.05% of SSR markers were found polymorphic. Assessment of genetic diversity based on SSR markers at molecular level was found to be more suitable owing to the greater ability to discriminate genotypes more precisely than the morphological diversity-based characterization. This information will be useful for collection, conservation and designing breeding programs.

Key words : Morphological diversity, Molecular diversity, SSR marker, NTSYS.

Introduction

In India, Mustard is the most important oilseed crop next only to groundnut. Indian mustard (*Brassica juncea*) is well adapted in cropping system of rainfed areas and accounts for more than 75% of the total area under rapeseed-mustard in India, thereby playing a major role in Indian economy by contributing about 26.57% of the total oilseed production (Directorate of Economics and Statistics, DAC and FW, 2018). Indian mustard has recorded significant increase in area and production during last few years. Their production increased from 9.12 MT In the year 2019-20 to 10.11 MT in the year 2020-21. Though an increase in production is noted, it is still not enough to suffice the domestic demand for rapeseed mustard (Directorate of Economics and Statistics, DA and FW, 2021). To overcome this lacuna, plant breeders aim to search for genotypes which possess maximum genetic diversity, which can be utilized to develop superior hybrids and cultivars with higher yield and stability. Genetic diversity can either be created or the existing variability in the population can be used. *Brassica juncea, being* a natural amphidiploid of *Brassica rapa* and *Brassica nigra*, is expected to harbour a large amount of available genetic variations. Genetic diversity in the population is the basis of selection, whether natural or human-directed. The degree of differentiation

or variability between or within species is the basis of all crop improvement programs therefore for initiation of any breeding strategy aimed at enhancing the yield potential, genetic diversity is prerequisite because it results into desirable transgressive segregants which can be used to develop superior hybrids and cultivars. This would also help in widening the genetic basis of the population thus ultimately producing stable genotypes harnessing useful variability. Mahalanobis' D^2 statistic is a suitable tool in determining the degree of divergence between germplasm at genetic level and provides an extent of association between geographic and genetic diversity based on generalized distance at morphological level. But now a day, molecular markers are widely used as tools to assist breedersowing to their precise nature. Microsatellite or simple sequence repeat (SSR) markers are considered most responsive for the assessment of genetic diversity, as they are multi-allelic in nature, highly informative, highly reproducible have co-dominant inheritance and provide extensive genomic coverage (McCouch *et al.*, 2002). They are able to detect great level of allelic diversity and have been used to identify genetic variation among mustard genotypes. Therefore, the present experiment was conducted to characterize seventy-three Indian mustard genotypes at morphological and molecular level and to conduct a comparative study between two tools.

Materials and Methods

Plant material

A total of seventy-three Indian mustard genotypes were grown in Randomized block design with three replications during *Rabi* season of 2016-17. The genotypes procured from various institutes were used in this experiment are listed in Table 1. For DNA extraction 21-day-old healthy leaves from all the seventy-three genotypes were collected and kept at -80^{0}C.

Morphological observations

Data were recorded on twelve morphological traits *viz.*, plant height, number of primary branches, number of secondary branches, main raceme length, number of siliqua on main raceme, siliqua length, number of seeds per siliqua, number of silique per plant, test weight, seed yield per plant, seed yield per plot and seed yield per hectare. The data for plot yield was recorded from plot

Table 1 : List of Indian mustard genotypes used for the investigation.

