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Polymorphic information and genetic diversity in *Brassica* species revealed by RAPD markers

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Abstract: Randomly amplified polymorphic DNA (RAPD) is a tremendously convenient approach used to discriminate between *Brassica* species owing to its accuracy and speed. RAPD primers generate adequate genetic information that can be used in the primer-marker system. In this work, twenty RAPD-PCR based markers were executed to generate polymorphic data, like polymorphic information content (PIC), mean resolving power (MRP), resolving power (RP), effective multiplex ratio (EMR), and marker index (MI) for the first time and genetic distance among and between six *Brassica* species were calculated. Our results indicated that 20 primers produced a total of 231 scored band and generated 87% polymorphic bands. Average PIC, MRP, RP, MI, and EMR values were 0.088, 0.65, 6.7, 0.78, and 8.9, respectively. PIC showed an overall negative correlation with MRP, RP, MI, and EMR, whereas MRP, RP, and EMR, were positively correlated with each other. Genetic identities ranged from 41.99% (between *Brassica napus* and *Brassica oleracea*) to 68.83% (between *Brassica campestris* and *Brassica oleracea*). Dendrogram results showed no clustering between species except between *Brassica campestris* and *Brassica nigra*. Nevertheless, these results will be helpful to acquire useful information about the markers and their use to determine the genomic structures of *Brassica* species. Further, based on genetic distance and polymorphic information, new hybrids can be developed for effective oilseed production.

Introduction

Brassicas are known as oilseeds, vegetables, and flavoring crops. Brassicaceae plants are in the 2nd position due to oil production in the world (Warwick, 2011; Raza *et al.*, 2020). Triangle of *Brassica* species created by crossing among six species, i.e., *Brassica nigra, Brassica compestris, Brassica napus, Brassica oleracea, Brassica juncea*, and *Brassica carinata* (Nagaharu and Nagaharu, 1935). Different Brassicaceae plants, mainly vegetables, play a vital part in human foods due to the existence of few chemical compounds such as vitamins, minerals, glucosinolate, and few phenolic compounds; and these plants are known as the leading origin of different polyphenols (Cartea *et al.*, 2011; Neugart *et al.*, 2018). Molecular breeding of significant oilseed crops to acquire good quality and high biomass production are the major causes of a reduction in the genetic differences in some essential plants (Iqbal *et al.*, 2015; Raza, 2020).

Genome mapping and identification of loci for the specific traits (yield, height, and weight, etc.) in crops are the main goals of plant geneticists and breeders. Knowledge of the co-segregation of alleles and their genetic linkages with the desired locus can be verified through the identification of loci using molecular marker systems. The number of loci, their positions, and their order within a chromosome was determined using various marker systems

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[(randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR), etc.)] (Nagaoka *et al*., 2010; Li *et al*., 2012; Amosova *et al*., 2014; Su *et al*., 2015). However, the efficacy of markers largely depends upon the number of amplified alleles and their comparative rate present on the genome. A perfect molecular marker system should be: (i) Polymorphic in nature, (ii) Evenly distributed throughout the genome, (iii) Generate several, independent and reliable loci, (iv) Simplest, fast, accurate, and cheap, (v) Have a link to unique phenotype, and (vi) Requires no prior genomic knowledge (Agarwal *et al*., 2008; Nadeem *et al*., 2018). This ideal molecular marker technique is a hard choice and has been compromised between trustworthy and ease of analysis, mathematical capacity, and courage to reveal polymorphisms.

Nevertheless, the degree of polymorphism is mostly evaluated by two indexes (measurement) (i) heterozygosity (H) having well-known evaluation method and variability formula (Nei and Roychoudhury, 1974; Nei and Li, 1979), and (ii) polymorphism information content (PIC) (Botstein *et al*., 1980) with associated values used for analyses of polymorphic information. The PIC indicates how good the marker/primer is and can specify the polymorphism among different populations based on the number and chronicity of the amplified bands (Botstein *et al*., 1980). The maximum value of PIC for dominant markers is 0.5. Furthermore, there are few associated parameters/values which help to select the effective primer-marker system. Effective multiplex ratio (EMR) is analyzed by several loci, which are polymorphic in the studied germplasm of an experiment (Nagaraju *et al*., 2001). Marker index (MI) is a statistical parameter that indicates the total effectiveness of the marker system, e.g., whether the used marker is suitable or not (Powell *et al*., 1996; Nagaraju *et al*., 2001). Resolving power (RP) is a parameter characterizing the capacity of the primer/marker integration to analyze the differences among a huge number of genotypes (Gilbert *et al*., 1999; Prevost and Wilkinson, 1999; Chesnokov and Artemyeva, 2015).

