ORIGINAL ARTICLE

Genetic Diversity Analysis of *Brassica* Species Using PCR-Based SSR Markers

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Abstract
Genetic diversity is an important measure for the improvement of many crop species including *Brassica*. This study evaluated the genetic divergence among six *Brassica* species using simple sequence repeats (SSR). Ten SSR markers produced overall 21 alleles with an average of 2.1 alleles per primer. Out of 21, 18 alleles showed polymorphism (85.71%) and 3 alleles showed monomorphism (14.28%). A similarity matrix was constructed using Popgen32 software. Genetic identity ranged from 33.33 to 76.19%. *B. nigra* and *B. campestris* showed the highest identity, while the lowest identity was observed between *B. campestris* and *B. oleracea*. Sizes of amplified alleles were ranged from 70 to 290 bp. Polymorphic information content (PIC) varied from 0.37 to 0.71, with an average of 0.66 per primer. A dendrogram classified the genotypes into two main clusters. Cluster-A is further divided into cluster-C, which consists of *B. carinata* and *B. oleracea*. *B. napus* and *B. juncea* each form an independent cluster. Cluster-B consists of *B. nigra* and *B. campestris,* meaning that these two species are closely related to each other. The results indicated that these species can be isolated from each other at the molecular level by using molecular markers.

Keywords Alleles · Dendrogram · Gel electrophoresis · Molecular markers · Polymorphic information content

Genetische Diversitätsanalyse von *Brassica*-Arten unter Verwendung von PCR-basierten SSR-Markern

Zusammenfassung
Die genetische Diversität ist ein wichtiges Maß für die Verbesserung vieler Kulturpflanzen einschließlich *Brassica*. In der vorliegenden Studie wurde die genetische Divergenz zwischen 6 *Brassica*-Arten unter der Verwendung von Mikrosatelliten („simple sequence repeats", SSR) untersucht. Dabei produzierten 10 SSR-Marker insgesamt 21 Allele mit einem Durchschnitt von 2,1 Allelen pro Primer. Von den 21 Allelen zeigten 18 Polymorphismus (85,71%), und 3 Allele zeigten Monomorphismus (14,28%). Mit der Popgen32-Software wurde eine Ähnlichkeitsmatrix erstellt. Die genetische Identität lag im Bereich von 33,33–76,19%. Die höchste Identität wiesen *B. nigra* und *B. campestris* auf, während die niedrigste Identität zwischen *B. campestris* und *B. oleracea* beobachtet wurde. Die Größen der amplifizierten Allele lagen im Bereich von 70–290 bp. Der polymorphe Informationsgehalt (PIC) variierte von 0,37–0,71, mit einem Durchschnitt von 0,66 pro Primer. Ein Dendrogramm klassifizierte die Genotypen in 2 Hauptcluster. Cluster-A wird weiter unterteilt in Cluster-C, der aus *B. carinata* und *B. oleracea* besteht. Jeweils einen unabhängigen Cluster bilden *B. napus* und *B. juncea*. Cluster-B besteht aus *B. nigra* und *B. campestris*, was bedeutet, dass diese beiden Arten eng miteinander verwandt sind. Die Ergebnisse zeigten, dass diese Spezies auf molekularer Ebene unter Verwendung molekularer Marker voneinander isoliert werden können.

Schlüsselwörter Allele · Dendrogramm · Gelelektrophorese · Molekulare Marker · „Polymorphic information content"

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Introduction

The genus *Brassica* includes 37 varying species and has economic gains (Tsunoda et al. 1980). The family Brassicaceae is not only an important agricultural crop but also has commercial value. Controversy exists in numbering of basic chromosomes of *Brassica*, as their chromosome number X ranged from 7–11. Molecular research does not support *Brassica* species with chromosome number (X = 7) due to their multiple origins (Abbas et al. 2009). *Brassica* is commonly recognized as a flavoring crop, oilseed, and vegetable. In oil production worldwide, *Brassica* secures the second position, because its seeds have the potential of producing high quality oil. The raw materials have significant importance in production of biodiesel. In order to achieve future edible oil needs, *Brassica* breeding is essential to enhance the seed quality (Riaz et al. 2001). Different *Brassica* species, especially vegetables, play an important role in the human diet due to the presence of many compounds like vitamins, minerals, glucosinolates, and some phenolic compounds. Vegetables and some other plants of the Brassicaceae family are known as a major source of polyphenols (Harbaum et al. 2007). Molecular-based breeding of oilseed plants for superior quality and huge production are major sources of reduction in genomic variation in a few essential plants (Iqbal et al. 2015).

