Int.J.Curr.Microbiol.App.Sci (2019) *8*(1): 2564-2574

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 8 Number 01 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.801.269

Genetic Diversity Analysis for Drought Tolerance in Indian Mustard (*B. juncea* L. Czern & Coss) using Microsatellite Markers

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Keywords

SSR primer, similarity coefficient, Polymorphism, cluster analysis and *Brassica juncea*

Article Info

Accepted:
18 December 2018
Available Online:
10 January 2019

A B S T R A C T

A total of 200 SSR markers from different *Brassica* species were used in this study. Out of 200 SSR markers analyzed for polymorphism in two parental *Brassica juncea* genotypes (RB 50, drought tolerant and Kranti, drought susceptible), 51 were polymorphic. The polymorphic markers were used to screen F_2 population. A total of 108 alleles were identified in the RB 50 and Kranti and the parental *B. juncea* genotypes. The PIC (polymorphic information content) values for various primers ranged from 0.340-0.505 with an average of 0.406. Similarity coefficient data based on the proportion of shared alleles using 51 SSR markers was used to calculate the coefficient values among the 157 F_2 plants of RB 50 × Kranti and parental *B. juncea* genotypes and subjected to UPGMA tree cluster analysis. All the 157 F_2 plants clustered in two major groups at the similarity coefficient of 0.53. Two parental varieties RB 50 and Kranti had low similarity coefficient. Genetic relationship was also assessed by PCA analysis (NTSYS-PC). Two dimensional and three dimensional PCA scaling exhibited that two parental genotypes were quite distinct whereas all 157 F_2 plants interspersed between the two parental lines with distribution of most plants towards RB 50.

Introduction

Brassica juncea, a well-known plant of family Brassicaceae grown widely as an oil crop is one of the major source of edible oil in India. *Brassica juncea* (2n= 36; AABB) is an amphidiploid derived from chromosome sets of low chromosome number species; *Brassica nigra* (2n= 16; BB) and *Brassica rapa* (2n= 20; AA) (Srivastava *et al.,* 2001). Indian mustard (*Brassica juncea)* is a naturally self-pollinated species but recurrent out crossing occurs in this crop with a percentage of 5 to 30 per cent depending upon the environmental conditions and pollinating insect population. The productivity of these crops is greatly subjective of abiotic stresses such as drought, salinity, frost and heat. Water stress causes serious yield losses in Indian mustard (17-94 %). Drought reduces yield by affecting plant

growth which is a genetic character. Mustard genotypes having drought tolerant traits, performed better under water limited conditions in comparison to genotypes without such traits. Abiotic stresses are known to turn on multigene responses resulting in changes in various proteins, primary and secondary metabolite accumulation. Water is the crucial limiting factor for photosynthesis, growth and net ecosystem productivity of plants in arid ecosystems (Luo *et al.,* 2014). Plants respond to drought stress through a series of physiological, cellular and molecular processes culminating in stress tolerance. Drought tolerance is a quantitative trait involving many genes with cumulative effects.

Breeding for drought tolerance is generally considered slow due to the quantitative and temporal variability of available moisture across years, the low genotypic variance in yield under these conditions, and inherent methodological difficulties in evaluating component traits (Ludlow and Muchow, 1990), together with the highly complex genetic basis of this trait (Turner *et al.,* 2001). Due to complex nature of drought tolerance trait and its laborious screening, there is a need to exploit molecular techniques. The long time to develop improved varieties using the conventional plant breeding methods therefore motivated breeders to find tools that help them achieve goals faster. Therefore, traditional plant breeding has not been successful in producing drought tolerant cultivars therefore, QTL identification and MAS for drought tolerance is of prime importance for developing tolerant varieties of *Brassica* using molecular approaches. Nearly all modern plant breeding relies on molecular markers and they have myriad uses. The advent of various molecular markers has made it possible to assess genetic variability, identify genotypes and perform phylogenetic analysis as well as to devise conservation strategies and perform marker-assisted selection and breeding (Cordoza and Steward, 2004).