Genetic diversity can be described as a group of independent individuals or populations in relation to other individuals and populations (Abdel-Mawgood, 2012). Evaluation of genetic diversity is supposed to be an important technique to develop new cultivars or hybrids depending on the genetic identity and distance (Raza *et al*., 2018; Raza *et al*., 2019a). DNA markers are executed to find out the genetic variations and DNA barcoding. DNA based molecular markers are acknowledged as helpful and applicable markers for different applications in crop molecular-breeding, as they are feasible, ample, phenotypically neutral, including time and location independent (Nadeem *et al*., 2018; Raza *et al*., 2019b; Raza *et al*., 2019c). RAPDs markers are widely used in genetic studies due to their simplicity and speed. Nevertheless, RAPD markers are superior when simplicity and cost were appraising. RAPD analysis is one of the most straightforward marker-based technique among all the accessible marker techniques which are executed to evaluate the composition, modifications, and advancement of genetic material (Nadeem *et al*., 2018; Raza *et al*., 2019a; Raza *et al*., 2019c). Previously, RAPD marker systems were successfully used to determine genetic diversity in *Brassica* species (Liersch *et al*., 2013; Iqbal *et al*., 2015; Harun-Or-Rashid *et al*., 2016). However, this technique is being used for the first time in this research to gather the genetic and polymorphic information about *Brassica* species in Pakistan. Furthermore, in this study, RAPD markers are used to evaluate the polymorphic information through statistically calculated PIC, RP, MRP, MI, EMR, and genetic distances among *Brassica* species in Pakistan. The finding of this study will help to have an understanding of the primer-marker system and making a new combination of *Brassica* breeding in Pakistan.

Materials and Methods

Study area

Current research entitled "Polymorphic information and genetic diversity in *Brassica* species revealed by RAPD markers" was performed in the CABB, UAF, Faisalabad, Pakistan. Faisalabad is situated at a latitude of 31.418715 and longitude of 73.079109 with the GPS coordinates of 31°22' 8.2812" N and 72°56' 33.1008" E, having a semi-arid environment.

Plant material

In this study, six *Brassica* genotypes (Tab. 1) were used to calculate the polymorphic information and genetic diversity, and these genotypes were furnished by the UAF, Pakistan. Healthy and young leaf samples were taken for DNA extraction from each species, grown in field conditions and stored at −80°C until DNA extraction.

DNA extraction from leaf samples

Leaf samples of six *Brassica* species (Tab. 1) were washed with double distilled water (d_2H_2O), detached from the plant, immediately wrapped in aluminium foil and put in liquid nitrogen. DNA from each collected sample was extracted using standard 2× CTAB (Sigma-Aldrich, St. Louis, MO, USA) protocol with slight modifications according to Rogers and Bendich (1989) and Sharma *et al*. (2003). Total DNA was extracted from 100 mg of leaf sample (homogenised) from 10 different plants of the same variety within species. DNA was run on 1.5% agarose gel to examine the DNA quality and quantity, which were measured with Nanodrop1000 (Thermo Fisher Scientific, Wilmington, DE,

TABLE 1

***Brassica* species and the cultivated varieties we used in this research**

Sr. No.	Species name	Variety name
1	*Brassica carinata*	Peela Raya
2	*Brassica nigra*	Black Mustard
3	*Brassica oleracea*	Capitata
4	*Brassica juncea*	Raya Anmol
5	*Brassica napus*	Punjab Canola
6	*Brassica campestris*	Toria

USA). 50 ng μL^{-1} DNA was used as a working concentration in PCR amplification.