Molecular markers have developed a huge range of applications in the field of molecular biology that may include phylogenetic study, evolution, ecology, population genetics, and study of complicated genomic traits in both plants and animals. These different applications are achievable due to rapid advances in biological and molecular techniques (Morin et al. 2004). DNA-based markers contribute towards the utilization of useful genes in plant genetic engineering (Agrama and Tuinstra 2003). Different markers can be used to estimate genetic diversity, like amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), single nucleotide polymorphisms (SNP), simple sequence repeats (SSR), and random amplified polymorphic DNA (RAPD; Pejic et al. 1998).

Different molecular markers have been used for the desired plant selection and to help plant breeders in selecting the plant having the desired traits directly or indirectly. DNA markers are recognized as useful markers for application in plant molecular breeding as they are usable, abundant, phenotypically unbiased, and time and stage independent (Hoshino et al. 2012). Microsatellites or SSR markers are the most popularly used PCR-based markers. These markers are successfully utilized across plant species for practical approaches as they are co-dominant, locus specific, hypervariable, multi-allelic, and robust. The genome-wide distribution of SSR markers makes them suitable markers for gene mapping and high-ranked genotyping, and helpful in marker-assisted breeding and gene mapping (Kong et al. 2000; Edwards et al. 2007; Murray et al. 2009). The objective of the current research was to estimate the genetic diversity and polymorphic information contents (PIC) among *Brassica* species using PCR-based SSR markers.

Materials and Methods

Experimental Site and Location

A screen house study entitled "Genetic diversity analysis of *Brassica* species using PCR based SSR markers" was conducted in the Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan. Faisalabad is located at a latitude of 31.421045 and longitude of 73.089294, having a semiarid environment.

Plant Material

The *Brassica* genotypes used in this research were provided by the University of Agriculture, Faisalabad (UAF). Six genotypes (Table 1) were sown in compost and sand (1:3) in the growth room of CABB, University of Agriculture, Faisalabad, Pakistan. Healthy and fresh leaves were taken and stored at –20 °C for good-quality DNA extraction.

DNA Extraction and Quantification

DNA was extracted from each collected sample using standard 2xCTAB extraction buffer as described by Ramos-Gómez et al. (2014). To confirm the DNA extraction, samples were run on 1% agarose gel and DNA quantification was measured by using Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). DNA dilutions (25 ng/μl) were prepared for PCR reaction.

Table 1 *Brassica* species and their cultivated varieties used in this study

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

Table 2 List of SSR primers and their features used in this study

SSR primer	Motif type	Primer sequence (5′–3′)	Expected allele size (bp)	Annealing temperature (°C)
BRMS-008	di TC/AG	F: AGGACACCAGGCACCATATA	145	55
		R: CATTGTTGTCTTGGGAGAGC		
BRMS-019	di GT/CA	F: CCCAAACGCTTTTGACACAT	220	55
		R: GGCACAATCCACTCAGCTTT		
BRMS-027	di GA/CT	F: GCAGGCGTTGCCTTTATGTA	205	56–57
		R: TCGTTGGTCGGTCACTCCTT		
BRMS-029	di GA/CT	F: AACAAATGACACACACCACACT	232	56
		R: ATTGAAAATCTTAACCGTGAAG		
Na10-E02	di GA/CT	F: TCGCGCATGTAATCAAAATC	136	55
		R: TGTGACGCATCCGATCATAC		
Na10-G10	di GA/CT	F: TGGAAACATTGGTGTTAAGGC	237	58–59
		R: CATAGATTCCATCTCAAATCCG		
Na12-B09	tri GGC/CCG	F: ACGGAAGATCAAACAGCTCC	208	59–60
		R: TGAGCGACCCATTCTTTAGG		
Na12-C07	di GA/CT	F: ACTCAACCCCACAAACCTG	184	58
		R: AGTTCCCCGGATCCGATTAG		
Ra2-D04	di GT/CA	F: TGGATTCTCTTTACACACGCC	176	55
		R: CAAACCAAAATGTGTGAAGCC		
Ra2-E03	di GA/CT	F: AGGTAGGCCCATCTCTCTCC	279	55–56
		R: CCAAAACTTGCTCAAAACCC		

F Forward primer, *R* Reverse primer, *SSR* simple sequence repeat

PCR-Based SSR Analysis and Agarose Gel Electrophoresis

SSR analysis was carried out using 10 primer pairs. A few important properties of these primers are displayed in Table 2. The PCR (Bio-Rad, California, USA) protocol was conducted in 25 µl of mixture using standard protocols reported by Tommasini et al. (2003). The PCR cycles consist of beginning denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 sec (based on primer), extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. SSR (PCR) amplification bands were analyzed by 3% (w/v) agarose gel electrophoresis at 120 voltage, and amplified bands were viewed on a gel documentation system (Bio-Rad GDS, California, USA).