Molecular markers have been used to produce genetic maps that represent the genome based on the recombination frequency of the polymorphic markers within a mapping population. Simple sequence repeat SSR/microsatellite markers are simple tandem repeat of di- to tetra-nucleotide sequence motifs flanked by unique sequences. They are valuable as genetic markers because they are co-dominant, detect high levels of allelic diversity and easily and economically assayed by PCR techniques. SSR markers can distinguish different alleles of a locus that make it more powerful. Therefore, SSR markers have become the markers of choice for a wide spectrum of genetic, population, and evolutionary studies (Agarwal *et al.,* 2008). Several researchers have developed the genetic linkage maps of *B. juncea* using various types of molecular markers such as RFLP, RAPD (Sharma *et al.,* 2002), AFLP (Lionneton *et al.,* 2002; Pradhan *et al.,* 2003; Ramchiary *et al.,* 2007). Identification of molecular markers for drought tolerance is difficult task as it influenced by various factors like days to flowering and maturity, early shoot growth vigor, yield, shoot biomass production, rooting depth, root length density, root to shoot ratio, total transpiration, and transpiration efficiency (Varshney *et al.,* 2011). Therefore, dissection of such complex traits into components and identification of tightly linked markers for such traits can enhance the heritability of such traits and facilitate MAS for introgression of these traits into the different genetic backgrounds. Once molecular markers (i.e. for trait QTLs) linked to specific drought tolerance component traits found, it is possible to move them into adapted cultivars or other agronomic backgrounds through marker-assisted breeding. Moreover, identification of QTLs for the key traits responsible for improved productivity under

drought could be helpful in accelerating the process of pyramiding of favourable alleles into adapted genotypes for better production.

The present investigation was done to evaluate the genetic diversity in Indian mustard genotypes for drought tolerance. Genetic diversity analysis will help in introgression of drought tolerant genes into other high yielding cultivars to combat from drought stress.

Materials and Methods

Plant Materials

The parental lines (RB 50 and Kranti) and 157 F_2 progeny lines of *Brassica juncea* were procured from the oilseed section, Department of Genetics & Plant Breeding, CCSHAU, Hisar. All the 157 F_2 lines were selfed to obtain $F_{2:3}$ progeny lines.

Genomic DNA isolation

Genomic DNA was isolated from young leaves using CTAB method (Saghai-Maroof *et al.,* 1984). The precipitated DNA was washed with 70% ethanol and dried overnight at room temperature. The dried pellets were dissolved in T.E. buffer (1M Tris, 0.5M EDTA and pH 8.0). The DNA quality and concentration were checked by electrophoresis in 0.8% agarose gel and UV spectrophotometer

PCR amplification

SSR markers were used to evaluate genetic variability among the Indian mustard genotypes. PCR amplifications were performed using T100TM thermocycler. The total volume of PCR reaction was 20 µl per sample, containing 1 µl DNA, 2 µl of 10X PCR buffer with $MgCl_2$, 0.4 µM each forward and reverse primers (Integrated DNA Technology, India),200 µM dNTP (G Biosciences) and 0.5U *Taq* DNA polymerase (G Biosciences). The PCR tubes were set on the wells of the thermocycler plate. Then, the machine was run accordingly as, initial denaturation at 95°C for 3 min; Denaturation at 94°C for 1 min; Annealing at 50-60°C for 1 min; Extension at 72°C for 1 min; completion of cycling program (40 cycles); Final extension at 72°C for 7 min and reaction were held at 4°C. The amplified products were separated on 6% polyacrylamide gels containing ethidium bromide. Molecular weight marker of 20 bp was run with the PCR products. DNA bands were observed on UVtrans-illuminator in the dark chamber of the Image Documentation System.

Data analysis

For molecular diversity analysis, data was scored as 1 and 0 for each of the SSR locus. The presence of band DNA markers run on agarose/ polyacrylamide gel was taken as one and absence of band was read as zero. The 0/1 matrix was used to calculate similarity genetic distance using simqual'sub-program of software NTSYS–PC (Rohlf, 1990). The resultant distance matrix was employed to construct dendrograms by the un-weighted pair-group method with arithmetic average (UPGMA) subprogram of NTSYS-PC (Numerical Taxonomy System for Personal Computer).

