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Characterization of *Brassica napus* (Canola) Germplasm Based on Microsatellite Markers

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MA and ZM did interpretation of data and design of author MA; Author SBH did revision and proofreading; Author MA did acquisition of data, and analysis; and author TN did acquisition of data and drafting, revision and proofreading of the author MA. All authors read and approved the final manuscript.

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ABSTRACT

Brassica napus L. is a major oilseed crop all over the world. The aim of this study was to investigate the genetic diversity of *B. napus* germplasm by using simple sequence repeats (SSR) markers. In the current study, ten SSR markers were used for studying genetic diversity of ten *Brassica* cultivars. Out of 110 total bands, 68 bands were polymorphic with 52.11% average polymorphism. Mean value of Nei's genetic diversity and Polymorphism Information Content was 1.7, and 0.2630, respectively. These mean values show that there are moderate allelic differences between *Brassica* cultivars. The Nei's genetic distance among various cultivars was 0.3281 and 0.125 which showed that germplasm of *Brassica* cultivars are different from each other, which is probably due to anthropogenic interventions and environmental factors. Thus, genetically different lines identified in this study could be employed in breeding programmes to develop higher-quality canola inbred varieties in future.

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1. INTRODUCTION

Mustard (*B. juncea*) and Rapeseed (*Brassica napus* and *B. rapa*) are major oilseed crop around the world. In Pakistan, the crop is cultivated on an area of 272,100 ha, yielding 230,000 tons per year with 812 kg/ha average yield, which account for more than 17% of Pakistan's total domestic edible oil production [1]. Among the genus, genetic diversity provides a significant variation source that may be exploited to change *Brassica* crop cultivars using a variety of ways. Analyses of genetic relations are critical for germplasm management, crop improvement programs, and conservation strategy development. Local cultivars of oil crops usually contain good flavour and quality, as well as exhibit high level of disease and pest resistance, and could be preferable than foreign materials. Therefore, plant breeding requires the development of genetically heterogeneous gene pools [2].

However, modern plant-breeders face the challenge of developing higher yielding, more nutritional, and ecologically friendly cultivars that increase our life quality without requiring the use of new habitats for agriculture [3]. Plant breeders cannot produce varieties that fulfil changing needs in terms of adaptability to growing circumstances, yield, tolerance to abiotic or biotic challenges, or specific requirements of quality unless they access huge database of heterogeneous material of plant [4]. Therefore, accessibility to the diverse genetic diversity pool is the most effective approach to increase the performance of different cultivars. Furthermore, information regarding the genetic diversity collections of *B. napus* germplasm can provide geneticists and breeders with valuable information about the diversity of alleles found in *B. napus* genotypes, as well as aid in the identification of genetically distinct pools to be used in cross-combinations for improving major agronomic traits and exploit heterosis [5].

For the introduction of new diversity into breeding lines of oilseed rape, morphological, agronomical, and phonological parameters have traditionally been used. Since the emergence of modern molecular biology technologies, molecular markers are being used to investigate phylogenetic relationships among as well as within species of *Brassica*. These markers consist of randomly amplified polymorphic DNA [6], restriction fragment length polymorphism (RFLP) [7], and simple sequence repeat (SSR) [8] *etc*. When compared to other molecular marker approaches, SSR markers are abundant, informative, highly polymorphic, co-dominant, reproducible, technically simple, and reasonably inexpensive when information of primer is available. Moreover, SSR markers are frequently found in the gene-rich genomic areas, implying that they could be useful for association studies of allele-trait in well-characterized regions of genome with quantitative-trait loci.

SSR markers have been frequently employed in tomato, maize, and rice for diversity studies [9-11]. SSR markers have been proved beneficial for studying genetic structure and diversity of *Brassica*. Using eighteen pairs of SSR primers, 112 polymorphic loci were discovered in Australian canola varieties, revealing their genetic diversity [12]. By evaluating the polymorphic alleles of eighteen SSR markers, Soengas et al. [13] was able to determine the genetic link between eight groups and investigate the genetic structure. Based on this, the present study was aimed to investigate the genetic diversity or polymorphism of different local varieties of *B. napus*, which would be useful for breeding program of *Brassica* in the future.