PCR amplification and RAPD assay

Twenty different random decamer/RAPD primers (Tab. 2) were used to amplify the DNA through the PCR machine (Bio-Rad, California, USA). The total PCR reaction mixture of 25 µL contained, 3 µL of DNA template (50 ng μL^{-1}), 0.2 µL of *Taq* polymerase (0.2 units), 2.5 µL of 10× *Taq* buffer, 3 µL $MgCl_2$, 5 µL of dNTPs (10 mM), 1 µL decamer primer (5 pmol) and 10.3 µL of double-distilled deionized water. Amplification was done in a thermocycler machine with the reaction conditions as follow: denaturation at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min for the total of 35 cycles. Amplified bands were separated according to size on 2% agarose gel.

Observation recording and statistical data analysis

The gel separated fragments were visualized with GDS system (Bio-Rad, California, USA). All amplicons from every single primer were noticed as "1" for present and "0" for absent for six *Brassica* species. An ambivalent band that may not be clearly described was not scored. The amplified bands were calculated from top to bottom of the lane to obtain binary data matrices.

The total number of scored bands (TNSB), a total number of the polymorphic band (TNPB), the total number of the monomorphic band (TNMB) and percentage of polymorphic bands (PPB) were obtained through the counting of reliable visible bands. PIC of every band was measured, as described by Anderson *et al.* (1993), Wangphanich *et al.* (2010), and Chesnokov and Artemyeva (2015).

$$\mathrm{PIC}_i = 1 - \sum_j f_{ij}^2$$

where f_{ij} is the frequency of the j^{th} pattern of the i^{th} band whether it is present or not, in case of dominant markers. After this, the PIC of every RAPD marker was measured as:

$$PIC = 1/n \sum_{i=1}^{n} PIC_i$$

where n is the TNPB for that marker.

MRP is a parameter which is used to characterise the capacity of the primer/marker integration to analyze the differences among a huge number of genotypes. For the calculation of RP, information of a band (BI) was measured as:

$$\mathrm{BI}_i = 1 - (2x|0.5 - p|)$$

where p is the percentage of the six species having bands.

TABLE 2

The measure of polymorphic information through RAPD primers used in the study

Primer	Primer Sequence (5'–3')	TNSB	TNPB	TNMB	PPB	PIC	MRP	RP	MI	EMR
RAPD L1	CGCAAGACCT	14	12	2	85.71	0.07	0.63	7.66	0.78	10.28
RAPD L2	TCGGCGTCAA	10	10	0	100.00	0.08	0.48	4.83	0.88	10.00
RAPD L3	CCAGCAGCTT	13	12	1	92.30	0.07	0.63	7.66	0.84	11.07
RAPD L4	GACTGCACAC	7	6	1	85.71	0.14	0.83	5.00	0.72	5.14
RAPD L5	ACGCAGGCAC	8	8	0	100.00	0.10	0.52	4.16	0.87	8.00
RAPD L6	GAGGGAAGAG	9	9	0	100.00	0.09	0.46	4.16	0.84	9.00
RAPD L8	AGCAGGTGCA	15	14	1	93.33	0.06	0.67	9.50	0.86	13.06
RAPD L9	TGCGAGAGTC	9	9	0	100.00	0.09	0.61	5.50	0.87	9.00
RAPD L10	TGGGAGATGG	8	8	0	100.00	0.10	0.47	3.83	0.85	8.00
RAPD L12	GGGCGGTACT	10	8	2	80.00	0.11	0.77	6.16	0.70	6.40
RAPD L13	ACCGCCTGCT	8	7	1	87.50	0.12	0.71	5.00	0.75	6.12
RAPD L14	GTGACAGGCT	11	9	2	81.81	0.09	0.77	7.00	0.73	7.36
RAPD L15	AAGACAGCGG	12	10	2	83.33	0.08	0.61	6.16	0.74	8.33
RAPD L16	AGGTTGCAGG	11	9	2	81.81	0.10	0.48	4.33	0.74	7.36
RAPD L17	AGCCTGAGCC	17	14	3	82.35	0.06	0.67	9.50	0.76	11.52
RAPD L18	ACCACCCACC	12	10	2	83.33	0.09	0.73	7.33	0.75	8.33
RAPD L19	GAGTGGTGAC	10	8	2	80.00	0.11	0.85	6.83	0.71	6.40
RAPD L20	TGGTGGACCA	22	18	4	81.81	0.05	0.75	13.66	0.77	14.72
RAPD K11	AATGCCCCAG	14	12	2	85.71	0.07	0.76	9.16	0.78	10.28
RAPD K12	TGGCCCTCAC	11	10	1	90.90	0.09	0.73	7.33	0.82	9.09
Total	—	**231**	**203**	**28**	—	**1.77**	**13.13**	**134.76**	**15.76**	**179.46**
Percentage	—	—	**87.87**	**12.12**	—	—	—	—	—	—
Average	—	**11.55**	—	—	**88.78**	**0.08**	**0.65**	**6.73**	**0.78**	**8.97**