Results and Discussion

Genomic DNA was isolated from the parental and 157 F_2 population plants using standard procedures and agrose gel electrophoresis of isolated DNA was done which showed distinct bands (Fig. 1). Subsequently, a DNA fingerprint database of RB 50 and Kranti was prepared using various SSR markers. Polyacrylamide/agarose gels showing allelic polymorphism for selected markers with parents are shown (Fig. 2). The polymorphic markers were used to screen F_2 population. A

total of 200 SSR markers from different *Brassica* species were used in this study. Out of 200 SSR markers analyzed for polymorphism in two parental *Brassica juncea* genotypes (RB 50 and Kranti), 51 SSR primers (Table 1) were polymorphic. These 51 SSRs were considered reliable due to their codominant nature (Fig. 3).

Similarity coefficient data based on the proportion of shared alleles using 51 SSR markers was used to calculate the coefficient values among the 157 F_2 plants of RB 50 × Kranti and parental *B. juncea* genotypes and subjected to UPGMA tree cluster analysis. The allelic diversity was used to produce a dendrogram (cluster tree analysis, NTSYS-PC), to demonstrate the genetic relationship (Figure 6). All the 157 F_2 plants clustered in two major groups at the similarity coefficient of 0.53. Two parental varieties RB 50 and Kranti had low similarity coefficient. Genetic relationship was also assessed by PCA analysis (NTSYS-PC). Two dimensional and three dimensional PCA scaling exhibited that two parental genotypes were quite distinct whereas all 157 F_2 plants interspersed between the two parental lines with distribution of most plants towards RB 50 (Figure 4 and 5 respectively).

PIC (polymorphic information content value) for various primers in our study led to polymorphism related information about various primers. In our study, the PIC (polymorphic information content) values for various primers ranged from 0.340-0.505 with an average of 0.406. BRMS-027 was found to be the most informative marker depicting the highest PIC value of 0.505; source of this marker is *Brassica rapa*. BRMS019 primer from *Brassica rapa* was found with lowest PIC value of 0.340 (Table 1). Several researchers have used SSR markers for diversity analysis in *Brassica species* (Abbas *et al.,* 2009). In our study, the average PIC values were found to be equal to that of reported by Turi *et al.,* (2012) in *B. juncea* (0.46). Gupta *et al.,* (2014) reported low PIC value 0.281; Sudan *et al.,* (2016) PIC values ranged from 0.12-0.61 with an average to 0.314. PIC values (0.38-0.96) observed by Avtar *et al.,* (2016) were found to be higher than that of our study. Lower number of alleles per locus and lower PIC values may be attributed either to the use of less informative SSR markers, or the presence of lesser genetic diversity among the tested genotypes.

Vinu *et al.,* (2013) evaluated the genetic diversity among 44 Indian mustard (*Brassica juncea*) genotypes including varieties/ purelines from different agro-climatic zones of India and few exotic genotypes (Australia, Poland and China). A and B genome specific SSR markers were used and phenotypic data on 12 yield and yield contributing traits was recorded. Out of the 143 primers tested, 134 reported polymorphism and a total of 355 alleles were amplified.

Molecular markers have been successfully employed for QTL mapping of drought tolerance. It has provided several dozen target QTLs in *Brassica* and the closely related *Arabidopsis* (Hall *et al.,* 2005). Many drought or salt-tolerant genes have also been isolated, like *BrERF4, BnLAS* and *AnnBn1* fordrought and salinity tolerance in *Brassica rapa* and *Brassica napus* respectively, some of which have been confirmed to have great potential for genetic improvement for stress tolerance (Zhang *et al.,* 2014).