S. no.	Name of genotype	S. no.	Name of genotype	S. no.	Name of genotype
1	GIRIRAJ	26	CS 54	51	NDRE 4
2	URVASHI	27	JMM 914 (ABR-5)	52	NPJ 112
3	PM 30	28	ASHIRWAD	53	MCN 10-11
4	KRANTI	29	RLM 619	54	HUJM 9901
5	RH 406	30	RGN 229	55	ISH 7-3-2
6	VARDAN	31	DRM 150-35	56	HUJM 08-12
7	PBR 541-4	32	DIBYA	57	HUJM 05-03
8	PUSA BOLD	33	MAYA	58	NDR 8501
9	PM 26	34	PRO 19	59	JMM 915 (MCN-6)
10	SEJ 2	35	JAGANNATH	60	RGN 73
11	NRCHB 101	36	HUJM 9903	61	RGN 298
12	JMM 08-01 (MCN 08-12)	37	KANTI	62	PUSA BAHAR
13	RGN 236	38	BASANTI	63	PITAMBARI
14	PM 28	39	VARUNA	64	SHIVANI
15	RLM 1359	40	RH 8813	65	RH 119
16	PBR 91	41	JM 3	66	RH 749
17	NDRE 8	42	RH 8814	67	RB 50
18	PAB 9511 (ABR-4)	43	PM 27	68	VASUNDHARA
19	RLC 2	44	RH 781	69	RH 30
20	HUJM 07-06	45	VAIBHAV	70	RVM 2
21	PAB 9534 (ABR-1)	46	DRMR 601	71	PUSA TARAK
22	JD 6	47	GM 2	72	PUSA VIJAY
23	NDR 8	48	RH 8812	73	HUJM 08-12
24	JMM 915 (MCN -6)	49	HUJM 9504		
25	SWARN JYOTI	50	NRCDR 02		

however other traits were recorded from five randomly selected plants of each genotype in each replication.

Molecular analysis

DNA from 21-day-old leaves were extracted using CTAB method (Doyle and Doyle, 1987) with few modifications and later the DNA quality was estimated using Bio-photometer plus. In total thirty-four SSR markers were selected for molecular analysis. Polymerase chain reaction (PCR) was performed using a Sure Cycler 8800 thermal cycler *in-vitro* to amplify a precise piece of genomic DNA to a billionfold (Mullis *et al*., 1986). Amplified PCR product using specific SSR primers were separated out for visualization on gel electrophoresis in 2.5 per cent agarose gel prepared in TAE buffer. The gel was imaged under gel documentation system (Gel DocTM XR+, BIO-RAD, USA) under UV light. Resulting images were stored for the future evaluation. A binary data matrix was utilized to generate genetic similarity data among the seventy-three lines of mustard genotypes.

Statistical analysis

The mean data over the replications were used for the analysis of variance using WINDOSTAT version 9.0. Mahalanobis' generalized distance (Rao, 1952) was used for predicting the genetic divergence. For estimation of molecular diversity, NTSYSpc version 2.02 was employed which uses the binary data of polymorphic SSRs generated by scoring the gel images (Rohlf *et al*., 1998). The Jaccard's dissimilarity coefficient was determined using SIMQUAL programme. The Polymorphism information content (PIC) (Anderson *et al*., 1993) of each primer was calculated as below:

$$PIC = 1 - \sum_{i=1}^{n} \left(P_{ij}\right)^2$$

Where,

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

PIC value suggests the informativeness and estimates the discriminatory power of SSR markers.

Results and Discussion

Analysis of Variance

The analysis of variance showed that genotypes exhibited highly significant variation for twelve characters suggesting existence of heritable variation between the genotypes (Table 2). For the traits number of secondary branches, siliqua length, number of siliquae perplant, test weight, seed yield per plant, seed yield per plot and seed yield replications mean sum of square were found significant indicating that the blocking carried out for replications was required or valid.

Table 2 : Analysis of variance for various characters studied.

Source	df	Plant height	Number of Primary branches	Number of Secondary branches	Main raceme length	Number of siliqua on main raceme	Siliqua length (cm)	Number of seeds/ siliqua	Number of siliqua/ plant	Test weight (gm)	Seed yield /plant (gm)	Seed yield/plot (gm)	Seed yield (kg/ha)
Genotype	72	959.12**	3.49**	28.04**	100.60**	103.10**	0.43**	20.70**	9617.49**	0.98**	11.06**	2620.97**	583096.67**
Replication	2	296.59	0.31	16.31*	76.44	19.08	1.33**	8.50	32198.50**	0.08*	9.86**	3218.85**	522520.29**
Error	144	182.30	1.59	4.18	38.99	30.88	0.17	6.02	1371.32	0.02	1.25	259.98	58569.32
CV		7.98	15.97	18.36	11.69	14.02	8.02	17.72	17.53	3.16	14.87	13.67	13.64

Table 3 : Distribution of seventy three Indian mustard genotypes into different clusters.

