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GENETIC STRUCTURE OF PAKISTANI OILSEED *BRASSICA* CULTIVARS REVEALED BY MORPHOMETRIC AND MICROSATELLITE MARKERS

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Abstract

Brassicaceae is a diverse family of economically important crop plants grown worldwide. The rapeseed and mustard are traditional oilseed crops and are being cultivated in Pakistan for centuries. The present study reports genetic divergence among Pakistani oilseeds Brassica cultivars using morphometric and microsatellite markers. Plant material comprised of 31 cultivars of four different Brassica species i.e. *Brassica carinata*, *B. juncea*, *B. napus* and *B. rapa* including two exotic cultivars. All the cultivars were evaluated under field and laboratory conditions. Significant morphological variation was observed for quantitative traits e.g. days to flowering initiation & completion, days to maturity, leaf size, plant height, 1000-seed weight, leaves per plant, primary branches per plant, main raceme length, silique length and seeds per silique etc. A significant positive association was found between 1000-seed weight, days to flowering initiation and plant height. Seed yield was positively correlated to period of flowering and positively, but non-significant correlation was observed with plant height and number of primary branches per plant. Cultivars, DGL, Pakola, NARC-22, Takwara, Shiralee, Con-III, Dure-NIFA, Abasin-95, Dunkeld, Con-I, Con-II, Chakwal-Sarson and PakCheen-89 were characterized as cultivars having the highest number of siliquae on main raceme with the largest and highly productive siliquae. The PCR-based DNA polymorphism among 30 cultivars revealed significant genetic variation and depicted dissimilarity among cultivars except Dacca-raya and Sultan-raya. Cultivars were classified into 2 major groups and 4 small sub-groups according to their morphological characteristics, origin of cultivars (institute/ research station) and related species. Cultivars like Khanpur-raya, Bahawalpur-raya and Toria-sathi formed no association with other cultivars and positioned independently in the dendrogram.

Keywords: *Brassica* oilseeds, Morphology, Microsatellites, Pakistani cultivars, Polymorphism

Introduction

The genus Brassica is composed of important oilseeds, vegetables, and forage crops. Having been cultivated for different agricultural uses, Brassica is considered as one of the diverse group of plant species with considerable global and economic importance. The Brassica includes some of the most nutritious crops i.e. broccoli, cabbage, cauliflower, kale, kohlrabi, brussel sprout, turnip, etc. The oilseed rape (*B. napus*) is considered as the most important edible oilseed species after soybean and one of the predominantly grown members of the family *Brassicaceae* (Liu *et al.,* 2016). According to archeological records practice of cultivation of brassica plants can be traced back to 1500 BC (Prakash, 1980). Also, Yan (1990) reported evidence suggesting cultivation of *Brassicaceae* crops as old as 5000 BC. U (1935) proposed the evolution of major crop species i.e. *B. carinata*, *B. napus* and *B. juncea* from three progenitor species, *B. rapa*, *B. nigra* and *B. oleracea.* These relatively recent species, i.e. *B. napus* (AACC), *B. juncea (*AABB) and *B. carinata*, (BBCC) are amphidiploids hybrid species, evolved through inter-specific hybridization between monogenomic diploids (U, 1935; Burges *et al*., 2006). This genetic variation has augmented the morphotype diversity in edible *Brassica* species and elevated its usage as a food in different forms like roots, stem, leaf, flower and seeds (Zhao *et al.,* 2007). In major species the enormous amount of genetic divergence is desirable for developing new cultivars (Iqbal *et al.,* 2014). However, for efficient crop improvement it is pre-requisite to elucidate genetic diversity within the genetic pool of the given crop and also to identify elite genotypes in the population for effective utilization in the crop improvement (Jan *et al.,* 2017[a]; Baig *et al.,* 2018; Jan *et al.,* 2018).

The assessment of genetic variation can either be directed for directly selecting genotypes with desirable phenotype or to be utilized as parent in hybrids development. The information on genetic diversity is also useful for crop preservation strategies, gene bank management, designing breeding programs, and solving taxonomic problems (Khan *et al.,* 2014). Germplasm collection evaluation for genetic diversity may reveal important facts about evolutionary history of the family such as finding new alleles at specific loci and also aid in the development of perfect marker alleles from QTLs or genes of interest. These genes or QTLs could then be introgressed into new varieties along with other linked desirable traits (Redden *et al.,* 2009). There is wide genetic diversity in the wild relatives of brassica crops which needs to be explored as potential source of pre-breeding. Tapping the genetic diversity for these wild broad genetic based populations of Brassica may offer new alleles for biotic and abiotic stress resistance (Jan *et al.,* 2016).

Plant geneticists employ various strategies to study genetic variation and relationship within members of plant populations, but morphological evaluation is regarded as initial step of description and classification (Arif *et al.,* 2015; Jan *et al.,* 2017[b]). Agronomic traits and numerical taxonomic techniques have been used for species classification and study of the genetic relationship among and within species and germplasm collection of crops (Gomez-Campo & Tortosa, 1974; Takahata & Hinata, 1986). Traditionally, agronomic, morphological and protein markers have been used as criteria to determine extent of genetic variation in crop plants (Shinwari *et al.,* 2011). Recently, molecular markers have revolutionized identification and characterization of novel germplasm (Channa *et al.,* 2018; Shinwari *et al.,* 2018). Molecular markers are unique DNA landmarks found in the genome at specific regions. These are genomic segments transmitted from one generation to next following the standard laws of inheritance in living organisms (Semagn *et al*., 2006). A vast array of these markers systems have been utilized by geneticists to study the genetic variation, mechanism of evolutionary pathways, genotyping, trait association and marker assisted selection in many crops. Some of the popular marker types include but not limited to; Restriction Fragment length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), Single Nucleotide Polymorphism (SNP), etc (Song *et al*., 1988; Divaret *et al*., 1999; Lombard *et al*., 2000; Plieske & Struss, 2001; Pradhan *et al*., 2003). SSR also known as microsatellite markers are short nucleotide tandem repeats of 1-6 bp found in the genomes of higher organisms that can be repeated up to 100 times in a genome. These markers show higher polymorphism both at inter and intra-specific level when number of repeats is ten or greater than ten (Queller *et al*., 1993). SSR markers have been utilized in number of ways including association mapping, varietal identification and genetic divergence estimation (Gupta & Varshney, 2000). Eujayl *et al*., (2001) has suggested that SSR markers are better than other marker system to investigate genetic variation. In the present study, we employed both morphometric and SSR markers to investigate genetic variation among 31 different oilseed brassica cultivars.

