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GENETIC DIVERSITY OF *Brassica rapa* GERMPLASM OF KHYBER PAKHTUNKHWA PAKISTAN REVEALED BY MOLECULAR MARKERS

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ABSTRACT

A total of 96 indigenous *Brassica rapa* accessions were collected from different locations of Khyber Pakhtunkhwa, Pakistan. Simple Sequence Repeats (SSR) markers were used to identify the most diverse genotypes among the collected lots. Twenty six (26) different SSR primers were used for (genetic) variability among collected genotypes. These primers were selected from literature based on their previous results. These primers produced 135 scorable bands of which 75 were polymorphic, with an average of 55.5% polymorphic loci, and reflected the broader genetic background of the collected genotypes. An average 2.88 polymorphic bands with an average PIC value of 0.49 was recorded. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) divided all genotypes into three main groups. Group one contained three clusters, while group two and three had four and two clusters each. Based on the UPGMA dendrogram, genotypes collected from Kohat, Bannu, Swat and Haripur showed considerable amount of variation. From the present study, it is concluded that SSR markers can be proved as the best tool for the genetic variability of other local and exotic *B. rapa* genotypes.

Key words: *Brassica rapa*, genetic variability, Khyber Pakhtunkhwa, polymorphic bands, simple sequence repeats, Pakistan

INTRODUCTION

Due to its variant group of crop plants, Brassicas have enormous economic value across the globe. A number of agronomically important crop types come under the umbrella of *Brassica* species and are cultivated in various environmental conditions. A total of 3000 species and 360 genera, organized into 13 tribes encompass the family Brassicaceae (Cruciferae). Southwestern and central Asia and the Mediterranean region are the main centers of diversity. Among the Brassicaceae family, the most economically important species used in the world for multipurpose included: *Brassica carinata*, *Brassica juncea*, *Brassica napus*, *Brassica nigra*, *Brassica oleracea* and *Brassica rapa* [Pires et al. 2004]. These species can be used for various purposes like edible and industrial oils, and also consumed as vegetables, condiments, fodder and forage. Among these, diploids species are *B. nigra*, *B. rapa* and *B. oleracea*, while amphidiploids species are *B. napus*, *B. carinata* and *B. juncea* [Axelsson et al. 2000].

A country can excel in its socio-economical and scientific development by exploring its hidden and

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ignored indigenous materials. Currently in perspective of WTO (World Trade Organization), geographic information of collected germplasm is important for further morpho-biochemical and molecular evaluation. Every country is tailoring policies to secure utmost benefit from world market by utilizing available genetic resources [Narain 2000]. Conventionally the agronomical, phenological and morphological characters served as criteria for introgression of novel variability into oilseed rape breeding lines. Presently, morpho-physiological, molecular and biochemical markers are utilized to assess genetic variability in plant species [Gawronski et al. 2017, Anjum et al. 2018, Jan et al. 2018]. In early studies mainly morphological based variability was used. In recent years, biochemical markers have received more consideration as the data produced by these markers show the real pictures of variability of evaluated genotypes because they are the direct products of genes and genes are not influenced by the environment [Perry and McIntosh 1991].

Several modern molecular techniques along with morphological markers are currently used to assess genetic diversity in species of various crops. Plant breeders are now enabled to study reproduction, speciation and spatial-temporal dynamics of populations in many crop species by using molecular markers in these areas [Xu et al. 2002]. The identification of novel genes and characterization of locally collected genotypes are evaluated with the help of molecular markers. In plant breeding, DNA-based markers proved to be a useful instrument for the evaluation and utilization of important characters due to its reliable and excellent results [Khan et al. 2016].