2. MATERIALS AND METHODS

2.1 Plant material

Seeds of ten different genotypes of *B. napus* (ACC-23633, ACC-24210, ACC-24214, ACC-24217, ACC-24254, ACC-24875, ACC-24880, ACC-24889, ACC-27396, and ACC-27405) were collected from the Pakistan Agriculture Research Council (PARC), Islamabad, Pakistan. The research was conducted at the Molecular Markers Application Laboratory at the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan. To explore the genetic diversity of these genotypes, the following techniques were used.

2.2 Extraction of Genomic DNA

Four week old seedlings were collected for extraction of DNA following the protocol of Doyle and Doyle [14] with minor modifications. 2mg leaf sample was homogenized in 500µl of extraction buffer [NaCl (100 mM), SDS (1%), Tris-base (100 mM), and Na_2EDTA (100 mM)] following

incubation in water bath for thirty minutes. After that, 500µl of phenol (25): chloroform (24): isoamyl alcohol (1) solution was added following centrifugation at 9000 rpm for 15 min. After centrifugation, aqueous layer was separated and 500µl chilled isoproponal was added for DNA precipitation following centrifugation at 9000rpm for 2 min. The pellet was washed with 70% ethanol after discarding the supernatant, and was dried for half hour at room temperature. After that, 50 µl TE-Buffer was added in the tubes and was stored at 4°C. The quality of DNA was checked using agarose gel electrophoresis, while its quantity was measured using UV-spectrophotometer at 260nm wavelength.

2.3 SSR Assays

Ten pairs of SSR markers i.e. BRASO-I-I, BRAS014, BRAS072A, BRAS078, BRAS084, CB-10026, CB-I-0028, CB-10092B, CBI-0-1-43, and CBI-0369 (Table 1) were used for the genetic diversity analysis of *B. napus*. SSR primers were obtained from http://www.brassica.info/resource/markers.php and were synthesized by Macrogen chill.co.pk (Table 1). PCR amplification of SSR loci was performed in 25 µL reaction mixture containing genomic DNA (20 ng/µL), 10 X PCR-reaction buffer, dNTPs (0.2mM), 0.5pmol of forward and reverse primer, Taq DNA polymerase (0.5U) and $MgCl_2$ (15mM). PCR was performed in PTC-100 thermo-cycler with the following temperature profile: 95 °C for 5 minutes (initial denaturation), following 35 cycles with 94 °C denaturation for 45 seconds, annealing for 60 seconds, primer elongation for 2 minutes at 72 °C and final elongation for 10 minutes at same temperature. The products of PCR were analysed on polyacrylamide gel electrophoresis containing 10% polyacrylamide using Bio-rad electrophoresis cell. The gel was stained by sliver staining following protocol of Zhang et al., [15].

2.4 Data Analysis

Bands expressing specific alleles on microsatellite loci were manually scored on gel pictures. The genotype of a specific locus was scored based on the allelic number having specific size at a certain locus. A band present on gel indicating the presence of a certain allele at one locus was labelled 1, whereas its absence was labelled 0. To create a single matrix, genomic data encompassing 10 SSR markers and 10 *Brassica* cultivars were entered into a Microsoft Excel spreadsheet. The number of effective alleles (N_E) was derived from this dataset for each SSR marker and polymorphism percentage was also estimated to frame genetic-profile of *Brassica* crops. Nei's genetic diversity (H_E) analogous to heterozygozity was also calculated because it