Materials and Methods

Phenotypic analysis: The oilseed *Brassica* cultivars used in the study comprised of 31 Pakistani cultivars of oilseed brassica species i.e. *Brassica carinata, B. juncea, B. napus* and *B. campestris* (Table 1). The germplasm was planted in an augmented field design at PGRI, NARC Islamabad in October 2015. The row length was 5m with 45cm space between rows. Each variety was planted in 6 adjacent rows where intra-variety distance was 30cm and inter-variety distance was kept at 60cm. To obtain healthy and rigorous plants, recommended agronomic cultural practices were followed from the plantation stage to the maturity of last genotype. The crop was not sprayed for any pesticide and also no fertilizer treatment was carried out. To record data for agronomic traits, 5 randomly selected competitive plants were tagged for each genotype and data were collected for 19 quantitative traits i.e. days to flowering initiation (DFI), days to 50% flowering (D50%), days to flower completion (DFC), period of flowering (PF), days to maturity (DM), leaf length (LL), leaf width (LW), leaf length/leaf width ratio (LL/LW), leaves per plant (LPP), plant height (PH), primary branches per plant (PBP), main raceme length (MRL), siliquae on main raceme (SMR), silique length (SL), silique width (SW), silique length/width ratio (SL/SW), seed per silique (SS), 1000 seeds weight (HSW), seed yield per plant (SYP).

Microsatellite Analysis: Seeds of all the morphologically characterized genotypes except Raya-2000 were grown in pots under greenhouse conditions. For isolation of total genomic DNA 2-3 fresh leaves of each genotype were used following Fulton *et al*., (1995) microprep DNA extraction protocol. A set of 20 microsatellite primer pairs was employed to screen 30 cultivars of 4 *Brassica* species for investigating marker polymorphism. After short screening, 11 primers were selected for estimating molecular marker polymorphism in cultivars (Table 4). Altogether, 11 primers included 7 from BRMS series i.e. BRMS 019, BRMS 020, BRMS 033, BRMS 036, BRMS 037, BRMS040, BRMS 042-2 (Suwabe *et al*., 2002), 3 Na12BO9, Na12DO4, Na12HO2 (Lowe *et al*., 2002) and Ni4B10 (unknown) amplified alleles and produced easily score-able banding pattern in brassica 30 varieties.

PCR (Polymerase Chain Reaction) was carried out in 0.5ml PCR strip tubes containing 25 µl of reaction mixture including 25ng of sample DNA, 1x PCR buffer, 0.2mM each dNTPs, 0.5µM of forward and reverse primer, 2mM $MgCl_2$ and 1 unit of *Taq* Polymerase (Fermentas). Amplification reactions were performed in Veriti thermocycler (Model # 9902 Applied Biosystems Inc, USA) and reaction profile was programmed as follows: 1 cycle (4 minutes) at 94ºC for separating strands, followed by 35 cycles of 40 seconds at 94ºC to denature the strands, 40 seconds at 55ºC for annealing, 1 minute at 72ºC for primer extension, 2 minutes at 72ºC for terminal extension and followed by soaking at 4ºC. PCR primers used are shown in Table 4. After amplification, PCR products were resolved on a 2% agarose gel with 1xTBE as electrode buffer in a horizontal electrophoresis chamber. Gene ruler (Fermentas) was used as fragment size standard stained with ethidium bromide and bands were visualized under UV geldoc apparatus (Bio-Rad).

Table 1. Oilseed *Brassica* cultivars used during present study.

No.	Cultivar name	Accession	Brassica species	Origin
1.	RL-18	22862	*Brassica juncea*	AARI, Faisalabad
2.	Bahawalpur-raya	22852	-do-	RARI, Bahawalpur
3.	S-9	19509	-do-	ARI, Tandojam
4.	Early-raya	23680	-do-	ARI, Tandojam
5.	Khanpur –raya	19510	-do-	ORS, Khanpur
6.	NIFA-raya	22854	-do-	NIFA, Peshawar
7.	BARD-1	19493	-do-	NARC, Islamabad
8.	Sultan-raya	19511	-do-	Unknown
9.	Dacca-raya	1676	-do-	Bangladesh
10.	Raya Anmol-87	22860	-do-	AARI, Faisalabad
11.	Poorbi-raya	1673	-do-	AARI, Faisalabad
12.	Canola-mustard	23695	-do-	NARC, Islamabad
13.	Raya-2000	24271	-do-	AARI, Faisalabad
14.	Chakwal-raya	22850	*Brassica carinata*	BARI, Chakwal
15.	Peela-raya	22863	-do-	AARI, Faisalabad
16.	DGL	22858	*Brassica napus*	AARI, Faisalabad
17.	Pakola	27452	-do-	NARC, Islamabad
18.	NARC-22	1323	-do-	NARC, Islamabad
19.	Dure-NIFA	22856	-do-	NIFA, Peshawar
20.	Shiralee	23633	-do-	Australia
21.	Dunkeld	23634	-do-	Australia
22.	CON-I	24172	-do-	NARC, Islamabad
23.	CON-II	24173	-do-	NARC, Islamabad
24.	CON-III	24193	-do-	NARC, Islamabad
25.	Abasin-95	22855	-do-	NIFA, Peshawar
26.	Chakwal-Sarson	22851	-do-	BARI, Chakwal
27.	PakCheen-89	23632	-do-	ARI, Mingora, Swat
28.	Takwara	22853	-do-	ARI, Dera Ismail Khan
29.	Toria-A	23630	*Brassica rapa*	AARI, Faisalabad
30.	BSA	23631	-do-	AARI, Faisalabad
31.	Toria-Sathi	22861	-do-	AARI, Faisalabad