Cluster	Number of genotypes	Name of genotypes
I	70	NDR 8501, RLM 1359, MCN-10-11, KANTI, RB 50, RH 406, PM 30, ISH-7-3-2, JD-6, RLM 619, PUSA TARAK, RH 30, VASUNDHARA, VARUNA, RH 8813, GM-2, RH 0749, JMM 914 (ABR-5), DRMR-150-35, JAGANNATH, PAB 9511 (ARB-4), HUJM-07-06, HUJM-08-01, BASANTI, RH 781, RGN 73, PM-27, HUJM-9804, DRMR 601, HUJM-05-03, RH 119, SHIVANI, PBR 541-4, RVM-2, NRCHB-101, MAYA, NRCDR 02, URVASHI, JM-3, VAIBHAV, RH 8812, PM 28, VARDAN, ASHIRWAD, RLC-2, SEJ-2, PM 26, PUSA BOLD, PUSA BAHAR, RGN 229, HUJM-9903, JMM-08-01 (MCN-08-12), PRO-19, KRANTI, PAB 9534 (ABR-1), DIBYA, CS-54, PBR 91, RGN 236, HUJM-08-12, SWARN JYOTI, RGN 298, GIRIRAJ, PUSA VIJAY, RH 8814, NDRE-8, HUJM-9901, NPJ 112, NDR-8, HUJM-9504
II	1	NDRE 4
III	1	MCN 6 (JMM-915)
IV	1	PITAMBARI

Mahalanobis D^2 analysis

The morphological divergence using Mahalanobis D^2 statistics were studied among seventy-three Indian mustard genotypes, which allocate Euclidean distance to form group of genotypes using Ward's method. Ward's method classified the seventy-three genotypes in four clusters. The cluster-I was the largest and possessed 70 genotypes, whereas other three clusters were solitary containing single genotype each (Table 3). Clustering pattern of seventy-three Indian mustard genotypes according to Tocher's method are shown in Figure 1. In a study sixteen Indian mustard genotypes were grouped into four clusters (Iqbal *et al*., 2014). In another study, seven clusters were resulted using forty-five Indian mustard varieties (Devi *et al*., 2017). Forty-six genotypes of Indian mustard grouped into seven clusters (Gangapur *et al*., 2010). In an experiment, 45 Indian mustard genotypes were evaluated for extent of diversity and eight clusters were formed (Pande *et al*., 2013). Researcher grouped sixty mustard genotypes into ten clusters during evaluation for morphological diversity (Pande *et al*., 2013).

The maximum intra-cluster distance (15.01) was noted in cluster-I 15.01 (Table 4). Other clusters recorded zero intra-cluster distances because of the fact that they contained single genotype. The maximum inter-cluster distance was noted between cluster II and cluster IV (68.85) followed by that between cluster III and cluster IV (60.16). The inter-cluster distance betweencluster III and cluster IV was 60.16, while it was 56.74 between cluster I and cluster IV. The inter cluster distance found between cluster I and cluster II was (53.99), cluster I and cluster III (39.45) and cluster II and cluster III (47.85). In an experiment, zero intra-cluster distance among the three clusters were recorded (Neeru *et al*., 2015). In another experiment two clusters having null intra cluster distance were reported (Pankaj *et al*., 2017).