Statistical Data Analysis

Bands scoring were done by using (1) for presence and (0) for absence of bands in order to obtain the genetic similarity matrix. Genetic similarities were estimated through Popgene32 software version 1.44 (Department of Renewable Resources, University of Alberta, Canada) (Yeh et al. 2002). The dendrogram was built based on the unweighted pair group method with arithmetic average (UPGMA) displaying genetic relationships between six genotypes. Polymor-

phism information content (PIC) for every marker was estimated using Power-marker software (Liu and Muse 2005).

Results

Level of Polymorphism

A set of ten SSR primers was used to evaluate the level of polymorphism among six *Brassica* species. Primers displayed a different level of polymorphism among all six genotypes. Overall, 21 alleles were generated by SSR markers with a mean of 2.1 alleles per primer. Out of ten, only three primers (BRMS-019, Na10-G10 and Na12-B09) gave both polymorphic and monomorphic bands while remaining seven showed only polymorphic band pattern. The highest number of alleles was produced by the two primers BRMS-019 and Na10-G10, while a lower number of alleles was produced by primer BRMS-027 (Table 3).

Polymorphic Information

For all SSR primers, the quantity of amplified alleles (QAA), quantity of polymorphic alleles (QPA), quantity of monomorphic alleles (QMA), percentage of polymorphic alleles (PPA), and polymorphism information content (PIC) were calculated. Out of the total, eighteen alleles showed polymorphism (85.71%) and three alleles showed

Table 3 SSR markers with analyzed data

S. no.	Name of primer	Observed size (bp)	QAA	QPA	QMA	PPA	PIC
1	BRMS-008	70–200	2	2	0	100	0.6589
2	BRMS-019	120–260	3	2	1	66.66	0.7193
3	BRMS-027	100–250	1	1	0	100	0.6990
4	BRMS-029	100–260	2	2	0	100	0.7193
5	Na10-E02	70–190	2	2	0	100	0.6800
6	Na10-G10	160–260	3	2	1	66.66	0.6990
7	Na12-B09	160–220	2	1	1	50.00	0.6990
8	Na12-C07	150–250	2	2	0	100	0.7193
9	Ra2-D04	150–190	2	2	0	100	0.7124
10	Ra2-E03	270-290	2	2	0	100	0.3750
Total	–	–	*21*	*18*	*3*	–	*6.6812*
*Percentage/Average**	–	–	*2.1**	*85.71*	*14.28*	*88.33**	*0.6681**

QAA quantity of amplified alleles, *QPA* quantity of polymorphic alleles, *QMA* quantity of monomorphic alleles, *PPA* percentage of polymorphic alleles, *PIC* polymorphism information content, *SSR* simple sequence repeat, * indicates the average values of QAA, PPA and PIC produced by each primer

Table 4 Similarity matrix (above diagonal) and genetic distance (below diagonal) based on SSR analysis of six *Brassica* species

Pop ID	*B. carinata*	*B. nigra*	*B. oleracea*	*B. juncea*	*B. napus*	*B. campestris*
B. carinata	–	0.4762	0.7143	0.6667	0.7143	0.5238
B. nigra	0.7419	–	0.3810	0.5238	0.4762	0.7619
B. oleracea	0.3365	0.9651	–	0.6667	0.6190	0.3333
B. juncea	0.4055	0.6466	0.4055	–	0.5714	0.5714
B. napus	0.3365	0.7419	0.4796	0.5596	–	0.6190
B. campestris	0.6466	0.2719	1.0986	0.5596	0.4796	–

monomorphism (14.28%). The percentage of polymorphic alleles was 88.33%. All primers produced specific, effective, and measurable alleles. The amplified alleles ranged from 70 to 290 bp. All primers displayed different levels of polymorphism among six *Brassica* species. Distinction capacities for every single genotype were determined using PIC values. PIC varied from 0.37 to 0.71, with a mean of 0.66 per marker. Nine SSR primers indicated PIC numbers more than 0.5, which means there is a huge genetic variation present among the six *Brassica* species (Table 3). PIC values indicated that primers were equally distributed in populations. Polymorphic information observed by SSR primers can be used for the production of hybrid genotypes.