Note: TNSB: Total number of scored bands, TNPB: Total number of polymorphic bands, TNMB: Total number of monomorphic bands, PPB: Percentage of polymorphic bands, PIC: Polymorphism information contents, MRP: Mean resolving power, RP: Resolving power, MI: Marker index, EMR: Effective multiplex ratio.

Then, the RP of each primer was measured as:

$$RP = \sum_{i=1}^{n} BI_i$$

where n is the TNPB for that marker (Prevost and Wilkinson, 1999).

Further, we calculated MRP for each marker as:

$$MRP = 1/n \sum_{i} BI_i$$

EMR was measured as a total number of polymorphic bands/per primer multiplied by the percentage of the polymorphic bands per their total number.

$$EMR = np\left(\frac{np}{n}\right)$$

where *np* is the number of polymorphic bands, and *n* is the total bands number. The high EMR value indicates the more efficiency of the primer-marker system.

MI is a statistical parameter used to calculate the total effectiveness of the primer-maker system. MI is the product of the PIC values (or expected heterozygosity, HE) and EMR. MI was measured as:

$$\text{MI} = \text{PIC} \times EMR$$

Besides, the relationships among PIC, MRP, RP, EMR, and MI were calculated. The binary data were analysed through Popgene32 software v1.44 (Department of Renewable Resources, University of Alberta, Canada) (Yeh *et al.*, 2002) for genetics distance among *Brassica* species. A dendrogram was generated after cluster examination for the similar coefficients with the unweighted pair group method of the arithmetic mean (UPGMA) (Sneath and Sokal, 1973). Besides, principal coordinate analysis (PCoA) was performed with genetic distances calculated by Popgen32 software present in *Brassica* species.

Results

Estimation of polymorphism

Twenty decamer primers were used to generate valuable information about polymorphism (Tab. 2). Bands generated by primer L6 and L16 are shown in Fig. 1. These primers generated, in total, 231 bands (TNSB) which could easily be scored. The total number of amplified fragments produced by every primer varied from 7 (RAPD-L4) to 22 (RAPD-L20) with a mean of 11.55 fragment per primer. The size of the amplified fragments was varying from 0.1–2 kb. Primers showed the different capacity to amplify polymorphic bands. A single primer scored a simple measure of primer variability based on a number of polymorphic fragments. Out of the total, 203 bands showed polymorphism (87.87%), and 28 bands showed monomorphism (12.12%) (Tab. 2). The highest number of polymorphic bands (18) were generated by primer RAPD-L20, followed by RAPD-L08, RAPD-L17 (Tab. 2). These primers could be used to calculate reliable genetic distance and diversity.

Measurement of effective polymorphic information

For each RAPD primer, TNSB, TNPB, TNMB, PPB, PIC, MRP, RP, MI and EMR were calculated (Tab. 2). Average PIC, MRP, RP, MI and EMR values for all primers were 0.088, 0.65, 6.73, 0.78, and 8.97, respectively (Tab. 2). Values of PIC with all primers indicated that polymorphism was unequally distributed in the population. The validity of the unequal distribution of markers was further observed in the strong negative correlation between PIC and TNPB (Fig. 2). Primers RAPD-8 and RAPD-20 were seemed more efficient for the primer-marker system as both had higher EMR value (Tab. 2). Good MRP and higher MI value indicated that all primers could detect the differences within and between subjected populations.