Table 4 : Intra and inter cluster distance between the different clusters.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1	15.01	53.99	39.45	56.74
Cluster 2		0.00	47.85	68.85
Cluster 3			0.00	60.16
Cluster 4				0.00

Molecular Diversity analysis

Molecular diversity in seventy-three Indian mustard genotypes was estimated using thirty-four microsatellite markers (SSRs). Out of the thirty-four SSRs used only sixteen revealed polymorphism (Table 5). A total of thirty-four alleles were generated ranging from 2 to 3 with the average of 2.12 alleles per locus. Presence of a smaller number of alleles per locus suggested that the markers used in study were less efficient. In a study while estimating the molecular diversity in 23 genotypes average of 2.37 alleles per locus was reported (Sudan *et al*., 2016). In an investigation average of 2.64 alleles per locus (Vinu *et al*., 2013), while in another investigation average of 2.74 alleles per locus (Nanjundan *et al*., 2015) were observed.

The primers Na10 A09 and Na10 D08 amplified three loci however other markers were polymorphic at two loci. The amplification patterns of Na10 B10 marker were shown in Fig. 2. The per cent polymorphism varied from 5.88 to 8.82 with average of 6.25 while the PIC value fluctuated from 0.39 (Na10 E08) to 0.66 (Na10 D08) with the average of 0.57. Ten SSR markers exhibited polymorphic information content greater than the average PIC value. The minimum resolving power was noted for Na10 B11 (1.29) and maximum for Na10 B10 and Na12