Note: AARI: Ayub Agricultural Research Institute; ARI: Agricultural Research Institute; BARI: Barani Agricultural Research Institute; NIFA: Nuclear Institute for Food & Agriculture; ORS: Oilseeds Research Station; NARC: National Agricultural Research Centre; RARI: Regional Agricultural Research Institute.

Statistical analysis

For morphological variation, mean values of each character were used for descriptive statistical analysis as per the procedure of Adair *et al*., (1973). Data of quantitative traits were utilized for cluster analysis according to Sneath & Sokal, (1973). Euclidean distance matrix was computed to evaluate the relationship between the entries. Likewise, Euclidean distances based dendrogram was generated using complete linkage method by statistical package NTSys PC v 2.1. For molecular analysis, each band/amplified allele was scored as 1 for 'presence' and 0 for 'absence' for each primer. The binary data was then used to generate similarity coefficients both for unique or common bands (alleles). Dice algorithm was used for calculating genetic similarity estimates (F) among all the cultivars which is similar procedure to Nei & Li (1979) i.e. Similarity (F) = 2Nab/(Na+Nb). UPGMA-based cluster analysis was performed and dendrogram was generated using these similarity coefficients, to study relationship among cultivars.

Population structure and cultivars' interrelatedness was inferred through Bayesian approach using software package STRUCTURE v 2.2 (Pritchard *et al*., 2000). As prior information on the pedigree was not available, admixture model was used implying that cultivars night had common ancestry as well as independent allele frequencies in subpopulations. The *k* or number of subpopulations was set from 1 to 10, while 10 runs were performed for each value (1-10). A burn-in of 200,000 iterations was followed, for each run, by additional 300,000 iterations for minimizing the effect of the starting configuration. The results were submitted to online platform STRUCTURE HARVESTER (Earl & Bridget, 2012) for calculating *k* value with the highest likelihood which corresponds to the number of subpopulations.

Table 2. Basic statistics for 19 quantitative traits of oilseed brassica cultivarscharacterized during 2015-16 under field conditions.

Trait	Mean ± SD	Range	CV(%)	Variance
Days to flower initiation (DFI)	90.5 ± 17.9	54 – 126	19.8	321.3
Days to 50% flowering (DF)	103.9 ± 17.3	68 – 141	16.7	300.8
Days to flower completion (DFC)	121.2 ± 12.0	99 – 157	9.9	143.4
Period of flowering (PF)	30.7 ± 8.2	22 – 48	26.6	66.9
Das to maturity (DM)	156.4 ± 12.2	140 – 197	7.8	148.9
Leaf length (LL)	8.7 ± 0.7	7.3 – 9.6	8.1	0.5
Leaf width (LW)	3.9 ± 0.6	2.7 – 5.0	14.5	0.3
Leaf length/width ratio (LL/LW)	2.3 ± 0.2	1.9 – 2.9	9.8	0.1
Leaves/plant (L/P)	11.1 ± 1.6	7.4 – 14.4	14.7	2.7
Plant height (PH)	151.9 ± 22.2	120 – 208	14.6	493.2
Primary branches/plant (PB/P)	9.6 ± 1.5	7.4 – 13.0	15.4	2.2
Main raceme length (MRL)	61.6 ± 7.5	49.2 – 81.4	12.2	56.5
Siliquae on main raceme (SMR)	43.1 ± 9.6	23.0 – 62.4	22.2	91.3
Silique length (SL)	51.5 ± 13.0	29.1 – 72.8	25.2	168.0
Silique width (SW)	2.9 ± 0.7	2.2 – 4.6	23.5	0.5
Silique length/width ratio (SL/SW)	17.7 ± 3.3	9.9 – 23.1	18.9	11.2
Seeds/silique (S/S)	14.1 ± 2.8	10.3 – 18.9	20.0	7.9
1000-seed weight (1000-SW)	5.1 ± 1.1	3.1 – 7.7	21.2	1.2
Seed yield/plant (SY/P)	9.0 ± 3.4	2.8 – 16.8	37.6	11.5

Results

Agro-morphological diversity: A considerable level of intra- and inter-specific variation was observed for majority quantitative traits of brassica cultivars. Pattern and extent of variation among the genotypes was different for various phenotypic characters. The largest variability was observed for days to flowering, days to maturity, pods/main raceme, plant height and silique length, whereas a low variation was detected in leaf size, primary branches/plant, pod size, 1000-seed weight etc. (Table 2). Pattern of variation among the cultivars of 4 brassica species was dissimilar for different traits of economic interest. Cultivars belonging to *B. juncea* and *B. rapa* groups were earlier in flowering and physiological maturity, *B. napus* was intermediate, while *B. carinata* cultivars were the latest in flowering and reaching maturity.

