Pak. J. Bot., 49(6): 2213-2220, 2017.

INVESTIGATION AND CONFIRMATION OF INTROGRESSION IN BRASSICA SPECIES USING MICROSATELLITE MARKERS

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Abstract

Microsatellite markers or simple sequence repeats (SSR) are believed to be a reliable tool in the assessment of genetic variation within genomes and are therefore, widely used in population studies. In the present study, microsatellite markers were employed to confirm the process of introgression in F_2hybrids derived from interspecific crosses of *Oleiferous brassica* species viz., *B. napus* (AACC genome- used as female parent) crossed with each *B. juncea* (AABB) and *B. rapa* (AA). A total of 31 SSR primers were utilized and 99 alleles were detected, with 69 (69.7%) being polymorphic. Polymorphism information content of the primer sets ranged from 0.24 to 0.75. In population of *B. napus* x *B. junceae*, pair wise similarity indices ranged from 4.4 to 9.2and from 4.2 to 8.2 in F_2 hybrids of*B. napus* x *B. rapa*. The deviation of Introgressed hybrids towards female parent in both populations revealed that C genome has played a vital role in the introgression process of brassica species under study. Our results stressed upon the use of SSR markers in assessment of genetic diversity at molecular level.

Key words: Brassica landraces, Genetic diversity, Microsatellite primers, Introgressive hybridization.

Introduction

Introgression being an important source of variation and speciation is ubiquitous phenomenon in all plant species. It is a sort of genome mixing that expresses composite combinations of parental genes, and hence is different from simple hybridization. The process may involve relocation of chromosomal segments regulating specific characters among gene pools. The process creates new gene groupings that may be beneficial in breeding programs. According to Ellstrand *et al.* (1999), globally most of the crops once domesticated may be cross-compatible with their ancestors, as they might have gone through this process and have derived genes from other species.

Brassica napus L., is an amphidiploid (AACC genome,2n = 38) species that is believed to be the result of inter-specific cross between "*Brassica rapa* L. (syn. *rapa*) (AA genome, 2n = 20) and *Brassica oleracea* L. (CC genome, 2n = 18)" (Nagaharu, 1935). Weedy*B. rapa*may be a derivative from cultivars that dispersed as a run-away plant from meadows in Japan (Tsuda *et al.*, 2014). It has been reported that *B. rapa* was brought to Japan in the 8th century, and later in the ninth century, *B. juncea*was conceded from China (Hoshikawa, 1998). Several investigators have extensively studied the crossing ability and introgression among species of Brassicaceae. The ability of crossing and frequency of natural crossing of *B. napus* and *B. rapa* has been described by El-Esawi (2015) and Tsuda *et al.* (2014).

Introgression (gene flow) from *B. napus* (donor) to *B. rapa* and *B. juncea* can be ranked as 1st and 2nd, respectively. Introgressive hybridity from *B. napus* to *B. rapa*has been tested and it has been observed that cultivated *B. napus* and *B .rapa* have the ability to hybridize and cross back naturally in meadows and/or in laboratory trials. Sometimes, F_1 plants set more seeds when crossed with *B. rapa* (Hauser *et al*., 1998) but much fewer in other cases (Jørgensen *et al*., 1996; Mikkelsen *et al*., 1996).It has been noticed that seed setting frequency of these introgressed hybrids greatly diverges in controlled environment trials (Jørgensen & Andersen, 1994), in fields (Landbo *et al*., 1996) and in terrestrial areas (Wilkinson *et al*., 2000). The possible reasons may be the difference in the frequencies of parents and resulting hybrids and variability in their pollen composition(Hauser & Jørgensen, 1998).

Despite of the fact that in Asia, among Brassicaceae, *B. juncea* is the major weed but gene flow from *B. napus* to *B. juncea* is negligible. Evidence of intended gene flow of *B. napus* to*B. juncea*, unintended gene flow of *B. napus* into other species via *B. juncea* to evaluate hybridity and gene flow between *B. juncea* and other Cruciferous species are lacking. Since, *B. juncea* is globally and widely distributed, this species has the potential role of acting as an intermediary of gene flow from *B. napus* to associates of the Cruciferae. However, indirect gene flow from *B. napus* to allied Cruciferae may occur, in which these two species i.e., *rapa* and/or *juncea* could work as a link species.