In the present study, DNA fingerprint database of 157 RB50 x Kranti F_2 plants representing the drought and its related traits variation was prepared using 51 polymorphic SSR markers. The NTSYS-pc UPGMA tree cluster analysis and two dimensional PCA scaling exhibited that two parental genotypes were quite distinct and diverse, whereas 157 F_2 plants were

interspersed between the parental *B. juncea* genotypes. This also indicates that the population is ideal for linkage mapping and QTL identification.

Thakur *et al.,* (2018) used SSR markers to unravel genetic variations in *Brassica* species. 100% cross transferability was obtained for *B. juncea* and three subspecies of *B. rapa*, while lowest cross-transferability was (91.93) was obtained for *Eruca Sativa*. The average percentage of cross-transferability across all the seven species was 98.15%. Neighbour-joining-based dendrogram divided all the 40 accessions into two main groups composed of *B. Carinata*/*B. napus*/*B. Oleoracea* using SSR primers. Our studies also clustered all the 157 F_2 plants in two major groups at the similarity coefficient of 0.53. Two parental varieties RB 50 and Kranti had low similarity coefficient. Genetic relationship was also assessed by PCA analysis (NTSYS-PC).

Table.1 DNA polymorphism in RB50 and Kranti varieties of Indian mustard (bp) used

Sr. No.	SSR Marker name	Marker source	PIC Value	No. of alleles	Amplified fragment size (bp)	
					RB50	Kranti
1	Ni4-F11	*B. nigra*	0.47	2	170	160
2	BRMS-037	*B. rapa*	0.49	2	125	120
3	BRMS-056	*B. rapa*	0.47	2	220	215
4	BRMS-048	*B. rapa*	0.46	2	180	185
5	BRMS-003	*B. rapa*	0.47	2	160	155
6	BRMS-005	*B. rapa*	0.46	2	150	155
7	BRMS-006	*B. rapa*	0.39	2	170	165
8	BRMS-008	*B. rapa*	0.50	2	120	115
9	BRMS-011	*B. rapa*	0.47	4	205	200
10	BRMS-015	*B. rapa*	0.50	2	140	145
11	BRMS-017	*B. rapa*	0.48	2	170	165
12	BRMS-018	*B. rapa*	0.50	2	140	135
13	BRMS-020	*B. rapa*	0.48	2	130	125
14	BRMS-027	*B. rapa*	0.505	2	225	230
15	BRMS-029	*B. rapa*	0.48	2	240	245
16	BRMS-031	*B. rapa*	0.44	2	180	185
17	BRMS-042	*B. rapa*	0.45	2	125	120
18	SSR Na10-B04	*B. rapa*	0.49	2	260	262
19	SSR Na12-D03	*B. rapa*	0.40	2	120	115
20	BRMS019	*B. rapa*	0.34	3	120	115

21	BRMS040	*B. rapa*	0.42	2	200	195
22	BRMS043	*B. rapa*	0.46	3	300	290
23	BRMS051	*B. rapa*	0.48	2	260	250
24	BRMS026	*B. rapa*	0.46	2	250	252
25	Br_Genomic664	*B. rapa*	0.49	2	190	180
26	Br_Genomic935	*B. rapa*	0.50	2	185	190
27	Br_Genomic946	*B. rapa*	0.50	2	160	155
28	GSS_Bn606	*B. rapa*	0.44	2	140	130
29	GSS_Bn622	*B. rapa*	0.49	2	170	180
30	GSS_Bn624	*B. rapa*	0.47	2	180	190
31	GSS_Bn629	*B. rapa*	0.43	2	190	180
32	U_Brapa421	*B. rapa*	0.44	2	160	155
33	U_Brapa244	*B. rapa*	0.47	2	260	250
34	ENA2	*B. rapa*	0.50	2	240	245
35	ENA6	*B. rapa*	0.47	2	120	115
36	ENA14	*B. rapa*	0.47	2	200	210
37	ENA28	*B. rapa*	0.49	2	300	290
38	EJU4	*B. rapa*	0.44	2	290	280
39	BRMS001	*B. rapa*	0.50	2	120	110
40	Br_Genomic697	*B. rapa*	0.49	2	200	195
41	BN_3F027	*B. rapa*	0.50	2	155	160
42	BN_3F132	*B. napus*	0.43	2	135	130
43	BN_3F003	*B. napus*	0.46	2	155	150
44	BN_3F170	*B. napus*	0.41	2	145	140
45	GSS_Bn583	*B. napus*	0.40	2	150	140
46	ENA19	*B. napus*	0.40	3	240	245
47	ENA10	*B. napus*	0.39	2	380	370
48	ENA9	*B. napus*	0.42	2	480	500
49	SSR Na12-H09	*B. napus*	0.41	2	255	250
50	SSR Na14-D09	*B. napus*	0.42	2	260	250
51	SSR Na14-G06	*B. napus*	0.40	2	120	110