Correlation studies: For all 31 genotypes, correlation was observed among 19 quantitative characters. Some traits showed highly significant association with other characters (Table 3). Highly significant positive correlation was observed between days to flowering initiation and days to flower completion (0.93**), days to maturity (0.72**), and 1000 seed weight (0.56**). Also, DFI had positive correlation with leaf width (0.36**) and plant height (0.42**). Similarly, significant positive correlation was found between days to maturity and leaf width (0.46**), plant height (0.60**) and primary branches per plant (0.45*). Plant height was observed to have highly significant positive association with primary branches/plant (0.61**) and significant positive correlation with main raceme length (0.35*). Number of primary branches/plant was highly significant positively correlated with characters e.g. silique length (0.65**), seeds per silique (0.62**) and main raceme length (0.48*). Main raceme length was found to have highly significant positive association with siliquae on main raceme (0.55**) while significantly positively correlated with silique length (0.48*). Highly significant positive correlation was found between number of siliquae on main raceme and silique length (0.57**), silique width (0.43*) and seeds per silique (0.51**). Silique length was highly significant positively correlated with seeds/silique (0.78**) and silique width (0.64**). Similarly, silique width was found to be highly significant and positively correlated with seeds/silique (0.57**) (Table 3).

Cluster analysis: A Euclidean distance based dendrogram was generated to classify 31 cultivars into group of similar characteristics (Fig. 1). The first group of cluster analysis comprised of 11 cultivars of which 3 cultivars, RL-18, Early-raya and NIFA-raya fall in the Cluster I of the first group having close resemblances, while the second cluster included Khanpur-raya, Sultan-raya, Dacca-raya, Raya-2000, BARD-1, Bahawalpur-raya, S-9 and Canola-mustard. These 11 cultivars, clustered in the first group were characterized with less seeds/silique (11.4), the smallest number of siliquae per main raceme (34.9), minimum number of primary branches/plant (8.3) having shortest main raceme (57.1 cm) with smallest siliques (38.4 mm).

Table 3. Correlation coefficients among pairs of agro-morphological traits of brassica cultivars.

Trait	DFI	DF	DFC	PF	DM	LL	LW	LL/W	L/P	PH	PB/P	MRL	S/MR	SL	SW	SL/W	S/S	TSW	SY/P
DFI	1.00																		
DF	1.00**	1.00																	
DFC	0.93**	0.94**	1.00																
PF	-0.83**	-0.80**	-0.57**	1.00															
DM	0.72**	0.74**	0.87**	0.31	1.00														
LL	0.22	0.33	0.35	-0.01	0.28	1.00													
LW	0.36*	0.24	0.51**	-0.07	0.46**	0.77**	1.00												
LL/W	-0.31	0.41*	0.09	0.09	-0.38*	-0.33	-0.85**	1.00											
L/P	0.11	-0.35	0.14	0.14	0.41*	-0.42*	-0.03	-0.25	1.00										
PH	0.42*	0.15	0.52**	-0.17	0.60**	-0.02	0.32	-0.39*	0.53**	1.00									
PB/P	0.26	0.47**	0.39*	-0.02	0.45*	-0.33	-0.04	-0.13	0.92**	0.61**	1.00								
MRL	0.05	0.32	0.02	-0.08	0.05	-0.50**	-0.35	0.13	0.52**	0.35	0.48**	1.00							
S/MR	-0.21	0.06	-0.34	-0.05	-0.38*	-0.63**	-0.53**	0.23	0.28	-0.06	0.24	0.55**	1.00						
SL	0.00	-0.19	0.01	-0.04	-0.07	-0.54**	-0.28	0.02	0.65**	0.08	0.65**	0.48**	0.57**	1.00					
SW	-0.05	0.05	-0.13	-0.14	-0.31	-0.40*	-0.27	0.10	0.14	-0.18	0.19	0.16	0.43*	0.64**	1.00				
SL/W	0.10	-0.02	0.10	0.10	0.33	-0.23	-0.05	-0.08	0.66**	0.30	0.67**	0.41*	0.22	0.61**	-0.24	1.00			
S/S	-0.28	0.13	-0.27	0.18	-0.28	-0.52**	-0.35	0.10	0.61**	0.05	0.62**	0.33	0.51**	0.78**	0.57**	0.38*	1.00		
TSW	0.56**	-0.24	0.50**	-0.59**	0.34	0.10	0.14	-0.09	-0.04	0.27	0.08	0.06	-0.08	0.12	-0.01	0.20	-0.18	1.00	
SY/P	-0.25	0.60**	-0.03	0.48**	0.07	0.23	0.38*	-0.28	0.12	0.21	0.09	-0.18	-0.29	-0.06	-0.16	0.16	0.01	-0.01	1.00

Note: DFI (Days to flower initiation), DF (Days to 50% flowering), DFC (Days to flower completion), PF (Period of flowering), DM (Days to maturity), LL (Leaf length), LW (Leaf width), LL/W (Leaf length/width ratio), L/P (Leaves/plant), PH (Plant height), PB/P (Primary branches/plant), MRL (Main raceme length), S/MR (Siliquae/main raceme), SL (Silique length), SW (Silique width), SL/W (Silique length/width ratio), S/S (Seeds/silique), TSW (1000-seed weight), SY/P (Seed yield/plant).