Along with the use of morphological markers to analyze genetic diversity, DNA based markers have changed the entire picture of biological sciences. In addition to DNA fingerprinting, these molecular markers are used as a handy tool for genome analysis. Simple sequence repeats (SSR) are pattern of repeated short nucleotide sequences that determine an individual's inherited traits and are useful in determining parentage. These exist in the entire eukaryotic genome with divergent characteristics for each species (Ahmad *et al.*, 2014). The SSR markers (co-dominant in nature) are able to detect both homozygous and heterozygous loci and are ideal for genetic analysis, as these are highly polymorphic and exist in abundance within genome (Ahmad *et al.*, 2014; Broun &Tanksley, 1996; Kresovich *et al.*, 1995).

Due to commercial significance of these markers, much emphasis has been given on their use in identification of genetic differences at molecular level (Kumar *et al.*, 2015). These have also been proved as reliable tool in the assessment of genome specific genetic diversity in Brassica species (Tian *et al.*, 2017)

The present research was designed to verify the gene flow among three related brassica species. The SSR markers were employed to analyze the genetic composition of hybrids after gene flow in Brassica interspecific populations.

Materials and Methods

Plant material: For this study, F_2 hybrids from two interspecific crosses derived from locally collected landraces viz., Bn-RWP x Bj-UCD-40 (*B. napus*x *B. juncea*) containing 12 F_2introgressed hybrids and Bn-RWP x Br-RL-18 (*B. napus* x *B. campestris*) having 9 introgressed hybrids were selected. Introgressed hybrids were plants that had phenotypic characters of both distinct parental species altogether. Same female parental line with two different male parents was hybridized in independent cross in order to investigate that which genome has taken more part in introgression process.

Molecular investigations: Leaf tissues of parental lines were collected at 4-leaf stage, while leaf tissues of F_2introgressed hybrids were collected after the assessment of morphological introgression in field. Small leaves were collected from the introgressed plants (as these contain comparatively less proportion of polysaccharides) and were stored at -80˚c for future use.

DNA extraction: Genomic DNA extraction was carried out using CetylTtrimethyl Ammonium Bromide (CTAB) DNA extraction protocol (Doyle &Doyle, 1987).

Selection of primers: A total of 74 SSR primers were tested on five samples, of which 31 primer sets gave positive results during initial screening. These 31 primers were used for polymerase chain reaction (PCR) analysis (Table 1). Different annealing temperatures (52-58ºC) were tested during the screening process to select the best annealing temperature for a specific primer set. These primer sets were selected from the published data of Suwabe *et al.* (2003), Lowe *et al.* (2004), Burgess (2006), Batley (2007), Hopkins (2007), who tested them on various genomes of *Brassica* to confirm their cross amplification across *Brassica* species.

Table 1. List of SSR primer sets used in the study.