Fig.1 Agarose gel showing genomic DNA of parents and 1-37 plants of RB50 x Kranti F2 plants L-lamda DNA, P1-RB50, P2-Kranti

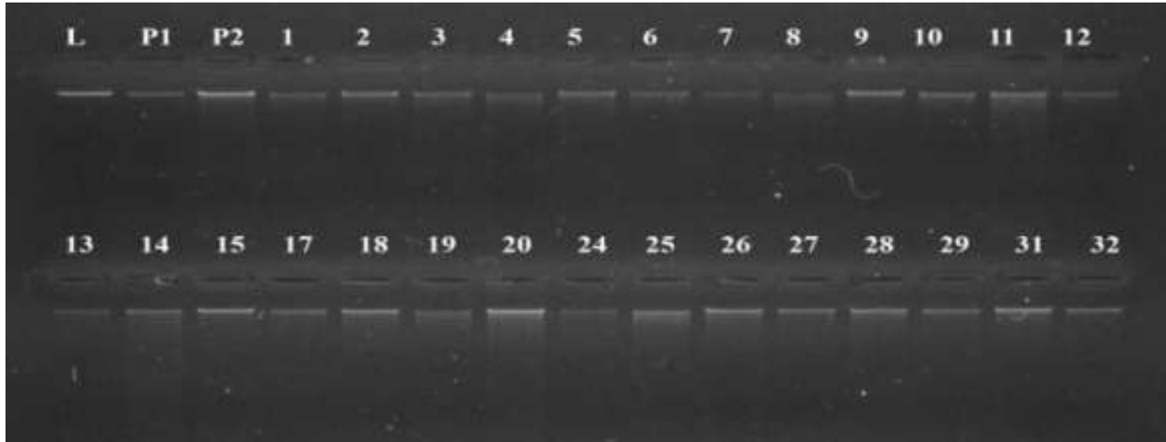


Fig.2 Polyacrylamide gel showing polymorphism among parents P1-Parent 1 (RB50), P2-Parent 2 (Kranti) and Lane L-20 bp ladder