Similarity Matrix and Cluster Analysis

Genetic similarity among and distance between the six *Brassica* species were estimated using Popgene32 software based on Nei's UPGMA (Yeh et al. 2002). Genetic similarity ranged from 33.33 to 76.19%. The similarity matrix clearly showed that *B. nigra* and *B. campestris* were closely related species (76.19%), and that *B. campestris* and *B. oleracea* were the most dissimilar species (33.33%). Likewise, *B. oleracea* and *B. carinata* were 71.43% similar, and thus the second most similar species. Results of

genetic similarity matrix and distance displayed that all six *Brassica* species are associated with each other (Table 4).

The dendrogram was constructed using Popgene32 software based on Nei's UPGMA based on SSR data. The dendrogram clustered the genotypes into two major clusters, A and B.

Cluster-A is further divided into cluster-C which consists of *B. carinata* and *B. oleracea*. *B. napus* and *B. juncea* each form an independent cluster. Cluster-B consists of *B. nigra* and *B. campestris,* meaning that these two species are closely related to each other (Fig. 1).

Discussion

In the present study, ten SSR primers were used to estimate the genetic diversity between six *Brassica* species. Overall, 21 alleles were generated by SSR markers, with a mean of 2.1 alleles/primer. Another study investigated a mean of 2 alleles per primer using 7 SSR markers for 31 *B. napus* cultivars (Uzunova and Ecke 1999); 3.9 alleles per primer were reported by Varghese et al. (2000) for 31 rapeseed winter genotypes using 6 SSR markers; and Tonguç and Griffiths (2004) noticed 4.44 alleles per primer among 54 *Brassica oleracea* genotypes with the help of

Fig. 1 Dendrogram of six *Brassica* species constructed from a similarity matrix based on Nei's UPGMA using ten simple sequence repeat (SSR) markers
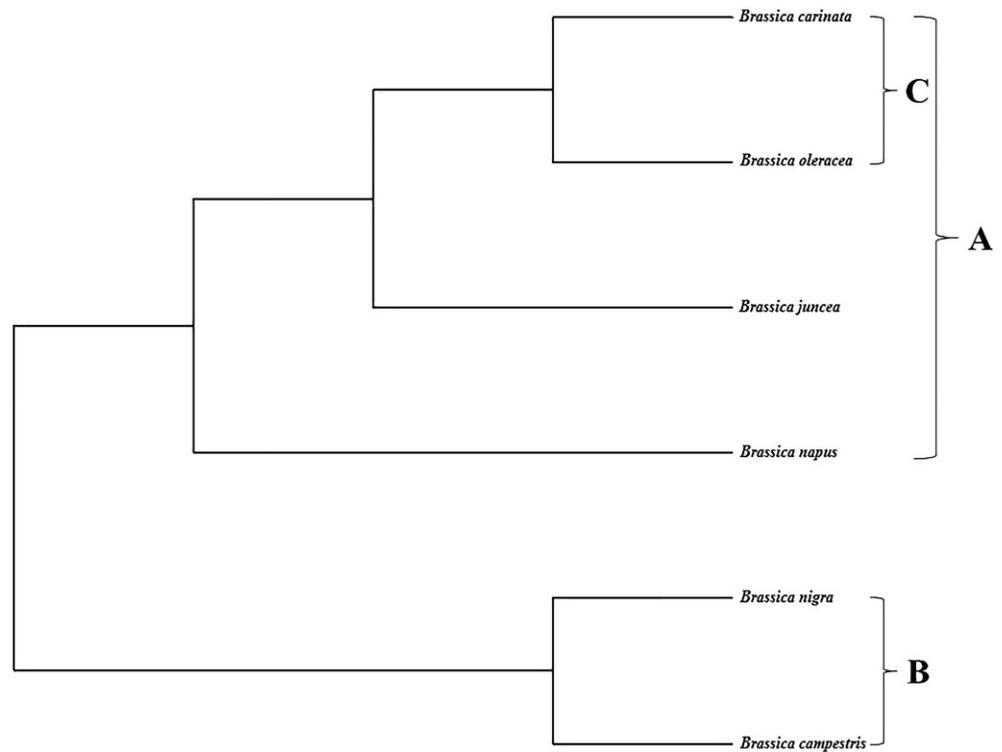
13 SSR primer pairs. In other research, 4 loci per primer were measured for *Brassica napus* cultivars using morphological markers noticed by Yu et al. (2005). However, 8.7 to 9.8 bands were observed in *Brassica carinata* by Teklewold and Becker (2006). These variations in number of bands might be due to the procedure or material used in those studies. In the current research, the mean number of alleles per primer was 2.1, which is closely related to the average number of alleles reported by Hvarleva et al. (2007). In 2003, Tang and Knapp (2003) performed an experiment for 47 accession of native American land races and wild genotypes of cultivated sunflower using 122 SSR markers. These markers produced 1341 alleles between the 47 genotypes with an average of 12 alleles per primer, the average number of alleles between wild genotypes was three time higher (9.7) then native American land races (3.5). In another latest research, Raza et al. (2018) observed 227 bands by 20 RAPD markers with a mean of 11.35 bands per marker and showed 86.34% polymorphism among ten cultivated sunflower genotypes.