SSR primer	Forward primer (bp)	Reverse primer (bp)
BRMS-008	AGGACACCAGGCACCATATA	CATTGTTGTCTTGGGAGAGC
BRMS-019	CCCAAACGCTTTTGACACAT	GGCACAATCCACTCAGCTTT
BRMS-027	GCAGGCGTTGCCTTTATGTA	TCGTTGGTCGGTCACTCCTT
BRMS-037	CTGCTCGCATTTTTTATCATAC	TACGCTTGGGAGAGAAAACTAT
BRMS-040	TCGGATTTGCATGTTCCTGACT	CCGATACACAACCAGCCAACTC
Na10-D03	ATGATTTGCCTTGAAATGCC	GATGAAACAATAACCTGAGACACAC
Na10-D09	AAGAACGTCAAGATCCTCTGC	ACCACCACGGTAGTAGAGCG
Na10-D11	GAGACATAGATGAGTGAATCTGGC	CATTAGTTGTGGACGGTCGG
Na10-E02	TCGCGCATGTAATCAAAATC	TGTGACGCATCCGATCATAC
Na10-F06	CTCTTCGGTTCGATCCTCG	TTTTTAACAGGAACGGTGGC
Na12-A02	AGCCTTGTTGCTTTTCAACG	AGTGAATCGATGATCTCGCC
Na12-A07	TCAAAGCCATAAAGCAGGTG	CATCTTCAACACGCATACCG
Na12-E02	TTGAAGTAGTTGGAGTAATTGGAGG	CAGCAGCCACAACCTTACG
Na14-D07	GCATAACGTCAGCGTCAAAC	CTGCGGGACACATAACTTTG
Ni2-B03	ACTTCTTGCCCTCCTCACC	AAATACTCACTGCAATACCCAGG
Ni4-D09	AAAGGACAAAGAGGAAGGGC	TTGAAATCAAATGAGAGTGACG
Ol09-A06	TGTGTGAAAGCTTGAAACAG	TAGGATTTTTTTGTTCACCG
Ol10-F11	TTTGGAACGTCCGTAGAAGG	CAGCTGACTTCGAAAGGTCC
Ol10-F12	TCCATGTTTCATGTTGGAGG	CTCTCCGGCTTCACTTTCC
Ol10-H02	AACAGGAAGAAACGACGAGG	AGAGAGCCATGAGAAGCACC
Ra2-A11	GACCTATTTTAATATGCTGTTTTACG	ACCTCACCGGAGAGAAATCC
Ra2-D04	TGGATTCTCTTTACACACGCC	CAAACCAAAATGTGTGAAGCC
Ra2-E03	AGGTAGGCCCATCTCTCTCC	CCAAAACTTGCTCAAAACCC
Ra2-E11	GGAGCCAGGAGAGAAGAAGG	CCCAAAACTTCCAAGAAAAGC
Ra2-E12	TGTCAGTGTGTCCACTTCGC	AAGAGAAACCCAATAAAGTAGAACC
Ra2-F11	TGAAACTAGGGTTTCCAGCC	CTTCACCATGGTTTTGTCCC
Ra2-G09	ACAGCAAGGATGTGTTGACG	GATGAGCCTCTGGTTCAAGC
Ra3-H10	TAATCGCGATCTGGATTCAC	ATCAGAACAGCGACGAGGTC
PBCESSRJU9	CCCTACCGCTGGCTAGACTT	GCATCATGACCAACTATCAACC
PBCESSRJU10	GCGGCGTAGGTACTGGAG	AGCCATCGAGCCATTCAG
BRMS-036	GGTCCATTCCTTTTTGCATCTG	CATGGCAAGGGGTAACAAACAT

Protocol for molecular marker analysis: Polymerase Chain Reaction (PCR) was performed in PCR tubes containing 20 µl reaction mixtures having 1 µl of genomic DNA, 15.5 µl of sterile autoclaved and deionized dd H_2O, 2 µl of 10 x Dream Taq buffer (with added 20 mM of $Mgcl_2$), 0.4 µl dNTPs (10 mM each), 0.5 µl of SSR primers (20 µm/20 pmol/µl), and 0.1 µl dream Taq DNA polymerase (5 U/µl) (Fermentas). DNA was amplified under the following thermal cycling conditions, 4 minutes (min) denaturation at 94°C, 35 cycles of annealing while each cycle having 1 min denaturation at 94°C, 1 min annealing at 52-58ºC depending on the primer set used, 2 min extension at72°C, and a final extension step of 72°C for 7 min.

Electrophoresis of amplified products: After the completion of PCR, 3 µl of 6x loading dye (10 mM Tris-Hcl (pH7.6), 0.15% orange G, 0.03% of xylene cyanol FF, 60% glycerol, 60 mM EDTA) was added to each PCR tube. The tubes were spun in microfuge for few seconds. 10 µl aliquot of PCR products mixed with loading dye was loaded in 3% agarose Hires (molecular biology grade, gene links). Electrophoresis was carried out in 1X TBE buffer (10 mM Tris- Borate, 1 mM EDTA) at 100 volts for 90 minutes. PCR products were separated by electrophoresis using 3% agarose gels and were visualized under UV light after staining with Ethidium bromide.

Data collection and analysis: Photographs of the gels taken under UV light by gel documentation system were used to score the bands of SSR primer. Each band (amplified allele) was considered as unit character and was scored as 1 for presence and 0 for absence for each sample-primer combination. Only clearly visible and distinguishable bands were considered for scoring. Allele size was determined in gel by comparing the band with known size of DNA marker. Data for band scoring was entered in binary data matrix using MS excel (2007) sheet.

Genetic similarity estimates (F) were calculated between all pairs of genotypes by the DICE algorithm according to Nei & Li (1979). These similarity coefficients were used to determine the relationships among the parents and introgressed hybridsthrough cluster analysis using unweighted pair- group method (UPGMA) and dendrograms were generated. All these calculations were carried out using software packages NTSysPC version 2.2 (Applied Biosystems Inc, USA) and Ms-Excel 2000.