The relationship between effective polymorphic parameters

PIC showed a negative correlation among all parameters (Fig. 2). The highest negative co-relation with TNPB indicated that higher the number of polymorphic bands generated by a primer lower would be the value of PIC. MI had a negative correlation with PIC (−0.20) and MRP (−0.62), but a positive correlation with the rest of the parameters. The relationships among MRP, TNMP, RP, EMR, and TNPB indicated positive correlation (Fig. 2).

Banding pattern, genetic distance, and cluster analysis among Brassica species

Twenty primers generated the highest number of 163 bands in *B. carinata* and a minimum number of 120 bands in *B. campestris* (Fig. 3). Five species, i.e., *B. nigra, B. juncea, B. campestris, B. napus, B. carinata* possessed unique private bands 1, 3, 1, 7, and 8, respectively (Fig. 3). Each primer developed a specific, effective and measurable band of changeable capacity. The level of polymorphism was different with different primers between six *Brassica* species. The highest number of bands (22) was displayed by primer

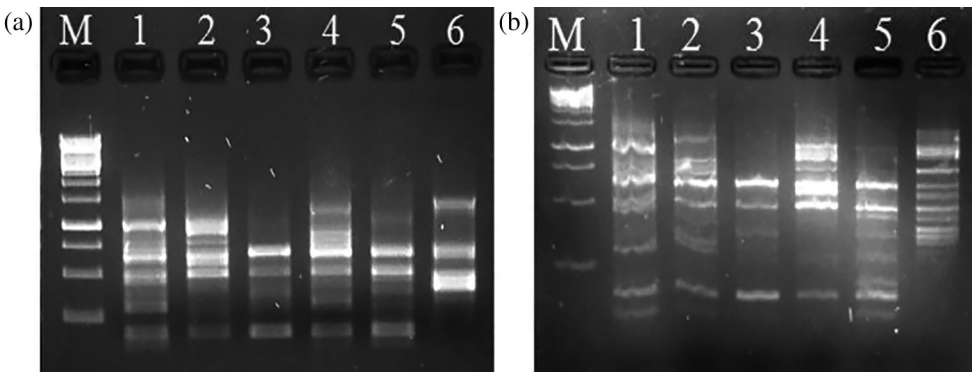

FIGURE 1. Amplification of bands using (a) Primer L6 and (b) Primer L16 on DNA of six *Brassica* species. Lane M: 1 kb Ladder; Lane 1: *Brassica nigra*; Lane 2: *Brassica juncea*; Lane 3: *Brassica campestris*; Lane 4: *Brassica napus*; Lane 5: *Brassica oleracea*; Lane 6: *Brassica carinata*.