Table 1. SSR primer for genetic diversity analysis of *Brassica* cultivars

Sr. No.	Primer information	
	Name	**Sequence**
1	BRASO-I-I F	TGG GAC GTA GTC AGT CAA CAA
	BRASO-I-I R	CCA AGT GCG AGA AGA GGA AG
2	BRAS014F	CCC AIT GAC AAC TCT TCT CIT
	BRAS014R	CTG TGT CGC CCA ITA TG
3	BRAS072AF	GCC ATC TAC ACA TTT ATC CC
	BRAS072AR	CAC TAA CCT TCT TGC TAC CGT
4	BRAS078F	ATT GGG TTC TGA CCI TIT CTC
	BRAS078R	CTT ITC CTC ATC GCT ACC AC
5	BRAS084F	ATT GGG TTC TGA CCT IIT CTC
	BRAS084R	TTT TCC TTC ATC GCT ACC AC
6	CB-10026F	TCG TTC TGA CCT GTC GIT AT
	CB-10026R	GGA AAT GGC TGC TCA TGC T
7	CB-I-0028F	CTG CAC ATT TGA AAT IGG TC
	CB-I-0028R	AAA TCA ACG CTT ACC CACT
8	CB-10092BF	TTG ATC CGA AAT CTC TGG
	CB-10092BR	AGG CAA GCA ATA GAT AAA GG
9	CBI-0-1-43F	CAT GGG AGG CTG TCT AAA
	CBI-0-1-43R	TTG CAC CCA TAC GTT TC
10	CBI-0369F	CAT CAC AGG ACC AGA GC
	CBI-0369R	CAA AGC CAA GAC ACC CAT

estimates genetic diversity of randomly breeding populations. POPGENE version 1.32 (http://www.ulaberta.ca/fyeh) was used to calculate Pair wise Nei's genetic-distance. The genetic relatedness of all ten *Brassica* cultivars was assessed by computing genetic distances for all samples using Nei's coefficient. Polymorphism Information Content (PIC) value was calculated using *PICi* =1 – $\Sigma_{j=1}^{n}$ $(PIJ)^2$ formula, i=marker, where n and pij = the number and frequency of alleles, respectively for that marker. Un-weighted pair group method with arithmetic average (UPGMA) was used to do cluster analysis [16].

3. RESULTS AND DISCUSSION

The study of genetic variation is beneficial to the evolution and conservation of species [17]. Molecular markers are frequently utilised in the genetic study of plants species due to their different properties such as high polymorphism, co-dominance, and reproducibility. Due to their multi-allelic character, these markers can easily detect a wide range of comparative allelic differences in germplasm [18].

In this study, SSR-DNA profile was created for investigating genetic relationship between 10 cultivars of *B. napus*. After amplification of PCR (Fig. 1), out of 110 total detected loci 68 appeared to be polymorphic with 52.11% average of polymorphism (Table 2). Out of ten successfully amplified SSR markers, eight markers showed polymorphism and created potentially score-able banding array. However, only one marker (BRAS072A) showed 100% polymorphism. N_E (no. effective alleles) ranged from 1.0000-2.000 with 1.7 mean values. Out of ten markers, BRAS084, CBI-0-1-43, and CBI-0369 showed 1.000 N_E values than all other markers. The Nei's genetic diversity or H_E value ranged from 0.000 to 0.48 with an average of 0.298, while the polymorphism information content value (PIC) ranges between 0.0000 to 0.3750 with an average of 0.2360 (Table 2).