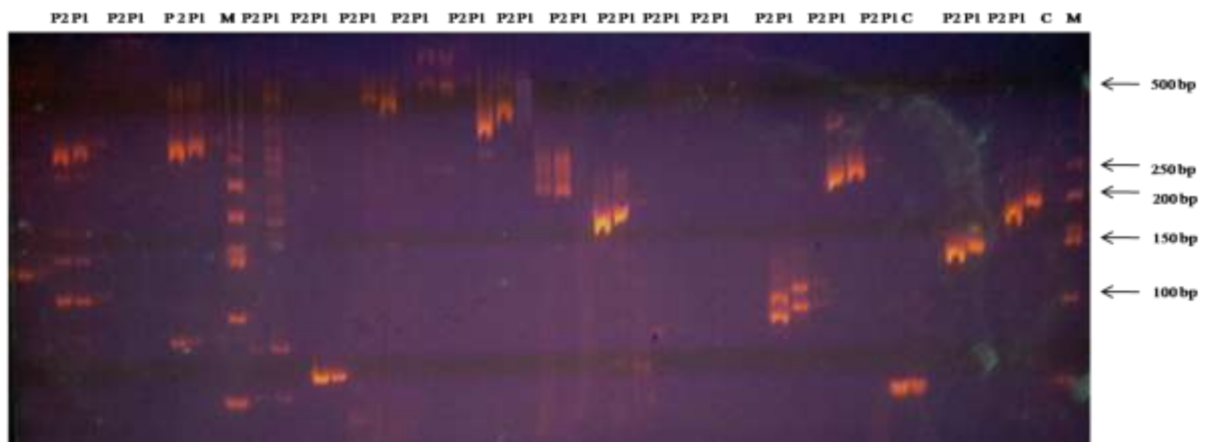


Fig.3 Polyacrylamide gel showing allelic polymorphism among F2 plants at BRMS-056 locus. Lane L-20 bp ladder, 1-42 F2 plants P1-RB50, P2-Kranti

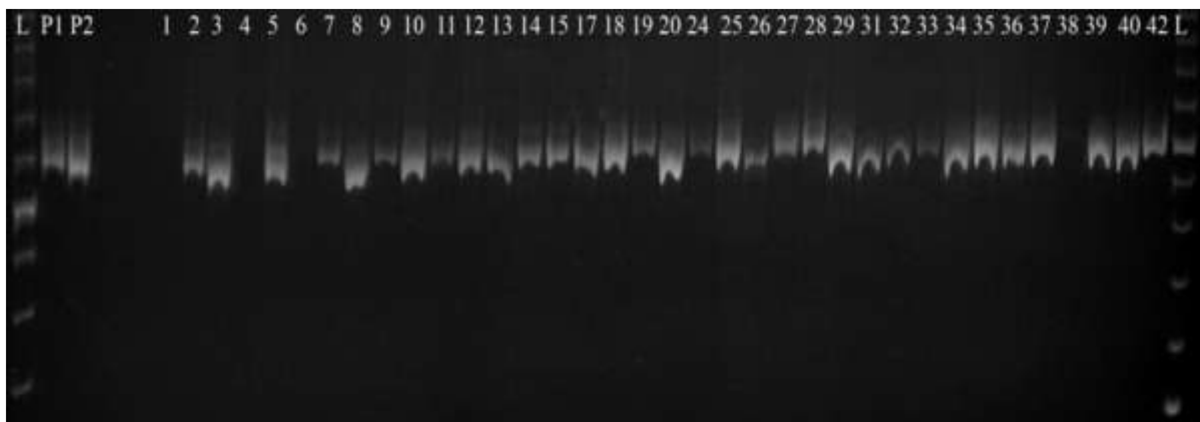


Fig.4 Two dimensional PCA scaling of 157 RB50 x Kranti F2 plants and parental genotypes based on SSR diversity analysis in Indian mustard