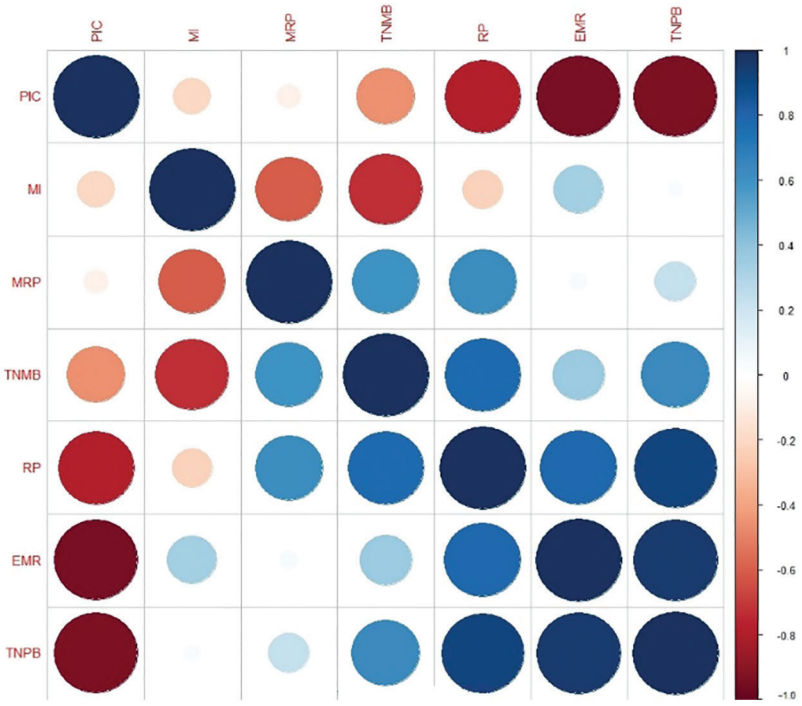

FIGURE 2. Correlation among parameters.
Blue is a positive correlation, while red is a negative correlation. Dark and light color shows the strength of the correlation, i.e., very-strong to very-low. TNPB: Total number of polymorphic bands, TNMB: Total number of monomorphic bands, PIC: Polymorphism information contents, MRP: Mean resolving power, RP: Resolving power, MI: Marker index, EMR: Effective multiplex ratio.

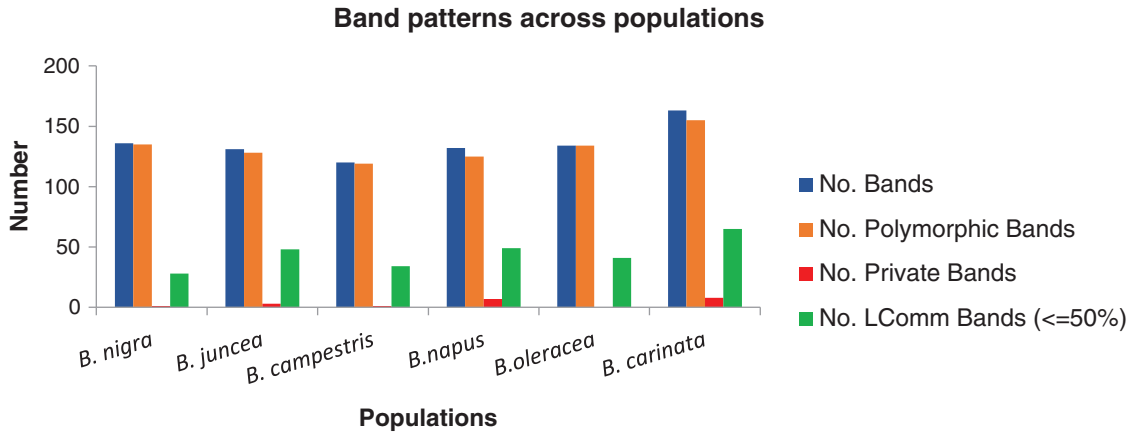

FIGURE 3. Graphical representation of the banding pattern of total number score bands (TNSB), monomorphic bands (TNMB), and polymorphic bands (TNPB), gained by six *Brassica* species.

L20 and the lowest number of bands (07) displayed by primer L4 (Tab. 2). The genetic identity ranged from 41.99% (between *B. napus* and *B. oleracea*) to 67.97%, which is between *B. nigra* with *B. campestris* and *B. oleracea* (Tab. 3). Based on dendrogram, results indicate that *B. campestris, B. nigra* and *B. oleracea* are closely related. These three *Brassica* species are the parents of the next three species. According to U's triangle, the *B. campestris* cross with *B. nigra* produces *B. juncea*, while *B. campestris* cross with *B. oleracea* gives rises to *B. napus*. The cross among *B. nigra* and *B. oleracea* give rises to *B. carinata* (Nagaharu and Nagaharu, 1935). The dendrogram showed the distance between parents and offsprings (Fig. 4). Association among *Brassica* species through principal coordinate analysis (PCoA) showed no grouping and were spread all over PCoA1 and PCoA2 (Fig. 5).