The second major group with 13 cultivars was further sub-divided into 2 sub-clusters. Cluster 1 had DGL, Pakola, NARC-22, Takwara, Shiralee and Con-III, while sub-cluster 2 had Dure-NIFA, Abasin-95, Dunkeld, Con-I, Con-II, Chakwal-Sarson and PakCheen-89. The second group had cultivars having maximum main raceme length (66.5 cm), the highest number of siquae on main raceme (51.3) with the largest (63.9 mm) and highly productive siliques (16.2 seeds/silique). The third group comprised of Raya-Anmol, Poorbi-raya, Toria-A, Toria-Sathi and BSA. These genotypes had the shortest flower completion period (102 days), early maturing (145 days), with short stature plants (140 cm) and higher seed yield (12.4g/plant). The fourth group had only 2 members i.e. Chakwal-raya and Peela-raya. These two cultivars were distinct from the other cultivars. These two cultivars had vigorous vegetative growth with maximum days to maturity (late maturing, 195.5 days), having the highest number of leaves/plant (14.1). These cultivars had maximum height (200cm) healthy seeds and the highest grain yield (13.7g/ plant) (Fig. 1).

Microsatellite studies: Fig. 2 shows the amplification profiles of 30 cultivars of Brassica oilseeds generated by marker BRMS 019. A total of 325 bands for 21 loci were detected by 11 pairs of SSR primers for thirty Pakistani brassica cultivars. Seventeen loci were found to be polymorphic (80.95%). The alleles number ranged from 1-3 bands per primer with average of 1.90 bands per primer among 30 cultivars. Of 11 primers used in the study 7 belonged to BRMS series of brassica microsatellite markers developed by Suwabe *et al*., (2002). These primers elucidated significant polymorphism regarding allele's sizes, presence/ absence or both. However, primers like BRMS-019, BRMS-033, and BRMS-040 also provided information weather the allele was homozygous or heterozygous. The product size of the BRMS primers ranged from 120bp (Na12Bo9) to 300bp (BRMS-019) (Table 4).

Besides BRMS series, 4 genome specific primers were also employed to detect polymorphism among cultivars. Na12DO4 'A' genome specific marker amplified alleles in *B. juncea,B. napus* and *B. rapa* varieties (*A* genome), while no marker was detected in *B. carinata* cultivars Chakwal-raya and Peela-raya. *C* genome specific SSR markers Na12BO9, Na12HO2 and Ni4B10 amplified all the *C* genome carrying cultivars (*B. carinata* and *B. napus* species). But interestingly, Ni4B10 amplified marker (194-220bp) even in cultivars devoid of 'C' genome (*B. juncea*) e.g. BARD-1, Dacca-raya, Early-raya, S-9, NIFA-raya, Sultan-raya, Raya-Anmol, Poorbi-raya and Canola-mustard. Similarly, Na12HO2 also generated allele (200-230bp) in all the cultivars including *B. juncea* and *B. rapa* (Table 4).

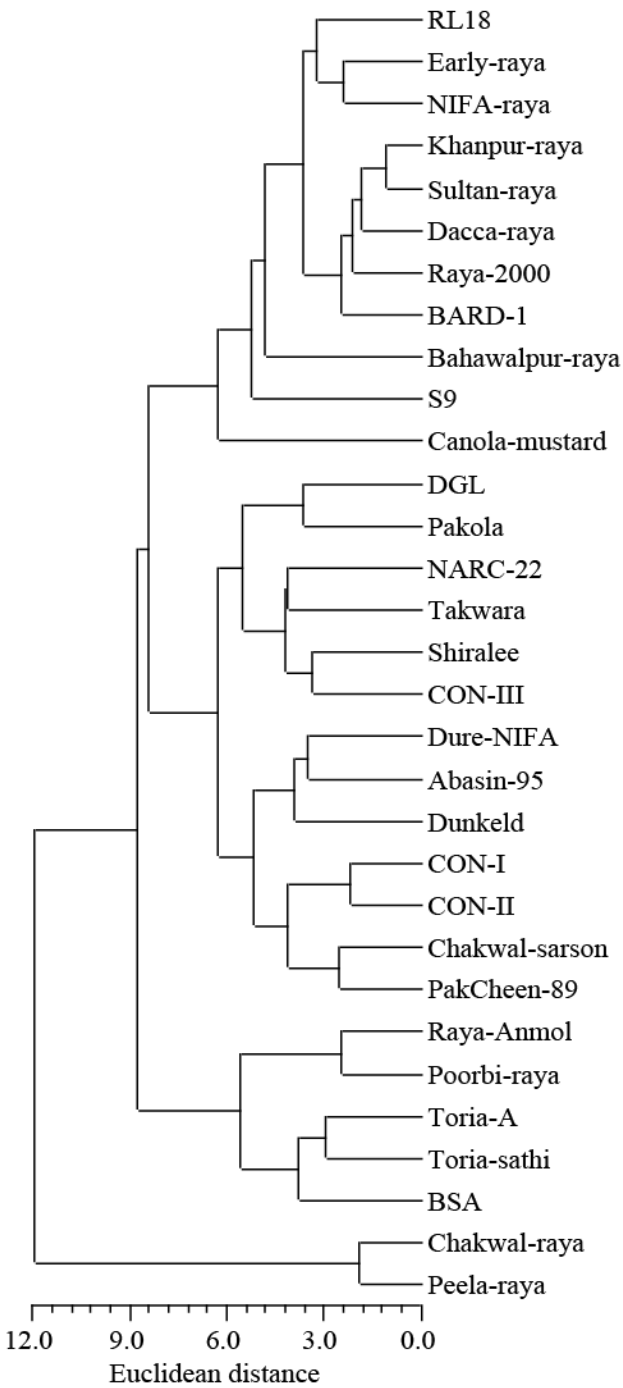

Fig. 1. Cluster analysis of oilseed brassica cultivars from Pakistan based on 19 morphological traits.