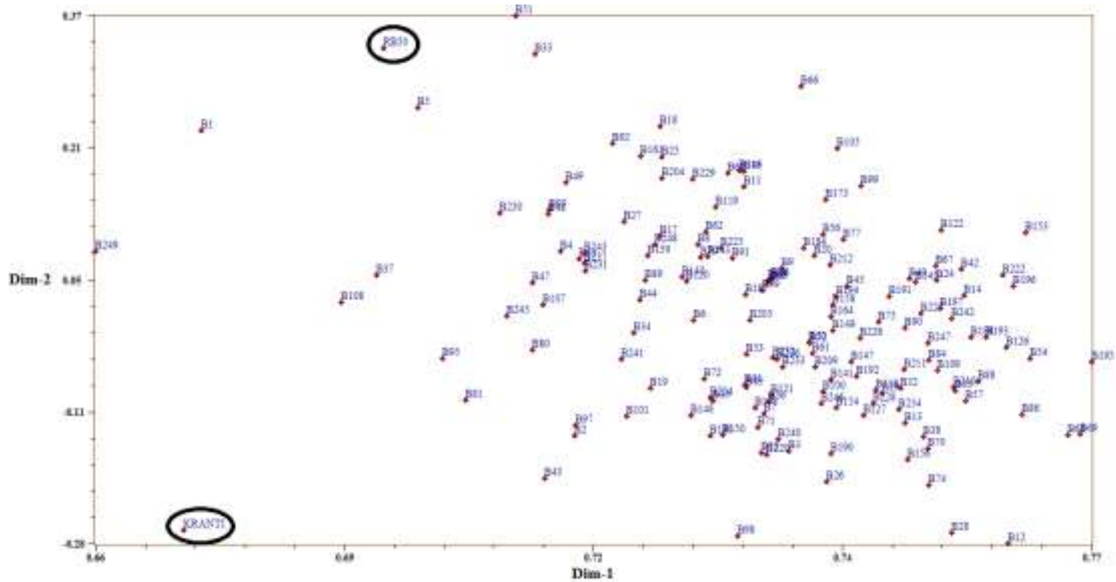


Fig.5 Three dimensional PCA scaling of 157 RB50 x Kranti F2 plants and parental genotypes based on SSR diversity analysis in Indian mustard

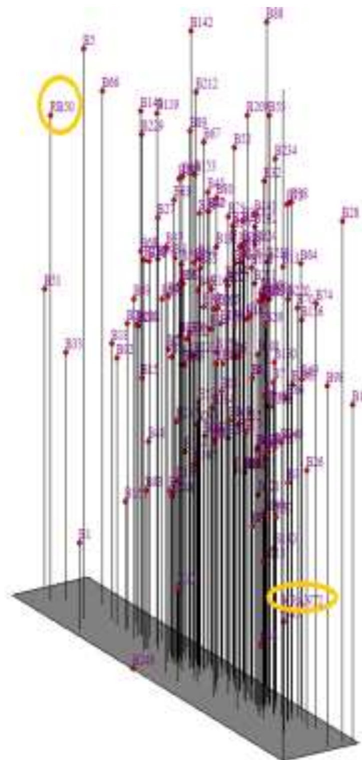
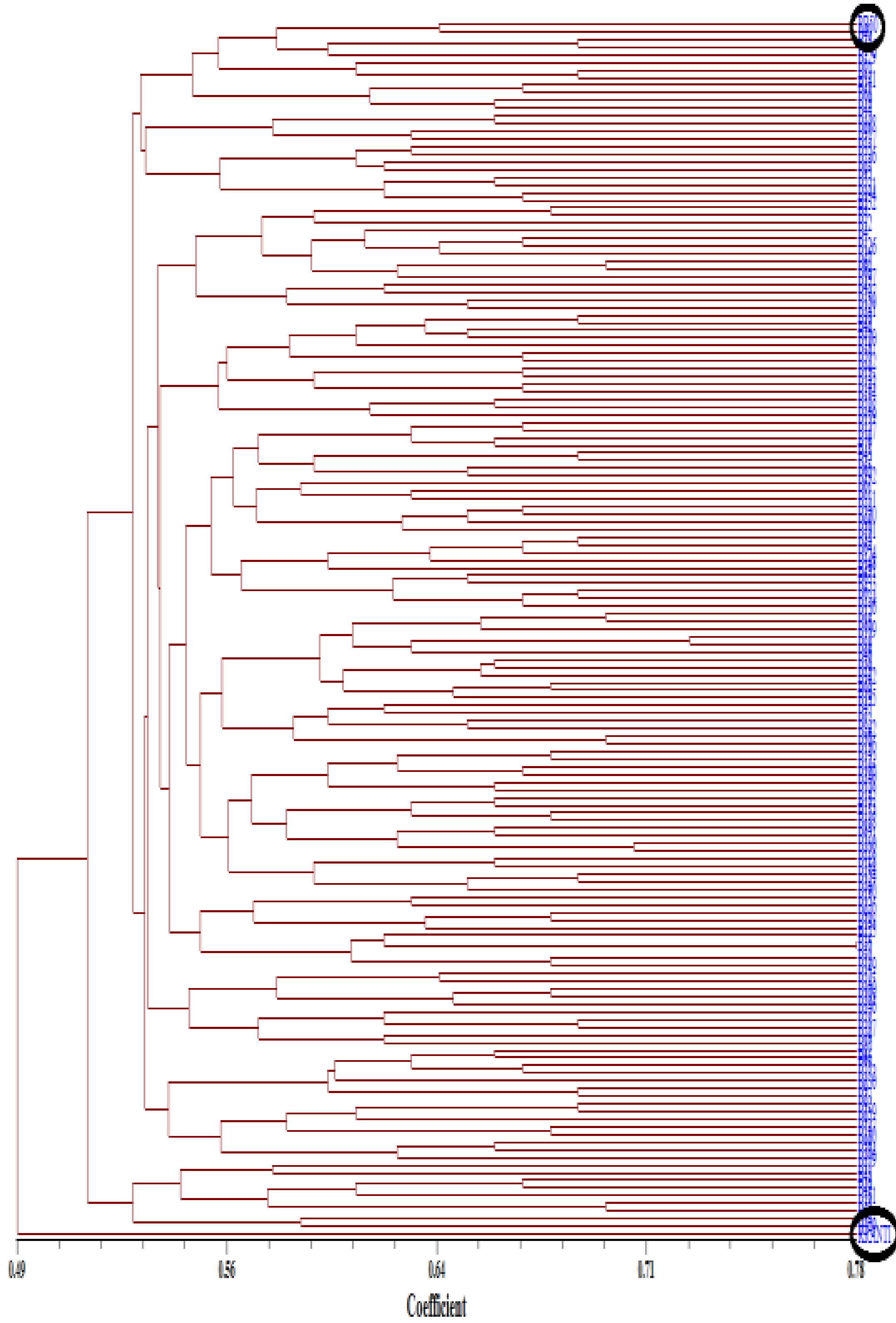