Discussion

The primary purpose of this study was to collect genetic information about Brassica species by the use of RAPD primers in Pakistan. Additionally, this research aimed at predicting the efficacy of RAPD primers in determining the genetic diversity present in six *Brassica* species in Pakistan. RAPD marker system was previously used to determine the genetic differences in *Brassica* species (Liersch *et al.*, 2013; Kumar *et al.*, 2015; Iqbal *et al.*, 2015; Harun-Or-Rashid *et al.*, 2016). However, none of them worked on the determination of RAPD primers efficacy in determining genetic diversity. In this experiment, additional parameters like PIC, MRP, RP, MI, and EMR, were calculated to determine the efficacy of RAPD primers along with genetic diversity in *Brassica* species in Pakistan. These parameters were used in the past for the genetic characterization of marijuana (Kayis *et al.*, 2010), and the PIC was used in *Brassica* (Fazeli *et al.*, 2008; Kanwal and Nawaz, 2014; Raza *et al.*, 2019b). RAPD primers generated data, which indicated the effectiveness of primers and successfully supported the use of the primer-marker system to detect genetic divergence among *Brassica* species in Pakistan. The same kind of attempts was made

TABLE 3

The genetic identity (upper diagonal) and the genetic distance (lower diagonal) for six *Brassica* species using RAPD markers

Pop. ID	*B. nigra*	*B. juncea*	*B. capmestris*	*B. napus*	*B. oleracea*	*B. carinata*
B. nigra	****	0.5714	0.6797	0.6277	0.6797	0.5541
B. juncea	0.5596	****	0.5368	0.4416	0.6234	0.5758
B. capmestris	0.3862	0.6221	****	0.5325	0.6883	0.4675
B. napus	0.4657	0.8174	0.6302	****	0.4199	0.4935
B. oleracea	0.3862	0.4726	0.3735	0.8677	****	0.5281
B. carinata	0.5904	0.5521	0.7603	0.7062	0.6384	****

Note: stars (****) within the table are used to differentiate between the upper diagonal (above stars) and the lower diagonal (below stars).

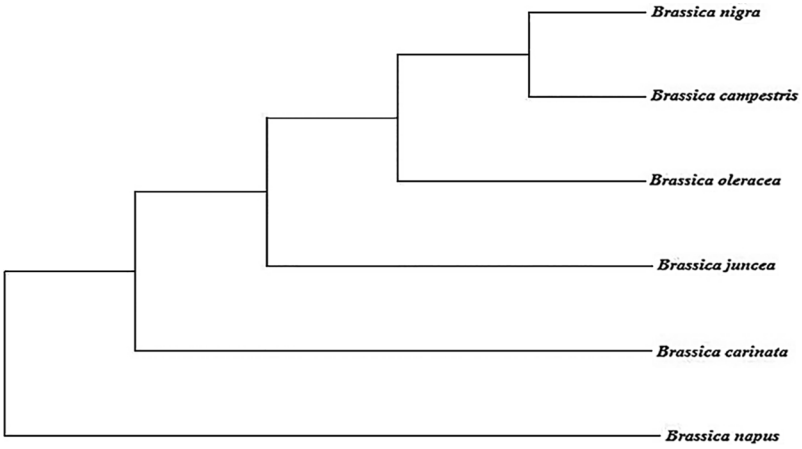

FIGURE 4. The unweighted pair group method with arithmetic means (UPGMA) dendrogram based on summarised data regarding differentiation among six *Brassica* species.