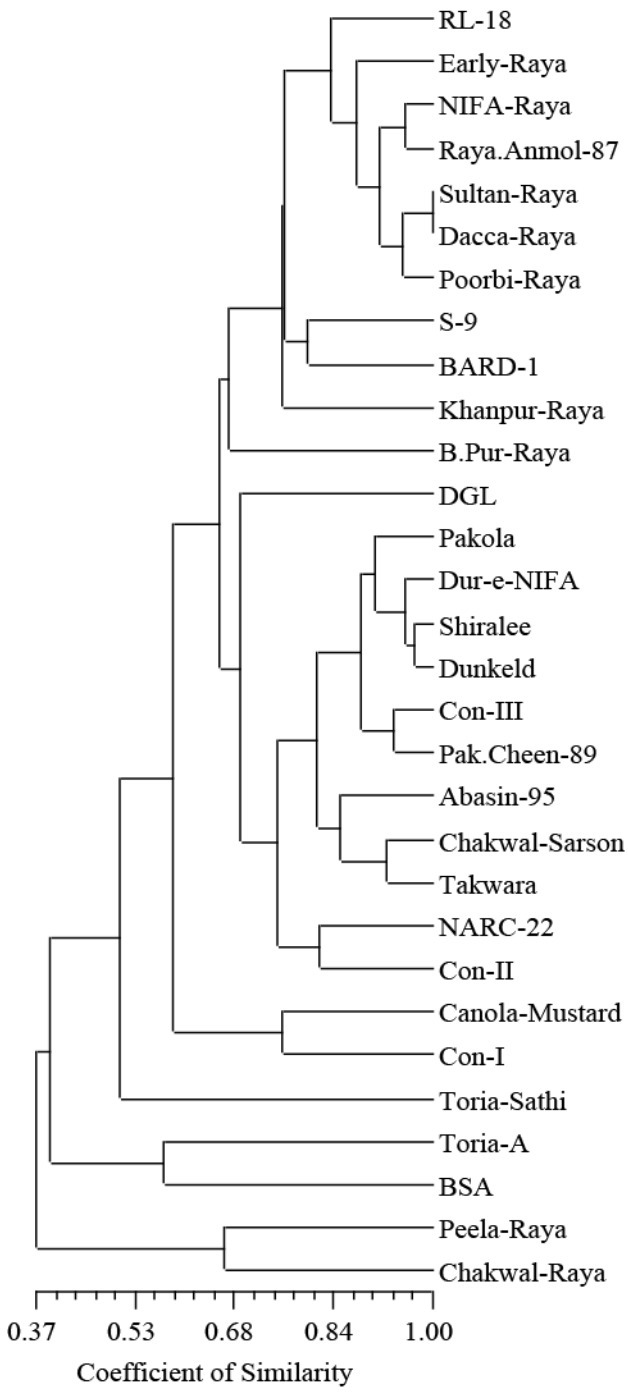

Fig. 3. Cluster analysis of 30 brassica cultivars from Pakistan based on SSR markers.

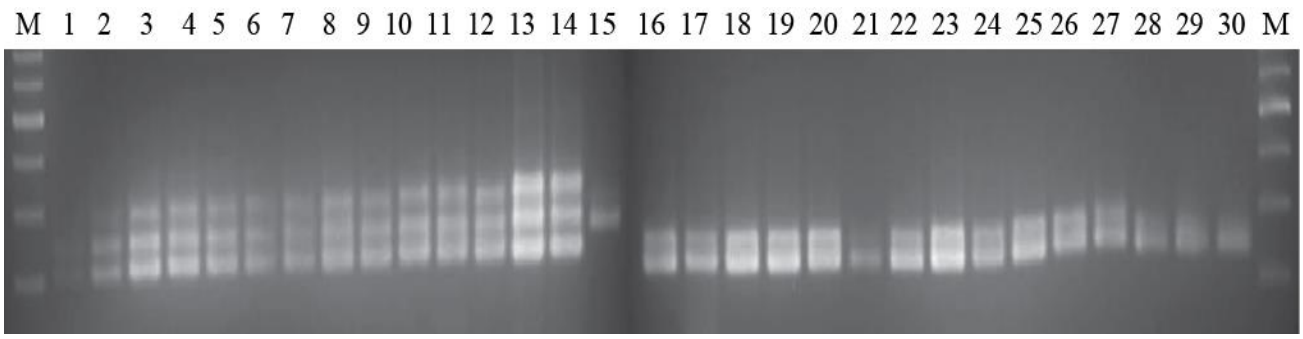

Fig. 2. SSR profile of 30 cultivars of brassica oilseeds using the marker BRMS 019. Lanes: 1-12 (*B. juncea*), 13-14 (*B. carinata*), 15-27 (*B. napus*), 28-30 (*B. rapa*), M is 100bp DNA ladder.

Table 4. Primer description and amplification of alleles.

S. No.	Primer name	Primer orientation	5' – 3'	Amplified fragments	Polymorphic fragments	Fragment size (bp)
1.	BRMS 019	F	CCCAAACGCTTTTGACACAT	3	2	220-300
		R	GGCACAATCCACTCAGCTTT			
2.	BRMS 030	F	TCAGCCTACCAACGAGTCATAA	2	2	212-230
		R	AAGGTCTCATACGATGGGAGTG			
3.	BRMS 033	F	GCGGAAACGAACACTCCTCCCATGT	2	1	225-250
		R	CCTCCTTGTGCTTTCCCTGGAGACG			
4.	BRMS 036	F	GGTCCATTCCTTTTTGCATCTG	1	1	140
		R	CATGGCAAGGGGTAACAAACAT			
5.	BRMS 037	F	CTGCTCGCATTTTTTATCATAC	2	2	154-200
		R	TACGCTTGGGAGAGAAAACTAT			
6.	BRMS 040	F	TCGGATTTGCATGTTCCTGACT	3	3	220-280
		R	CCGATACACAACCAGCCAACTC			
7.	BRMS 042-2	F	AGCTCCCGACAGCAACAAAAGA	1	1	150
		R	TTCGCTTCCTTTTCTGGGAATG			
8.	Na12Bo9	F	ACGGAAGATCAAACAGCTCC	2	2	120-130
		R	TGAGCGACCCATTCTTTAGG			
9.	Ni4B10	F	GTCCTTGAGAAACTCCACCG	2	1	194-220
		R	CCGATCCCATTTCTAATCCC			
10.	Na12HO2	F	CTATGGTTCATCTTTCGCCG	2	2	200-230
		R	GCTGCACATCCATCTCTCG			
11.	Na12DO4	F	ACGGAGTGATGATGGGTCTC	1	0	281
		R	CCTCAATGAAACTGAAATATGTGTG			
	Total			**21**	**17**	