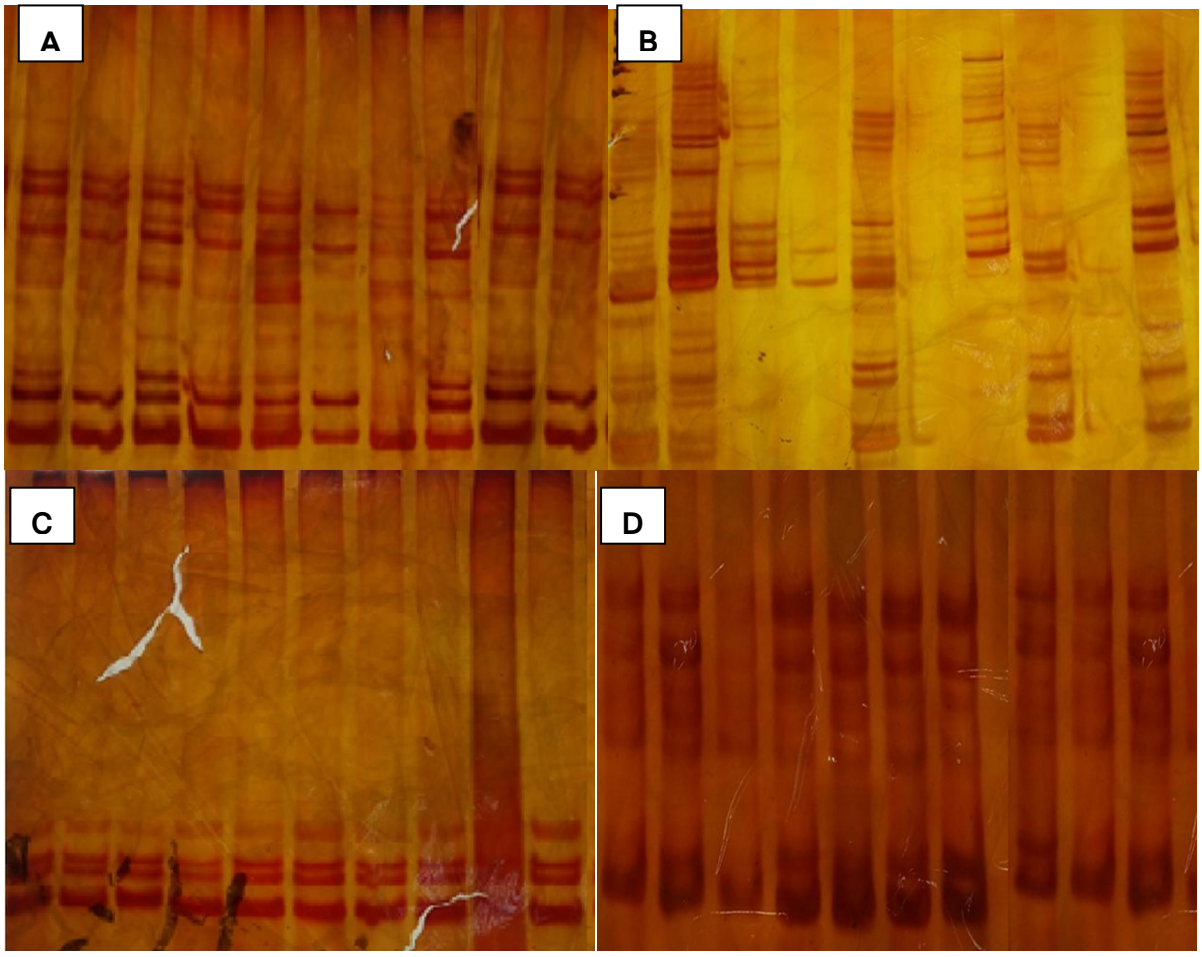

Fig. 1. Amplification results of the primer (A) BRASO-I-I; (B) BRAS014; (C) BRAS072A; (D) BRAS078

The PIC value predicts the efficacy of any marker in genetic evaluation, linkage mapping, and molecular lineage. Allelic differences are more likely to be shown by markers with greater PIC values [19]. In this study, maximum value of PIC value was 0.3750 using CB-10092B marker, while the minimum was 0.000 with BRAS084 and CBI-0369 (Table 2), this could be attributable to inadequate coverage of genome or a low degree of genetic heterogeneity among cultivars. Since no marker in this study has a PIC value of 1, this indicates that there is no specific gene in the genome of *Brassica* cultivars. Average H_E and PIC scores in the moderate range imply that there is moderate allelic variation across all the tested *Brassica* cultivars.