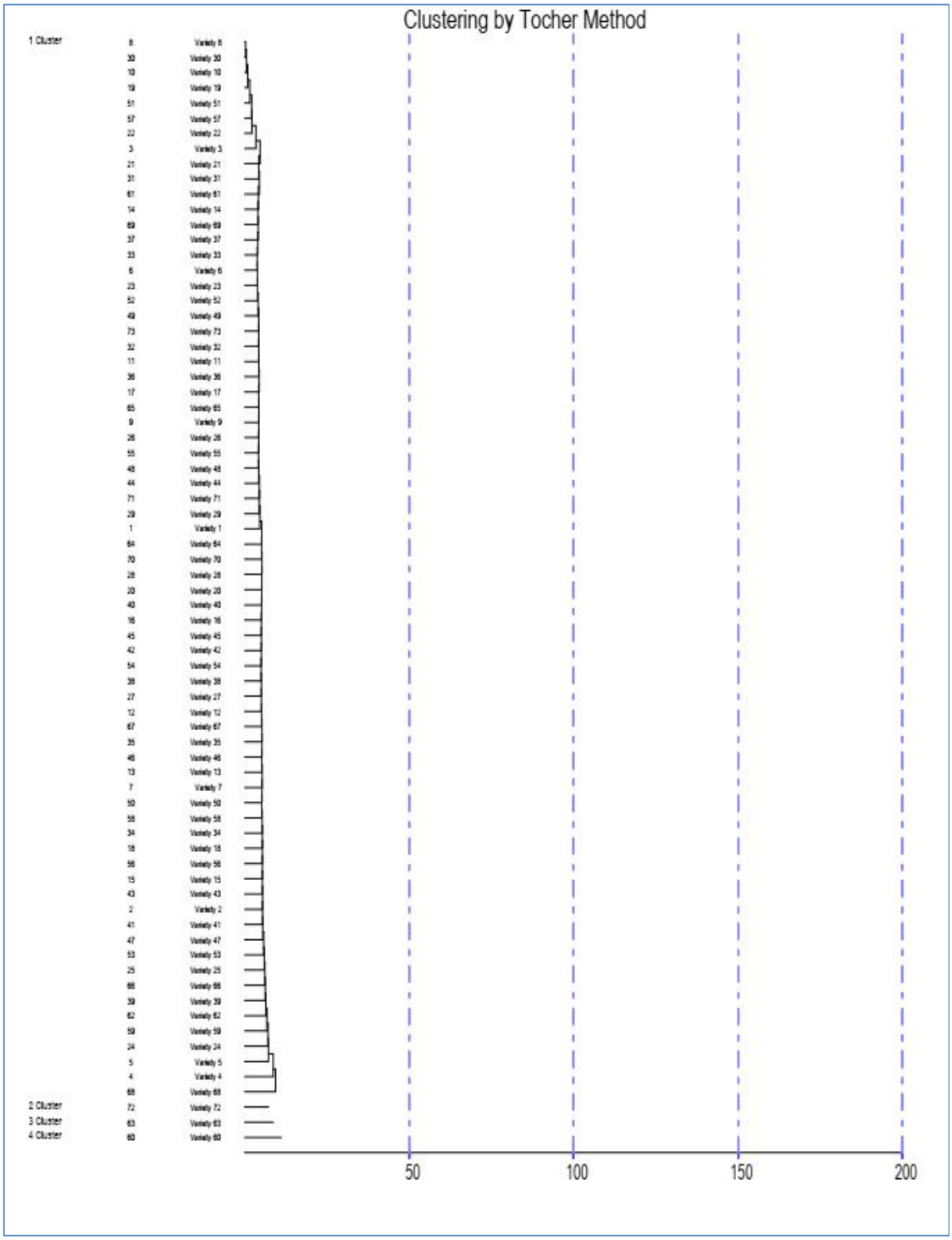

Fig. 1 : Clustering of seventy-three Indian mustard genotypes based on Mahalanobis' D^2 method.

A01 (2.00) with average of 1.72. In previous studies, the PIC value ranged from (0.39 to 0.72) (Fayyaz *et al.*, 2014), (0.04 to 0.77) (Sudan *et al.*, 2016) and (0.42 to 0.73) (Patel *et al.*, 2018). The primers Na10-B07, Na10-B1, Na10-D07, Na10-E02, Na10-B04, Na10-D08, Na10-D09 and Na12-B09 were efficient in detecting polymorphism according to PIC value.

The UPGMA based clustering of seventy-three Indian mustard genotypes revealed only two major clusters at 50 per cent similarity coefficient (Fig. 3). However, the two major clusters subdivided into five different clusters at 63 per cent similarity coefficient. Cluster-I comprised of highest number of genotypes (23), while cluster II comprised of least number of genotypes (2). The cluster III, IV and V consisted of 16, 17 and 15 genotypes, respectively (Table 6). In an earlier study, thirty-nine

Table 5 : Attributes of molecular diversity using different polymorphic SSR markers.

S. no.	Primer	Primer Sequence	No. of alleles	Per cent polymorphism	PIC value	Rp
1.	Na 10 A09	TCTTGAGCAAAGAAACTTGG CAAACTGAGCCATACACAAAGG	3	8.82	0.59	1.78
2.	Na 10 B07	GCCTTAGATTAGATGGTCGCC ACTTCAGCTCCGATTTGCC	2	5.88	0.64	1.62
3.	Na 10 B10	GTCGGGTTTGAGTGAGTTGG CATCGCAGATCCTTCTCTCC	2	5.88	0.49	2.00
4.	Na 10 B11	TTTAACAACAACCGTCACGC CTCCTCCTCCATCAATCTGC	2	5.88	0.61	1.29
5.	Na 10 D07	CTACTTTGATGGACACTTGCC TCTGAAGTTGATTAGTCGGTCC	2	5.88	0.65	1.51
6.	Na 10 E02	TCGCGCATGTAATCAAAATC TGTGACGCATCCGATCATAC	2	5.88	0.62	1.73
7.	Na 10 F06	CTCTTCGGTTCGATCCTCG TTTTTAACAGGAACGGTGGC	2	5.88	0.53	1.73
8.	Na 12 A01	GCATGCTCTTGATGAACGAA GCTTCAACCTCTCAATCGCT	2	5.88	0.45	2.00
9.	Na10-B 01	CAAGTGTCTGCTAGGTGGGG TCGATCGAAGAAACCAGACC	2	5.88	0.59	1.78
10.	Na10-B 04	GCGTCGAGAGAGATCGAGAG CTCACCGTCACTGCTTCATC	2	5.88	0.60	1.75
11.	Na10-D 08	TCCATTCATTAAAATCGGCG TTCTGATCCCTTTCTCTCCC	3	8.82	0.66	1.70
12.	Na10-D 09	AAGAACGTCAAGATCCTCTGC ACCACCACGGTAGTAGAGCG	2	5.88	0.65	1.37
13.	Na10-D 11	GAGACATAGATGAGTGAATCTGGC CATTAGTTGTGGACGGTCGG	2	5.88	0.52	1.95
14.	Na10-E 08	TCGGGGTTTGTTGTGAGGGA GGAGGATGCTAAGAGTGAGC	2	5.88	0.39	1.92
15.	Na12-B 09	ACGGAAGATCAAACAGCTCC TGAGCGACCCATTCTTTAGG	2	5.88	0.63	1.70
16.	Na12-E 06A	TTGGGTTGACTACTCGGTCC CCGTTGATTTGGCTAAGACC	2	5.88	0.55	1.70
Total			**34**			
Average			**2.12**	**6.25**	**0.57**	**1.72**