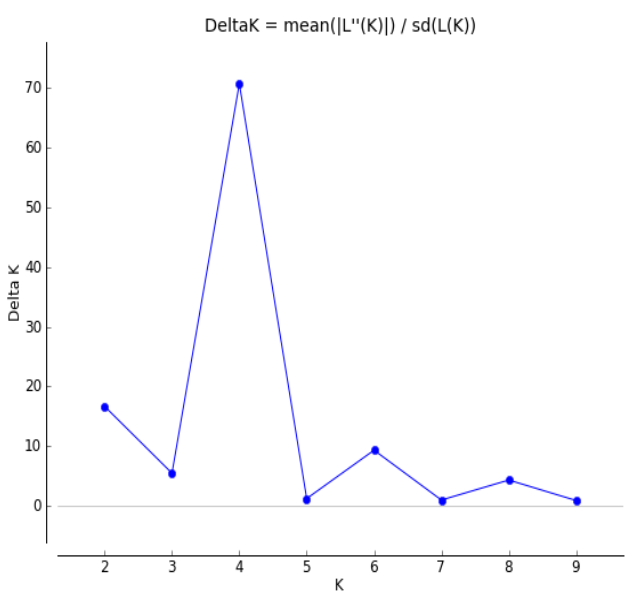

Fig. 4. The highest peak showing Δk =4 which corresponds to number of sub-populations.

Cluster analysis: SSR scoring data was subjected to calculategenetic distances which were utilized to perform cluster analysis. The subsequent dendrogram classified 30 cultivars into different groups. Two larger groups (group I and group II) were formed, besides 4 small clusters with 2, 2 genotypes each. Similarly, 3 cultivars remained independent as they did not fall in any group or cluster (Fig. 3). The group I included 7 cultivars i.e. RL-18, Early-raya, NIFA-raya, Raya-Anmol, Sultan-raya, Poorbi-raya and Dacca-raya. NIFA-raya and Raya-anmol had maximum similarity coefficient of 0.96 in this group. Members of this group had larger leaves, small and less productive siliquae. All the cultivars in this group belonged to *B. juncea*. Group II comprised of 12 cultivars i.e. DGL, Pakola, Dure-NIFA, Shiralee, Dunkeld, Con-III, PakCheen-89, Abasin-95, Chakwal-Sarson, Takwara, NARC-22, and Con-II. Shiralee and Dunkeld were found to have 0.97 similarity coefficient. All these cultivars were found to produce higher number of siliquae on main raceme (average 50), maximum number of primary branches, larger and productive siliquae (16.3 seeds/ silique) with long main raceme which is also a yield component trait. All these cultivars belonged to *B. napus*. Other small clusters observed were of 2, 2 members each. These comprised S-9 and BARD-1, Canola-mustard and Con-I, Toria-A and BSA, and Peela-raya and Chakwal-raya. The association between cultivars S-9 and BARD-1 may be the result of common ancestors as the former was developed in Agricultural Research Institute (ARI), Tandojam, while the later in NARC, Islamabad. These two institutes worked closely in a joint collaboration on developing oilseed brassica cultivars in the past. Also, Canola-mustard and Con-I paired in a single cluster and Toria-A and BSA were grouped in the same cluster which is reasonable because the first two cultivars were developed in NARC, Islamabad and the later two were

developed by Ayub Agricultural Research Institute (AARI), Faisalabad. Hence common origin of these cultivars resulted in their similar genetic make up which was further confirmed by clustering closely. Chakwal-raya and Peela-raya clustered in a single group because both belonged to *B. carinata* and these cultivars were found to be late maturing with vigorous vegetative growth, while cultivars like Khanpur-raya, Bahawalpur-raya and Toria-Sathi formed no association with other cultivars and positioned independently in the dendrogram. These cultivars had moderate grain yield with good vegetative growth (Fig. 3).

Population Structure: A total of 325 bands generated by 11 primers in 30 *Brassica* cultivars were used as dominant markers for population structure analysis. The result of STRUCTURE HARVESTER suggested Δk=4 (the sharpest peak see Fig. 4). Hence studied population of 30 cultivars can be conveniently divided into 4 subpopulations as shown in the cluster (Fig. 5). Cluster differentiated all the 30 cultivars into 4 distinct (more than 50%) or mix colours. The first group (light green) included 6 cultivars from *B. juncea, B. napus* and *B. rapa* from diverse locations i.e. Faisalabad, Bahawalpur, Khanpur, Islamabad and Peshawar. The red denoted large group included 12 *B. juncea* cultivars from Tandojam, Peshawar, Faisalabad and Islamabad. The yellow group comprised of 12 *B. napus* cultivars from Faisalabad, Chakwal, Islamabad, Swat, D.I Khan, Peshawar and Australian cultivars. Two *B. rapa* cultivars were distributed in blue group. *B. napus* cultivars i.e. DGL, NARC-22 and Con-II had significant admixture implying gene flow and recombination within cultivars.