Polymorphism information content (PIC): The PIC value utilizes both the number of alleles at a specific locus and their relative frequencies in a population. It illustrates the value of a marker for detecting level of polymorphism within a population (Bostein *et al*., 1980) and depends on the number of amplified alleles and their frequency distribution (Anderson *et al*., 1993).PIC value for each SSR primer set was used to measure allele diversity at each locus. It was calculated by the formula proposed by Tonguç & Griffith (2004).

$$PIC = 1 - \sum (pi)^2$$

Where *pi* states the frequency of *i*th allele in a population.

Results

Microsatellite marker availability across interspecific hybrids: Each of the selected primer varied greatly in their ability to identify the deviation of F_2hybrids from the parental genotypes or otherwise (Figs. 1 & 2). Divergent patterns of polymorphism were observed. The product size of each primer set was compared with that of expected size given on Brassica database domain (www.Brassica.info). In total, 99 alleles were amplified, of which 69 were polymorphic. The highest allelic polymorphism was exhibited by the primer sets Na10-D03 (Fig. 1) and Na10-D11 (Fig. 2) which produced five amplification products, while varying polymorphic alleles were specified by other primer sets. The proportion of polymorphic loci was 69.7%. The number of amplified products ranged from 1 to 5 with an average of 2.39 discrete DNA per primer**.** The PIC value for these polymorphic primer sets ranged from 0.24 to 0.75. Microsatellite primer Na10-E02 had the highest PIC value. Most of the primers had PIC value more than 0.40. Range of product size and PIC value of each polymorphic primer set are given in Table 2.

Phylogenetic relationship between parents and introgressed hybrids: In heterospecific population (*Bn*-RWP x *Bj*-UCD-40), pair wise matching indicators ranged from 4.4 to 9.20. Parents were quite divergent and had small resemblance index (5.5). F_2 hybrids were significantly matching with female napus parent. The similarity range of F_2 and *B. napus* parent was 6.4-8.9. Out of two main clusters, cluster-1 had close similarity indices with female (*Bn*-RWP*)* parent (Fig. 3). As "A" genome is common in *B. napus* (AACC) and *B. juncea* (AABB), yet the banding profile of both species was remarkably different. The possible reason might be the fact that "B genome" exists in *B. juncea* which makes it divergent from others.

The pair wise similarity indices ofpopulation *Bn*-RWP x *Br*-RL-16ranged from 4.2 to 8.2. The similarity index for both parents was nearly 5.74. One F_2introgressed population was placed in the same cluster with male parent *Br*-RL-16, showing that male parent (*B. rapa*) has also played minor role in process of gene flow. The highest resemblance was recorded between the F_2 populations and *B. napus* parent (Fig. 4).

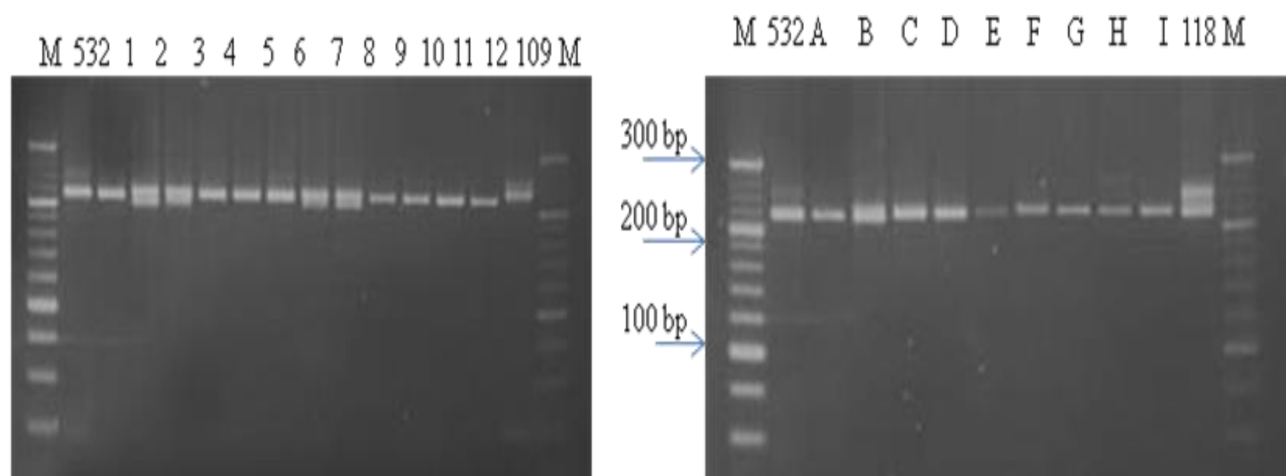

Fig. 1. SSR banding array generated by primer Na10-D03for F_2 introgressed hybrids and their parents.