In another study, researchers used 12 SSR primer pairs to evaluate the diversity in 90 genotypes of interspecific crosses among *B. napus* and *Brassica campestris*. Total 33 allels were produced by 12 SSR primers with a mean of 2.66 alleles per primer. Out of these, 32 alleles showing 95.83% polymorphism were reported by Kanwal and Nawaz (2014).

A total of 38 SSR primers were used to estimate the genetic diversity among 28 sunflower cultivars and 102 al-

leles with a mean of 2.32 alleles/primer were observed. Genetic similarities varied from 0.25 to 0.90, while this study noticed similarities ranged from 33.33 to 76.19. PIC ranged from 0.09 to 0.62 with a mean of 0.41 as reported by Darvishzadeh et al. (2010).

However, a few researchers have described that genotypes cannot be characterized based on their origin. Some outcomes have been noticed in Ethiopian bananas by Birmeta et al. (2002), Ethiopian and Eritrean sorghum (Ayana et al. 2000), green mustard (Rabbani et al. 1998), Chinese vegetables *Brassica* (Ren et al. 1995), and rapeseed (Mailer et al. 1994). Important heredity differences were observed among Chinese and European cultivars of *Brassica napus* because of variation in genetic material as indicated by Shengwu et al. (2003).

In this research, the observed allele size ranged from 70 to 290 bp, and this outcome is quite close to the band size measured by Cartea et al. (2003) and Teklewold and Becker (2006). Out of the total (21), 18 alleles were polymorphic and showed 85.71% polymorphism among amplified alleles, and this high value of polymorphism indicates the huge genetic diversity among genotypes. However, primers with highly polymorphic alleles are more useful to evaluate the genetic distances and polymorphisms among genotypes investigated by Pradhan et al. (2004) and Sobotka et al. (2004). The present research observed 85.71% polymorphism, while Yu et al. (2005) observed 56% polymorphism in 11 genotypes of oilseed rape, and

Teklewold and Becker ([2006]) observed 20–83% polymorphism among 43 Ethiopian mustard verities by using fifty RAPD markers.

PIC is the important feature for molecular research and the PIC value is a function of allele number and frequency. 9 out of 10 primers display PIC values higher than 0.5, which indicates the uniqueness of the SSR fingerprint. These values were comparable with the results assessed by Tonguç and Griffiths ([2004]), who used 13 SSR markers and obtained the same PIC values for 54 *Brassica* genotypes. In another study, PIC varied from 0.04 to 0.80 with a mean of 0.50 in *Brassica napus*, as observed by Plieske and Struss ([2001]). The PIC value in this study varied from 0.375 to 0.719, with a mean of 0.66. These results are very similar to Kanwal and Nawaz ([2014]), who noticed PIC varying from 0.395 to 0.726 with an average of 0.584 for each primer.

Conclusion

The conducted research outlines that genetic diversity and polymorphism exist among the six studied *Brassica* species. The allele number is directly and positively related to the genetic diversity and PIC values. The difference in PIC values with the reported values is due to the different genotypes and sample sizes. Results could be used to recognize different species, which could be utilized for future breeding programs aimed at creating genetic diversity in the local *Brassica* germplasm.

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