Although all molecular markers play a significant role in the genetic diversity of different crop species, Simple Sequence Repeats (SSR) markers have several advantages. They are extremely polymorphic, informative, codominant, and simple technically. Additionally, they are reproducible and are relatively economical where primer information is available. Moreover, SSR markers frequently occur in gene-rich genome regions, escalating potential applicability for allele trait association studies. Among various cruciferous species SSR have been efficiently used to explore genetic diversity. SSRs have been widely used to explore the hidden genetic relations, cultivars identification, exploring genetic associations and diversity in Brassica species [Lanner et al. 1996, Turi et al. 2012, Thakur et al. 2018]. To date no complete report is available focusing the molecular based diversity study from Khyber Paktunkhwa (KP) regarding identification and characterization of elite *B. rapa* accessions. Keeping in views these gaps, an effort was made in this study to check the variability in different *B. rapa* accessions collected from diverse locations of Khyber Pakhtunkhwa Province of Pakistan using SSR markers.

MATERIALS AND METHODS

Plant materials. In the present study five random selected plants from 96 accessions of *Brassica rapa* were collected from farmer fields of various locations of Khyber Pakhtunkhwa, Pakistan (Tab. 1, Fig. 1) during 2016. The seeds of these landraces and candidates lines were bulked together and submitted to National Genebank of Pakistan (NGP), National Agricultural Research Centre (NARC), Islamabad, Pakistan

Microsatellite marker analysis. During the first phase of the study, seeds of the collected genotypes were sown in pots in greenhouse. Three weeks old young seedlings were used for DNA extraction using a standard protocol [Doyle and Doyle 1990]. DNA was extracted from five individual plants for each accession. Nano Drop ND-2000 Spectrophotometer (Wilmington, USA) instrument was employed to examine extracted DNA concentration and purity from collected material. To get working concentration of 200 ng/µl for microsatellite study, the extracted DNA samples were diluted with TE