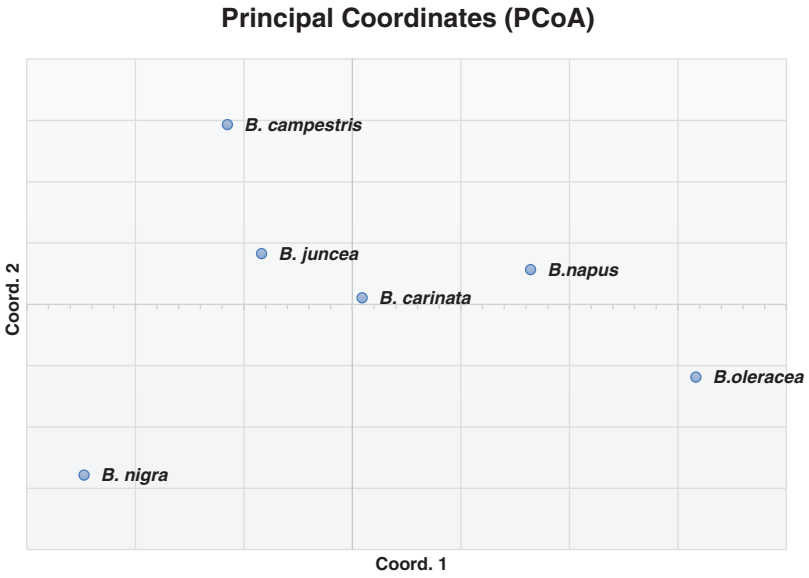

FIGURE 5. Principal component analysis (PCA) among six *Brassica* based on genetic distance.

in the past by Kayis *et al.* (2010); estimation of PIC was calculated by the number of the researcher (Fazeli *et al.*, 2008; Raza *et al.*, 2019b), while very few researchers measured rest of the parameters.

In a recent study, Raza *et al.* (2019b) observed PIC values varying from 0.375 to 0.719, with a mean of 0.66 between six *Brassica* species using SSR markers. While Fazeli *et al.* (2008) measured PIC values varying from 0.29 to 0.48 between different *Brassica napus* genotypes collected from various regions of the world using RAPD markers. In this experiment, low PIC value was observed, whereas the observed values for RP and EMR were high for the primers, which produced maximum numbers of TNPB. High RP in RAPD primers indicated the effectiveness of primer to use for the determination of genetic distance (Kayis *et al.*, 2010) and also determined MRP, RP, and MI values (Kayis *et al.*, 2010) and the means of these parameters are approximately near to current results. Matrix correlation was mostly used for phenotypical characters, but with the use of a molecular marker, it was used to evaluate the dissimilarities of RAPD

and ISSR marker (Santhosh *et al*., 2009; Kayis *et al*., 2010). However, the correlation with low PIC value, high RP, and EMR values means that primer is reliable to generate a huge number of polymorphic bands.

In this research, 20 RAPD markers were used to calculate the polymorphic information and genetic diversity among six *Brassica* genotypes. Overall, 231 bands were reported by these markers with a mean of 11.55 bands per marker and showed 88.78% polymorphism. In another research, Fazeli *et al.* (2008) noticed 86 bands using 9 random RAPD primers with an average of 9.55 bands per primer and showed 93.02% polymorphism. Harun-Or-Rashid *et al.* (2016) measured 23 amplified bands with a mean of 7.66 bands per marker revealed by 3 RAPD markers and gave 91.3% polymorphism, and these outcomes gave strong support to current results. Several researchers used SSR markers for the genetic diversity of Brassica species, but only a few reports are available on RAPD markers. For the first time, we evaluated the genetic distance, polymorphic information, and some other useful parameters (PIC, MRP, RP, MI, and EMR) to determine the efficacy of RAPD markers in Brassica genotypes.

Molecular markers are now acknowledged as a very convenient and high-quality technique for the assessment of genetic diversity at a DNA level. Molecular breeding of major oilseed crops, for good quality and high production, results in a decrease of the genetic divergence in various important plants. Molecular markers, morphological parameters, and pedigree studies are the primary source to measure genetic differences. The molecular marker provides knowledge about the better evaluation of genetic differences, as these markers are self-determining for confounding results of environmental factors (Iqbal *et al*., 2015).

Conclusion

The current study describes that genetic diversity and polymorphism exists among and between six *Brassica* species. Nevertheless, before the selection of a specific primer-marker system, it is required that RAPD primers should be evaluated statistically for their efficiency in assessing the polymorphic information. The calculation of statistically significant associations, the correlation among morphological and molecular parameters are the critical points in the final selection of markers. The diversity in PIC values with the reported values is due to the variation in genotypes and sample sizes. Although PIC values indicate the goodness of the markers based on the polymorphisms, here, some primers show fewer PIC values due to a negative correlation between PIC and polymorphic bands. Thus, based on the findings of this study, higher MRP is another crucial element to determine the primer efficiency. It is shown that RAPD is a very economical method and can give substantial information regarding diversity among any plant.

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