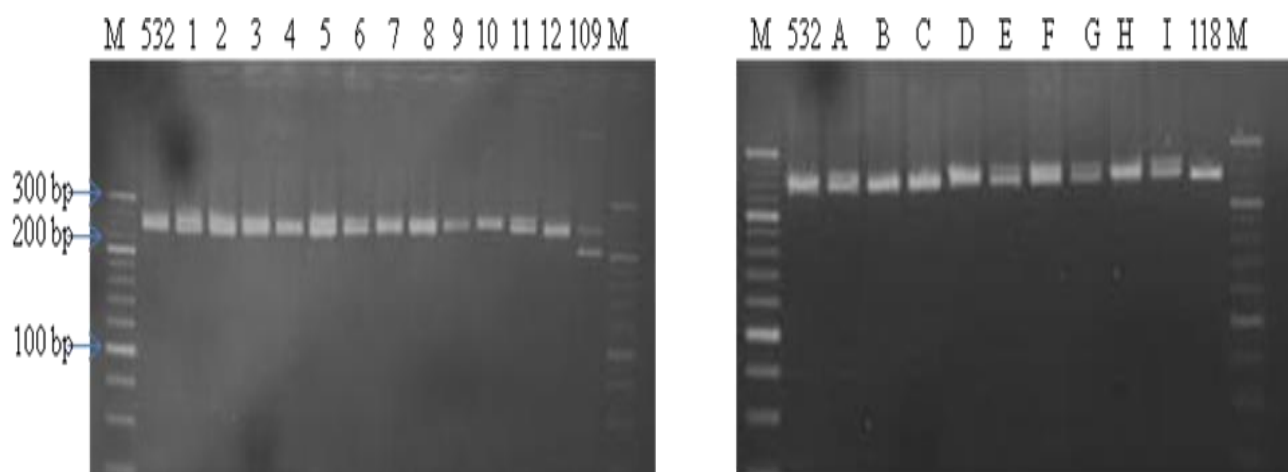

Fig. 2. SSR banding array generated by primer Na10-D11for F_2introgressed hybrids and their parents.

M = Ladder,
532= *Bn*- RWP(*B. napus*), 109 =*Bj*-UCD-40 (*B.juncea*), 118= (*Br*-RL-16)
1-12 = (12 F_2 introgressed hybrids from *Bn*-RWP x *Bj*-UCD-40),
A-I = (9 F_2introgressed hybrids from *Bn*-RWP x *Br*-RL-16)

Discussion

Microsatellite markers have been extensively used to calculate genetic diversity indices of diverse brassica populations. Polymorphism information content (PIC) of primers used in our study was in the range of 0.24 to 0.75. The results were in consistence with Tonguc &Griffith (2004) who detected 1 to 8 alleles. In their study, the PIC values were in the range of 0.25-0.86 for thirteen SSR primers that were utilized to differentiate broccoli, cauliflower and cabbage cultivars. Louarn *et al.* (2007) observed PIC value of 0.5 or above in 11 SSR primer pairs. Hasan *et al*. (2006) amplified 220 alleles at 51 polymorphic loci by using 30 SSR primer sets. Our findings support the research work of Yuan and Chao (2007) who detected a total of 21 alleles after using five SSR primers having PIC range between 0.25-0.92. The results of Sadia *et al.* (2010) further strengthened our results as they tested 25 microsatellite-specific primer pairs on 75 Brassica species and detected 2 to7 alleles per microsatellite locus. Researchers have detected up to 14 alleles (Zhou *et al*., 2006), while analyzing Chinese and Swedish rapeseed (*Brassica napus*) genotypes with 41 SSR primers.

The number of alleles detected was in range of 1-5 with an average of 2.39 alleles per locus. The results were in consistence with the findings of Ofori *et al*. (2008), who detected 2 to 8 alleles per SSR marker across different Brassica cultivars. Hasan *et al*. (2006) detected an average of 4.31 alleles per locus, while assessing 96 European rapeseed genotypes. Xioe *et al*. (2012) reported a mean of 3.4 alleles in 192 inbred lines. The results of Gyawali *et al*. (2013) also supported our findings as their research covered 169 rapeseed lines collected from diverse origins that were having 7.3 alleles per locus. Channa *et al.* (2016) identified194 fragments with an average of 3.46 alleles per locus after using 56 SSR primer set, while analyzing the genetic diversity among 77 rapeseed accessions. An average of 10.37 alleles with PIC value 0.52 was reported by Chen *et al*. (2017), who identified 311 alleles in brassica collections from diverse regions.