buffer. Micro-satellite markers that resided in A genomes of *B. rapa* were selected [www.Brassicainfo.com]. A total of 26 SSR primers were used in the study (Tab. 2). The Hasan et al. [2006] protocol was used for SSR analysis with minor modifications. The thermal cycler was optimized by using an initial denaturation step of 5 minutes at 94°C, followed by a second step of denaturation with 35 cycles of 1 minute at 94°C. It was followed by 1 minute annealing step at temperature 55°C–65°C and 2 minutes at 72°C. The different annealing temperatures were tested for different markers. The final extension step of 7 minutes at was performed at 72°C. The desired amplified products were stored in refrigerator at –20°C.

Table 1. List of *Brassica rapa* accessions used in the study

Sr. No.	Accession No.	Location	Sr. No.	Accession No.	Location	Sr. No.	Accession No.	Location
1	Kt1	Kohat	33	Si13	Swabi	65	Br1	Bunner
2	Kt2	Kohat	34	Si14	Swabi	66	Br2	Bunner
3	Kt3	Kohat	35	Si15	Swabi	67	Br3	Bunner
4	Kt4	Kohat	36	Mn1	Mansehra	68	Br4	Bunner
5	Kt5	Kohat	37	Mn2	Mansehra	69	Md1	Malakand
6	Hr1	Haripur	38	Mn3	Mansehra	70	Md2	Malakand
7	Hr2	Haripur	39	Mn4	Mansehra	71	Md3	Malakand
8	Hr3	Haripur	40	Mn5	Mansehra	72	Md4	Malakand
9	Hr4	Haripur	41	Bm1	Baatagram	73	Mr1	Mardan
10	Hr5	Haripur	42	Bm2	Baatagram	74	Mr2	Mardan
11	Hr6	Haripur	43	Bm3	Baatagram	75	Mr3	Mardan
12	Hr7	Haripur	44	Bm4	Baatagram	76	Ca1	Charsada
13	Hr8	Haripur	45	Sa1	Shangla	77	Ca2	Charsada
14	Hr9	Haripur	46	Sa2	Shangla	78	Ca3	Charsada
15	Hr10	Haripur	47	Sa3	Shangla	79	Km1	Kurram
16	Hr11	Haripur	48	Kn1	Kohistan	80	Oi1	Orakzai
17	Hr12	Haripur	49	Kn2	Kohistan	81	Tk1	Tank
18	Hr13	Haripur	50	Kn3	Kohistan	82	Tk2	Tank
19	Hr14	Haripur	51	St1	Swat	83	Na1	Nowshera
20	Hr15	Haripur	52	St2	Swat	84	Na2	Nowshera
21	Si1	Swabi	53	St3	Swat	85	Na3	Nowshera
22	Si2	Swabi	54	St4	Swat	86	Na4	Nowshera
23	Si3	Swabi	55	St5	Swat	87	Na5	Nowshera
24	Si4	Swabi	56	St6	Swat	88	Hu1	Hangu
25	Si5	Swabi	57	St7	Swat	89	Hu2	Hangu
26	Si6	Swabi	58	St8	Swat	90	Hu3	Hangu
27	Si7	Swabi	59	St9	Swat	91	Bu1	Bannu
28	Si8	Swabi	60	St10	Swat	92	Bu2	Bannu
29	Si9	Swabi	61	Cl1	Chitral	93	Bu3	Bannu
30	Si10	Swabi	62	Cl2	Chitral	94	Bu4	Bannu
31	Si11	Swabi	63	Cl3	Chitral	95	Bu5	Bannu
32	Si12	Swabi	64	Cl4	Chitral	96	Bu6	Bannu