Fig.6 Dendrogram (NTSYS pc, UPGMA) of 157 RB50 x Kranti F2 plants and parental genotypes based on SSR diversity analysis in Indian mustard



Genetic diversity analysis was performed among F_2 plants of the cross RH 30×CS 52 in Indian mustard (*Brassica juncea*) (CS 52 is salinity tolerant and RH 30 is salinity susceptible) using SSR markers. Out of 358 SSR markers, 42 were found polymorphic and 154 were monomorphic.

A total of 225 alleles, ranging from 2 to 4 were amplified. The PIC (Polymorphic Information Content) value ranged from 0.427-0.730 of Jaccard's similarity coefficients was generated between these F_2 populations (Patel *et al.,* 2018). Present study also showed 51 polymorphic primers out of 200 used for polymorphism analysis with total alleles 108 in F_2 population of *Brassica juncea*.

In conclusion, a total of 200 SSR markers from different *Brassica* species (87 from *Brassica rapa*, 88 from *B. napus,* 4 from *Brassica nigra,* 8 from *Brassica oleoracea* and 13 from *Arabidopsis)* were used to screen parental genotypes (RB50 and Kranti) in this study. Out of 200 SSR markers analyzed for polymorphism in two parental *B. juncea* genotypes (RB 50 and Kranti), 51 (25.5 %) were polymorphic.

Subsequently, a DNA fingerprint database of 150 RB50 x Kranti F_2 plants using 51 SSR (40 from *B. rapa*, 10 from *B. napus* and 1 from *B. nigra*) markers to assess the genetic diversity. Diversity analysis by NTSYS-PC software program showed widely diverse nature of both the parental genotypes and all the progeny lines were interspersed between the parents (RB 50 and Kranti) showing wide diversity in population. The population was screened with co-dominant subset of 51 putative polymorphic SSRs. Data for SSR markers was obtained in the form of ABH scoring which can be then used for map construction and QTL analysis for further cultivar development and analysis in *Brassica* species.

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How to cite this article:

Monika, Ram C. Yadav, Neelam R. Yadav, Summy, Ram Avtar and Dhiraj Singh. 2019. Genetic Diversity Analysis for Drought Tolerance in Indian Mustard (*B. juncea* L. Czern & Coss) using Microsatellite Markers. *Int.J.Curr.Microbiol.App.Sci.* 8(01): 2564-2574.
doi: https://doi.org/10.20546/ijcmas.2019.801.269