Genetic diversity assessment is fundamental knowledge that allows us to not only identify similarities and differences among plant species, but also help us to recognize environmental challenges on populations. Microsatellite markers are employed in genotyping of several species due to their co dominant, highly polymorphic nature [20], and their multi-allelic character allows to detect allelic variants across a wide variety of genetic resources [21]. In the current study, 10 SSR markers on 10 *Brassica* cultivars were utilized in an efficient way for studying genetic diversity. All amplified markers, resulted in 68 polymorphic bands out of 110 clear bands. These results showed high efficacy of microsatellite or SSR markers and thus, can be an efficient and suitable tool for investigation of genetic variations of many species of plant [22,23]. Previous researchers have examined the differences between winter and spring oilseed of Europe and China accessions using SSR markers [24,25]. Several other studies investigated genetic diversity of rapeseed germplasm and cultivars which showed similar results as observed in the current study [26-28]. Furthermore, understanding the genetic relationships and diversity of germplasm among local varieties and primary breeding lines might be an effective tool in agricultural improvement methods.

On the basis of Nei's genetic distance, cluster analysis was done using UPGMA that divided all cultivars into three clusters (Table 3). Cultivars within the same cluster share genetic characteristics, indicating that they are near relatives. Cultivars in one cluster are genetically

Table 2. Parameters of genetic diversity of 10 *B. napus* samples with 10 SSR markers

Primers	No. of loci		Polymorphism %	Allele frequency	N_E	H_E	PIC
	Total	Polymorphic					
BRASO-I-I	10	7	70%	0.7	2.000	0.42	0.3318
BRAS014	5	1	20%	0.9	2.000	0.18	0.1638
BRAS072A	20	20	100%	0.8	2.000	0.32	0.2688
BRAS078	8	4	50%	0.6	2.000	0.48	0.3648
BRAS084	4	0	0%	1.000	1.000	0.000	0.000
CB-10026	6	3	50%	0.6	2.000	0.48	0.3648
CB-I-0028	17	14	82.35%	0.7	2.000	0.42	0.3318
CB-10092B	19	18	94.73%	0.52	2.000	0.500	0.3750
CBI-0-1-43	11	1	9.09%	0.9	1.000	0.18	0.1638
CBI-0369	10	0	0%	1.000	1.000	0.000	0.000
Total	110	68	52.11%	0.772	1.7	0.298	0.236

Table 3. Frequency based Nei's genetic distance

Cultivars	ACC-23633	ACC-24210	ACC-24214	ACC-24217	ACC-24254	ACC-24875	ACC-24880	ACC-24889	ACC-27396	ACC-27405
ACC-23633	0									
ACC-24210	0.3281	0								
ACC-24214	0.25	0.2344	0							
ACC-24217	0.2656	0.25	0.1719	0						
ACC-24254	0.2188	0.3125	0.2656	0.3125	0					
ACC-24875	0.3594	0.3281	0.2188	0.25	0.375	0				
ACC-24880	0.1953	0.2891	0.1484	0.2109	0.2109	0.2891	0			
ACC-24889	0.1328	0.3203	0.2266	0.2109	0.2734	0.3359	0.2031	0		
ACC-27396	0.1797	0.3359	0.1641	0.2578	0.2891	0.2891	0.1875	0.1406	0	
ACC-27405	0.125	0.3438	0.2344	0.2813	0.1719	0.3281	0.1797	0.1484	0.1953	0

more similar than cultivars in another. Each cluster is sub-divided into smaller groups called sub-clusters. There are two cultivars in Cluster I (ACC-27405 and ACC-23633) and Cluster II (ACC-27396 and ACC-24889). However, in Cluster III the germplasm ACC-24217 is sub-divided into two cultivars (ACC-24214 and ACC-24880). Moreover, the three germplasm; *i.e.* ACC-24875, ACC-24210 and ACC-24254 form out boundaries from all the other cultivars. Thus, the results obtained in this study showed that SSR markers are beneficial and effective for evaluating the genetic variation of *B. napus* germplasm. Several other researchers have come to similar results about using SSR markers in rapeseed breeding [27,29].

4. CONCLUSION

The present study is the first preliminary research that utilized microsatellite or SSR markers for reporting the diversity of *B. napus* using a small number of cultivars. For further genetic variations and phylogenetic relationships of this species in the future, a detailed study with a high sample size is recommended.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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