Amplified products electrophoresis. The amplified products (10 µl) were run on different concentrations of agarose gel by using 20 bp of DNA ladder and the gel was visualized with help of UVI-Doc Gel Documentation System (JICA, JAPAN).

Allele scoring and data analysis. The PCR products produced several bands after running on agarose gel. A DNA ladder of known size was used for the comparison of bands. Bands were scored as bi-variate (1–0) data matrix, where 1 designate the occurrence of

Fig. 1. Geographical locations of collected Brassica rapa germplasm used in the study. The circles denote the areas from where seed samples were collected. The green color shows different districts of Khyber Pakhtunkhwa, while the blue color highlights former Federally Administrated Tribal Areas (FATA) of Pakistan

Table 2. Details of SSR markers used, indicating Brassica primer name, locus, forward and reverse sequence, motif type, polymorphic alleles and polymorphism information content (PIC)

Primer name	Locus	Forward primer	Reverse primer	Motif	No. of polymorphic alleles	PIC value
S004C14-2	HBr201	AAAGCTGACATTGGGATTGC	CCACTTCCCCGATATTTGAA	TA	3	0.34
niab003	niab003	TGTGTCGCTCGTCTACGTCT	ACCATCGACTTCGTGGAAAC	TC	3	0.56
niab004	niab004	GGAGCTTGTTCAATCCTCCA	TGTACCTGCGGCTTCTTTCT	T	4	0.40
niab009	niab009	TTCCCAAGCTTGCTGGTACT	GAGATTTCCCTCGCTTGATG	AG/CT	2	0.37
niab013	niab013	GGAACCGTCCTTACTTTCTCTGT	AGGATTGTGTTTTCCACATTGTC	AT	3	0.54
CNU246	CNU246	AAAGCCATCCATCCATCAAGC	GATGCAACATTTGACTGTGTTAGAGC	TC	1	0.32
CNU250	CNU250	CAGATTTTCGAAAGGTGGTTGG	CCATCACCCGAAAATCCAAA	AG/AT	3	0.39
CNU253	CNU253	CCCAAAACATCCAAACTCCTCA	CCCCAAAGAGATACAGAACAAAGC	GA	2	0.51
CNU254	CNU254	AAGCTTGAGCTTCCAGCCTTC	ATCAGTGCCGGCCTTGAATA	AT	5	0.78
CNU256	CNU256	TTGAAATACATGATACCCCAACCA	CCGTTTTCAGGGCACAGTTT	TA	3	0.61
CNU257	CNU257	TGCATGATGTTCATGTCTTGTAAA	TCCTTCTGTAAACCGGTTGTAATTT	TA	3	0.48
CNU268	CNU268	TCATTGGTGAAGAACCCACAAA	GCGACCATAAAAAGAGAGTGAGAA	AT	2	0.37
CNU270	CNU270	TCGATGATTAGTTTAGTTATTTCACG	CCTCAAACCAAGGAAGATTTCA	AT	3	0.45
CNU286	CNU286	AGTTGCCCCTATTCATGCAC	AATGCGTTCATGTGGGGATA	TC	2	0.55
CNU288	CNU288	GCGTTTCGTCCTCTTCTCAC	TTACCCACCTTGGCTTCATC	AG	3	0.51
niab063	niab063	GAAGAAACTCGGTGGGGAGT	AAAGAGTTCCGAAAATGGGC	AT	3	0.48
niab090	niab090	GCTGATTTCTCCGCTATCAC	AAGACACCGTTTGTGAATTT	AT	4	0.50
niab096	niab096	CAAAAAGAGCGTTACCTCCA	GATGAAGCTCTGAAGACCGA	AG	2	0.29
niab097	niab097	TTCTTTGGAGATGGTGTGGT	CAATCTTGTGGTGAGGGAAG	AAG	3	0.49
niab113	niab113	CAAAAAGTTGCGGTCAATCT	CCTCCAAAGCTCAATCACTG	AT	2	0.41
niab115	niab115	CGGTGTATACCGAACGAGAA	AAACCCAATCAACCCCTTTA	AT	3	0.56
niab116	niab116	CTGAACCGAATTGGCTAAAA	TAAACAGGGGAACTGAGCTG	AG	4	0.75
niab123	niab123	GGATCTAGAAACCCCTTCACA	ATCTTGTGTCGGGCAGATAA	AG	3	0.51
niab127	niab127	AGGCAGAGCAGTGTGCAT	GTGCCCTGATATGTGCAA	AT	3	0.60
niab129	niab129	CTTCTCGATGGTTTTTCTCG	TCGGAGACAGCGAATGAG	AG	3	0.51
niab131	niab131	ACCAACCAAACCCCAAAC	TTGTTTTCAGTGCTTCGGA	AT	3	0.62
Total					75	12.9
Average					2.88	0.49