mustard accessions were distributed into five different groups (Shu *et al*., 2016), while in other research, seventy-seven accessions were grouped into five major clusters (Channa *et al*., 2016). Molecular diversity in thirty *Brassica* genotypes operating UPGMA method resulted five major groups (Saini *et al*., 2019). The genetic diversity among thirty-seven genotypes were evaluated which grouped them into five clusters (Chandra *et al*., 2016). In a study 217 *Brassica napus* genotypes were classified using 37 SSR markers by UPGMA analysis into two major group only (Qu *et al*., 2012).

All the genotypes of cluster III seem distinct at 55% similarity coefficient while cluster II seem distinct at around 59% similarity coefficient. Three genotypes MCN10-11, HUJM 99-01 and HUJM 08-12 acquired similar position because of 100% similarity between them. Four genotypes Vardan, Pusa Bold, Swarn Jyoti and Ashirwad also possessed 100% similarity (Fig. 3). In present study, nearly 47.05% of SSR markers detected

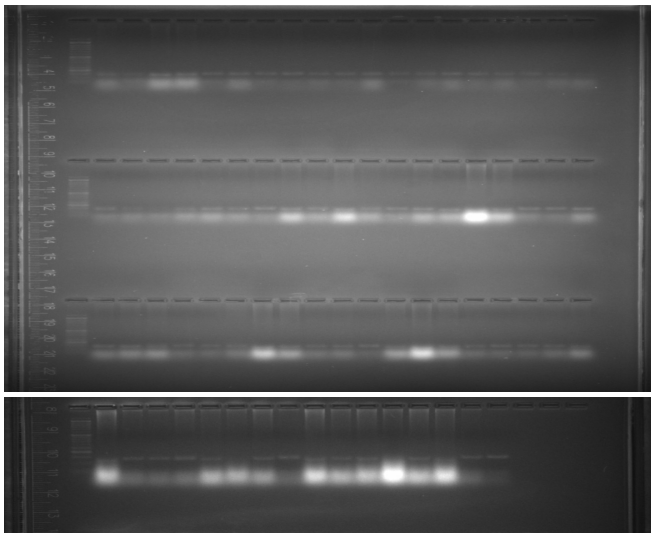

Fig. 2 : Banding pattern of seventy-three genotypes using the SSR "Na10 B10".

germplasm accession Pitambari had unique morphological characters from rest of the genotypes but this uniqueness was not detected in molecular diversity and was found in the cluster III having sixteen genotypes and closely related with RH 119 and RGN 298. Similar results were obtained for NDRE 4, which was included in cluster II with no other genotype when morphological diversity was taken into account while it was included in cluster III along with sixteen genotypes and showed close relation with NPJ 112 according to molecular diversity. This may be justified by the fact that few random SSRs were considered for the present studies which were not linked to the morphological traits considered for study. The presence of high genetic similarities among the genotypes used for study may be another reason. This finding indicates that the primers used in the study did not relate to the phenotypic feature and therefore could not capture

Table 6 : Clustering of seventy-three genotypes based on molecular diversity.