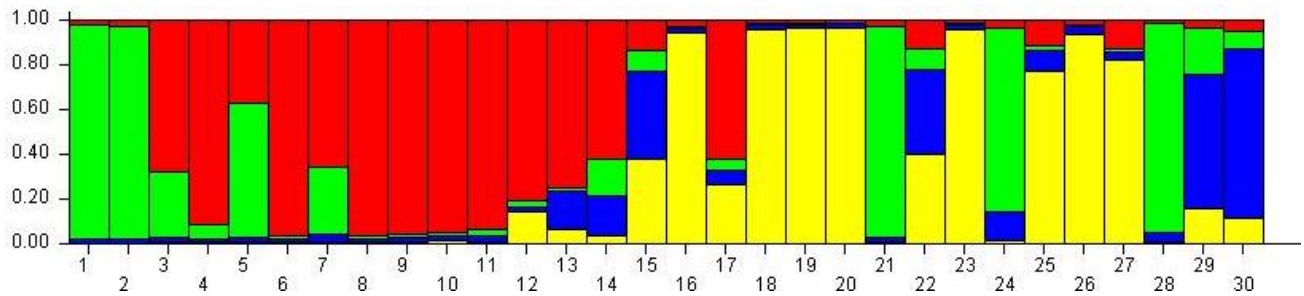

Fig. 5. Population structure distributing 30 *Brassica* oilseeds cultivars into 4 sub-populations.

Discussion

The correlation coefficients matrix revealed a positive association between days to flowering initiation, 1000-seed weight and plant height. Similarly, days to flowering completion had highly positive correlation with plant height, leaf width and 1000 seed weight, while yield had significantly positive correlation with plant height and number of primary branches per plant. The study was supported by findings of Singh (1974) and Ozer *et al*., (1999). An important yield character, number of seed per silique was observed to be positively correlated to characters like number of primary branches per plant and siliquae per main raceme etc. These results revealed similar pattern as previously reported (Ali *et al*., 2003; Rao *et al*., 2003). Greater phenotypic variation was observed between species, however lesser genetic variation was observed by morphological studies within species. This was further confirmed by previous studies. Rabbani *et al*., (1998) studied 52 *B. juncea* accessions for morphological characterization and reported lesser genetic variation. Therefore, it is concluded that further morphological characterization of the brassica cultivars is required, while also devising new strategies to preserve the existing genetic diversity among the germplasm.

The primers in our study generated less number of alleles than initially suggested by authors e.g. in BRMS series of primers, Suwabe *et al*., (2002) detected 4,5,4,6,3,9 and 8 alleles for BRMS-019, BRMS-030.BRMS-033, BRMS-036, BRMS-037, BRMS-040 and BRMS-042-2, respectively, while in our study these primers amplified 3,2,2,1,2,3,1 alleles, respectively. However, the differences in the number of amplified allele have been reported in the earlier studies also as Louarn *et al*., (2007) observed variation in number of alleles for primers by detecting six alleles in cauliflower cultivars for SSR marker, while Tonguc & Griffiths (2004) detected 4 alleles for the same primer. In the present study 2 species specific SSR primers amplified alleles but also not only in their focal species in all the species. This may be assumed that the presence of alleles in all the varieties may be due to their common ancestor or genes flow. However, the specificity of these primers has been found to be varying by curators as Lowe *et al*., (2002) used 16 primer pairs of which 13 amplified alleles in not just in focal but also non-targeted brassica species. However, the author suggested high polymorphism and increased size of amplified fragment in focal species than transfer specie. Specificity of these markers should be checked and reviewed as the PCR was re-performed to verify the amplification. For all the primers used no cultivar-specific allele was detected which could be used as an informative marker for identification of that cultivar among the studied population. However, significant molecular polymorphism was observed among genotypes studied which could be further used for cultivars screening. Sadia *et al*., (2009) also observed SSR based polymorphism in the brassica accessions of Pakistani origin.

Westman & Stephen (1999) evaluated brassica germplasm from four different regions and concluded that specific pattern was observed between the genotypes from the same region i.e. genotypes from the

same origin clustered together; while in the present study for all cultivars, no grouping pattern was observed for geographical distribution of genotypes rather, genotypic affinities corroborated with species of the cultivars and their morphological characteristics. This highlights the fact that genetic base of the Pakistani oilseed cultivars is narrow as suggested by Rabbani *et al*., (1998). However, cultivars developed at common research stations/institute clustered together in dendrogram because of common ancestors and similar genetic make up. Previous studies supported these findings as Soengas *et al*., (2006) observed grouping pattern in a set of 25 *B. napus* cultivars using SSR markers. We evaluated genetic structure of 30 cultivars from 4 different *Brassica* species using Bayesian statistical model. The approach has been earlier applied by Xiao *et al*., (2012) to study genetic structure in rapeseed. Zhao *et al*., (2007) employed STRUCTURE based statistical model to study population of 160 *B. rapa* accessions and observed 4 distinct subpopulations on the basis of AFLP fingerprinting.

Conclusion

It has been observed that cultivars developed by common institute/ seed company, clustered together. From our study we infer that cultivars reflect their genetic variation on the basis of their origin. In the present study significant molecular variation had been revealed by SSR primers. Both morphological and molecular markers were efficient to explicitly discriminate cultivars on the basis of species origin. However, we recommend further molecular analysis by employing SNP markers of genotyping by sequencing (GBS) to report genetic diversity and genotype identification in Pakistani oilseed *Brassica* populations.

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