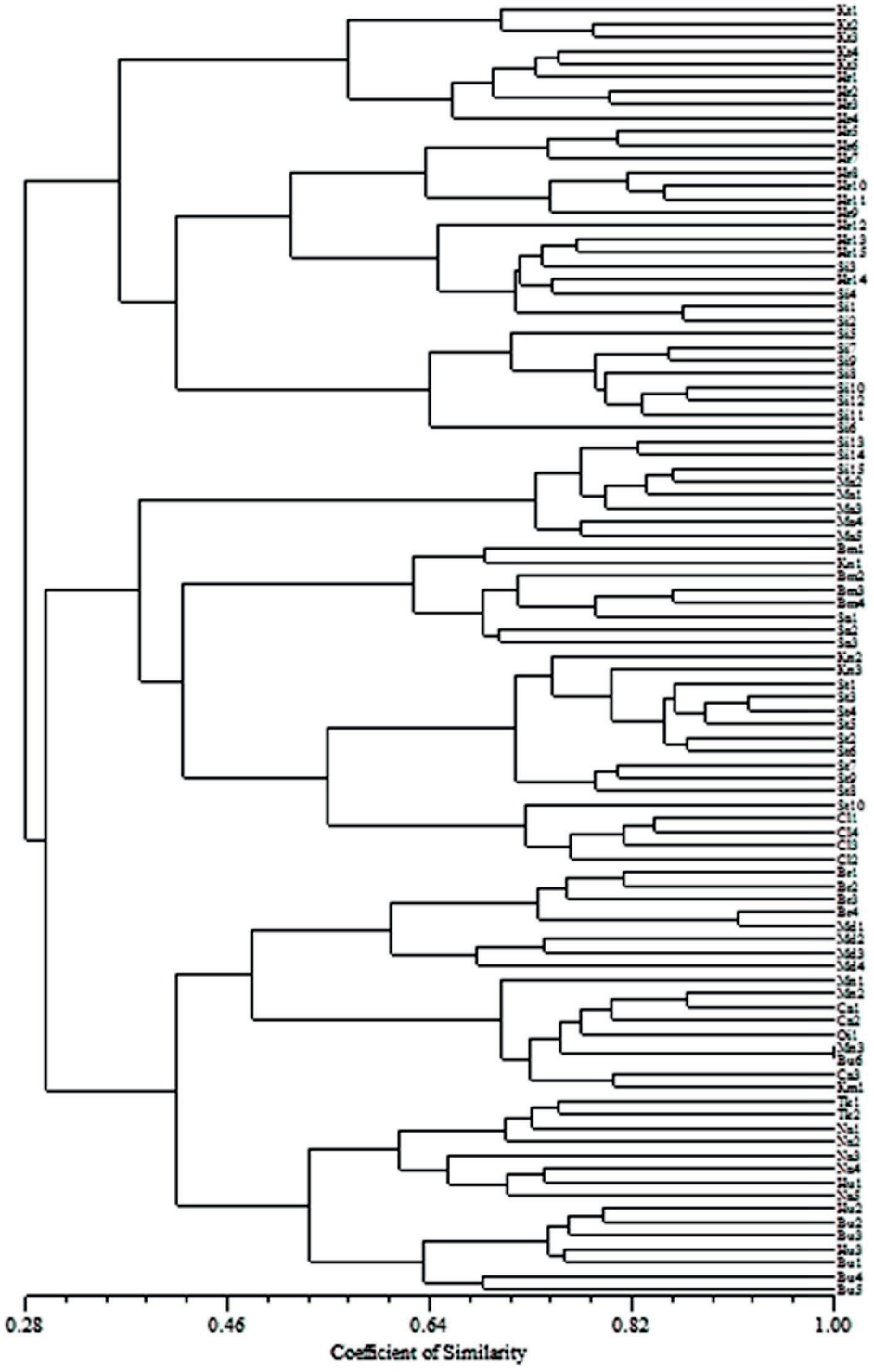

Fig. 2. UPGMA generated dendrogram shows molecular based variability among 96 diverse *B. rapa* germplasm of Khyber Pakhtunkhwa, Pakistan

allelic band and 0 is used for absence of allelic band. Primers producing more bands were selected for further study. The Anderson et al. [1993] method was used for the calculation of the polymorphism information content (PIC) value of each marker used in the study. Average allele numbers, PIC values, and genetic similarities were calculated. Pair-wise associations of the genotypes based on the percentage of exceptional and common amplification products (alleles) were used to measure the genetic similarity by Dice coefficients using PAST program [Hammer et al. 2001, Turi et al. 2012]. The Nei and Li [1979] method was used to calculate the genetic similarities (F) between all pairs of genotypes. Genetic tree was constructed dendrogram using UPGMA based method pair-group method.