Cluster	Number of genotypes	Name of Genotypes
I	23	GIRIRAJ, URVASHI, HUJM 05-03, VARUNA,RH 8813, JM 3, PUSA BAHAR, SHIVANI,RB 50, RH 30, RH 749, RVM 2, VASUNDHARA,PUSA TARAK, MCN 10-11, HUJM 9901,HUJM 08-12, ISH 7-3-2, NDR 8501, HUJM 9804,RGN 73, PUSA VIJAY, HUJM 08-1
II	2	PAB 9511 (ABR 4), RLC 2
III	16	PM 30, JAGANNATH, VAIBHAV, NRCDR 02,JD 6, HUJM 07-06, PAB9534(ABR 1), NDRE 4,NPJ 112, MAYA, PRO 19, KRANTI, DRMR 601,RGN 298, RH 119, PITAMBARI
IV	17	RH 406, VARDAN, PUSA BOLD, SWARN JYOTI,ASHIRWAD, PBR 541-4, PM 26, SEJ 2, NRCHB 101,CS 54, RGN 229, DIBYA, DRM 150-35, NDR 8,JMM915(MCN 6), JMM914(ABR 5), RLM 619
V	15	JMM08-01(MCN 08-12), PBR 91, PM 28, RGN 236,HUJM 9504, RLM 1359, PM 27, GM 2, RH 781,NDRE 8, HUJM 9903, KANTI, BASANTI, RH 8814,RH 8812

polymorphic using the template from seventy-three Indian mustard genotypes. The low polymorphism in present investigation may be due to the high relatedness of the genotypes used in the experiment. In different studies 80.48 (Ghosh *et al*., 2019), 93.70 (Vinu *et al*., 2013) and 50.00 (Fayyaz *et al*., 2014; Sudan *et al*., 2016) per cent polymorphic markers were reported.

Relationship between morphological and molecular diversity

The pattern of grouping was found significantly different while studying the morphological diversity and molecular diversity. Morphological diversity clustered the genotypes into four major clusters where cluster I alone comprised 70 genotypes representing limited variability existing in the germplasm studied. On contrary, grouping of genotypes based on molecular diversity resulted five clusters having almost even distribution of genotypes. The the variability. It may also be possible that the phenotypic variation might have arisen due to the phenotypic reaction of the genotype to the environment of its adaptation without any changes at molecular level. Add references for these types of studies, if available. Clusters formed based on molecular diversity has more justified distribution of genotypes in comparison with the clusters formed based on morphological diversity where maximum genotypes were placed into single cluster. Greater admissible distribution of genotypes in molecular diversity-based clustering may be due to the fact that DNA based markers (SSR) were used for study which are not influenced by environment and also considers the non-coding DNA regions hence shows the genuine genetic diversity. Present study concludes the fact that marker-based diversity estimation is more equitable and reliable as compared to the morphological diversity owing to the