RESULTS AND DISCUSSION

Significant level of variation was observed among the tested genotypes using SSR markers. These differences among the genotypes were calculated on the basis of presence and absence of bands. By using 26 primers, 135 bands were produced of which 75 were polymorphic. On average, 2.88 polymorphic bands were produced by each primer. On the basis of overall results of the primers used in the study, 15 primers produced high level of polymorphism (Tab. 2) and were recommended to be used in the genetic diversity of oilseed Brassica crop. After compilation of the SSR results, primer CNU254 was the most polymorphic, producing 5 polymorphic bands, whereas primer CNU246 was the least polymorphic, generating 1 allelic band. Polymorphism produced by SSR markers was in the range of 29 to 78%, having an overall average of 55.5% (Tab. 2). Our results support the previous findings of Stephanie et al. [2009], Gupta et al. [2014] and Thakur et al. [2018], they also reported significant level of polymorphism in different species of oilseed Brassica by using SSR markers. These results will help the future scientists and researchers who want to study the genetic diversity in local or exotic collected Brassica species. Although all the primers used in the study produced significant results, but some produced high level of polymorphism, so the primers CNU254, CNU256, niab116 and niab131 are selected for future studies as they demonstrate the highest polymorphism.

Based on the results, genotypes collected from Kohat, Bannu, Swat, and Haripur showed maximum polymorphism as compared to the collection of other parts of province. The main reason for high level of diversity in these accessions might be due to as these genotypes are not previously evaluated or ignored in any breeding program. These finding are supported by the previous results of Turi et al. [2012], who studied genetic variability in locally collected Brassica species from different regions of Pakistan (Punjab, Islamabad, Gilgit and Khyber Pakhtunkhwa) with the help of SSR markers and reported high variability in genotypes of these areas as compared to the rest of the country. Maximum sub-species level agro-morphological and molecular variability was also recorded by Thakur

Table 3. Distribution of the studied genotypes into various groups and clusters along with their salient features

Main group	Sub-cluster	Number of genotypes	Main features
Group I	CI	9	Disease resistant, more pods per plant and early maturing one
	C2	15	
	C3	8	
Group II	C1	8	Larger pod length, more seeds per pod, more branches per plant but late in maturity
	C2	8	
	C3	11	
	C4	6	
Group III	C1	16	Tall plants, more branches per plant and early to medium maturity
	C2	15	

et al. [2017]. Therefore, there were some relations among those accessions and *B. rapa* species. So it is recommended that these genotypes should be used in further study to evaluate it for other important traits. In order to study the relatedness among the collected genotypes a similarity matrix based on the proportion of shared SSR fragment was established. Polymorphic information content (PIC) values among genotypes ranged from 0.13 to 0.78 with an average PIC value of 0.49 (Tab. 3).

Cluster analysis divided the studied genotypes into three major groups. These major groups were further sub-divided into various clusters (Fig. 2). Group one had three clusters and comprised of 32 accessions from Haripur, Swabi and Kohat areas. Genotypes included in this group were disease resistant, more pods per plant and early maturing one. Group two was constituted of four clusters having 33 genotypes and majority of these genotypes belonged to Swabi, Mansehra and Battagram. The main features of this group were longer pods, more seed per pod, more branches but late in maturity. The third group contained 31 genotypes from Bannu, Hungu, Kurram agency, Nowshera, Malakand, Tank, Nowshera, Bunner, Charsada and Mardan. These genotypes having tall plants, more branches per plant and early to medium in maturity (Fig. 2, Tab. 3). Similar findings were obtained by Das et al. [1999] and by Cansian and Echeverrigaray [2000], who noted similar range of genetic dissimilarities in *B. campestris* and cabbage genotypes. Similarly, Shat et al. [2018] reported maximum genetic variability among Iranian, Indian, Turkish, Ethiopian, Pakistani and Egyptian fenugreek accessions using biochemical markers.

CONCLUSIONS

In large reasonable amount of variations were observed in the evaluated genotypes. But specifically genotypes collected from Kohat, Bannu, Swat and Haripur showed high level of variation as compared to the rest of locations. These genotypes showed unique banding pattern than other ones and highly diverged UPGMA based cluster dendrogram. The main reason for high genetic diversity in the mentioned locations is that as the genetic resources of these areas are less explored as compared to other areas. So it is recommended that the genetic diversity of these locations should be further explored in order to broaden the genetic background of available germplasm and to be used these genotypes in future breeding programs.

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