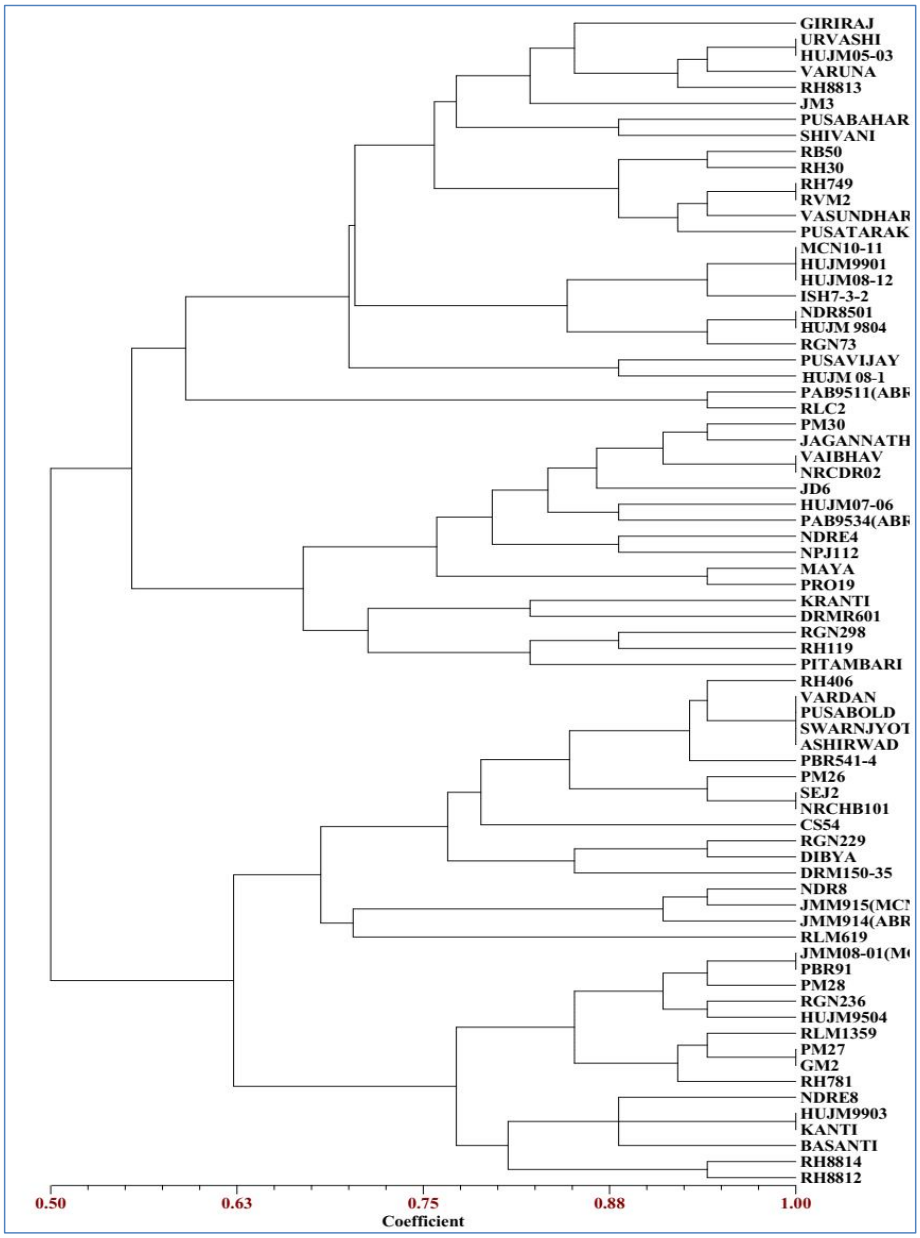

Fig. 3 : Clustering of seventy-three Indian mustard genotypes based on Molecular diversity.

preciseness of SSR markers *i.e.*, its ability to detect number of alleles per locus and number of markers used for the study. A study reported similar results and concluded that SSR marker are more reliable and stronger tool compared to quantitative traits in discriminating *Brassica juncea* genotypes (Vinu, 2013).

Conclusion

The results of estimation of morphological diversity and molecular diversity were found critically different in the study. The clusters made based on molecular diversity were more trustworthy than the morphological diversity. The SSR markers were better tool in discriminating the genotypes compared to the morphological traits. The genotypes Urvashi, NDRE 8, RH 8812 and HUJM 9903 were reported most diverse along with high seed yield per hectare which may be utilized for hybridization in future breeding program. Saying this one cannot ignore the fact that morphological-based diversity analysis when done in controlled environment supported with precision in data recording will improve the efficiency of molecular-based diversity analysis.

Conflict of interest

Each author certifies that they are all in agreement with the guidelines and declare no conflict of interest.

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