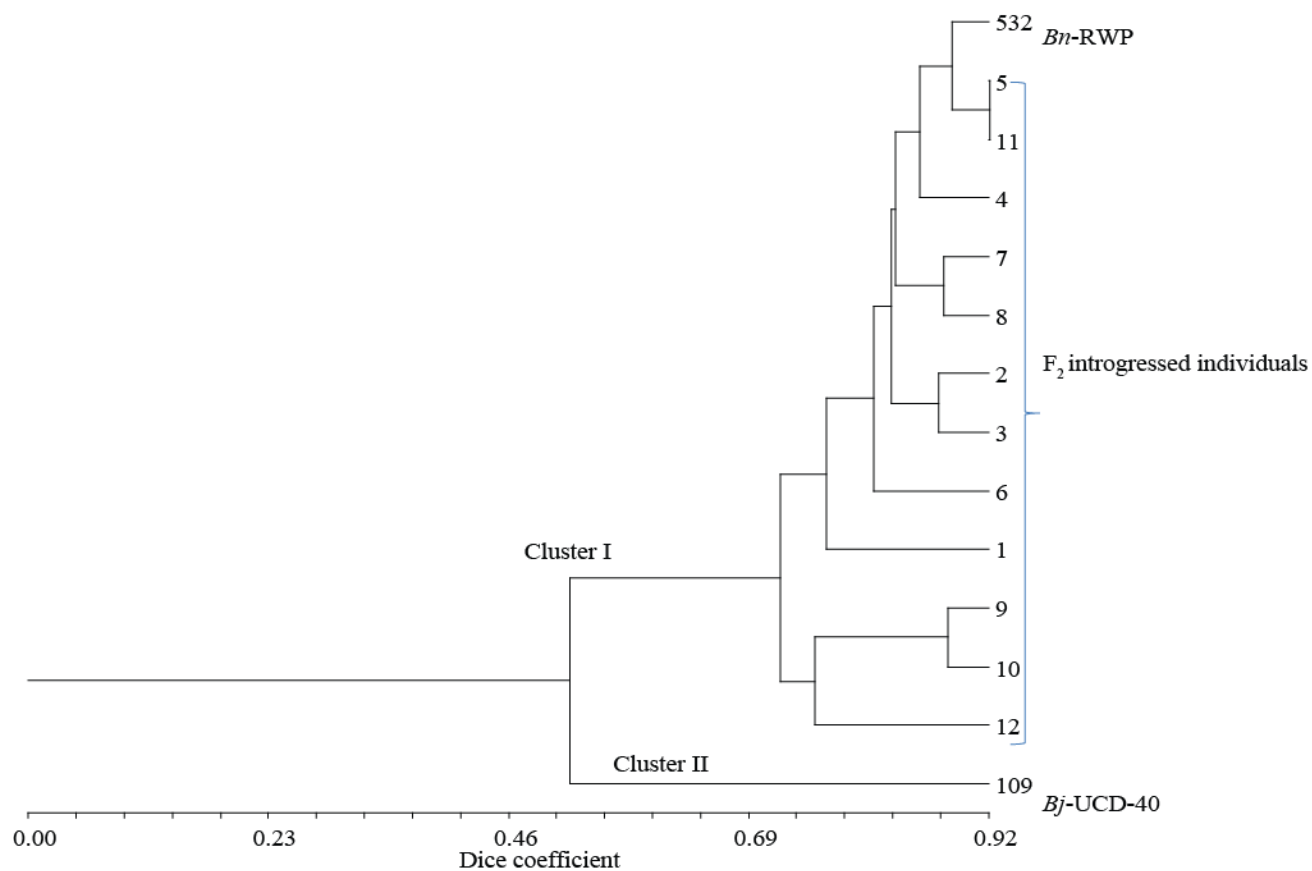

Fig. 3. The relationship among parental lines and F_2introgressed hybrids (*Bn*-RWP x *Bj*-UCD-40) based on SSR marker scoring.

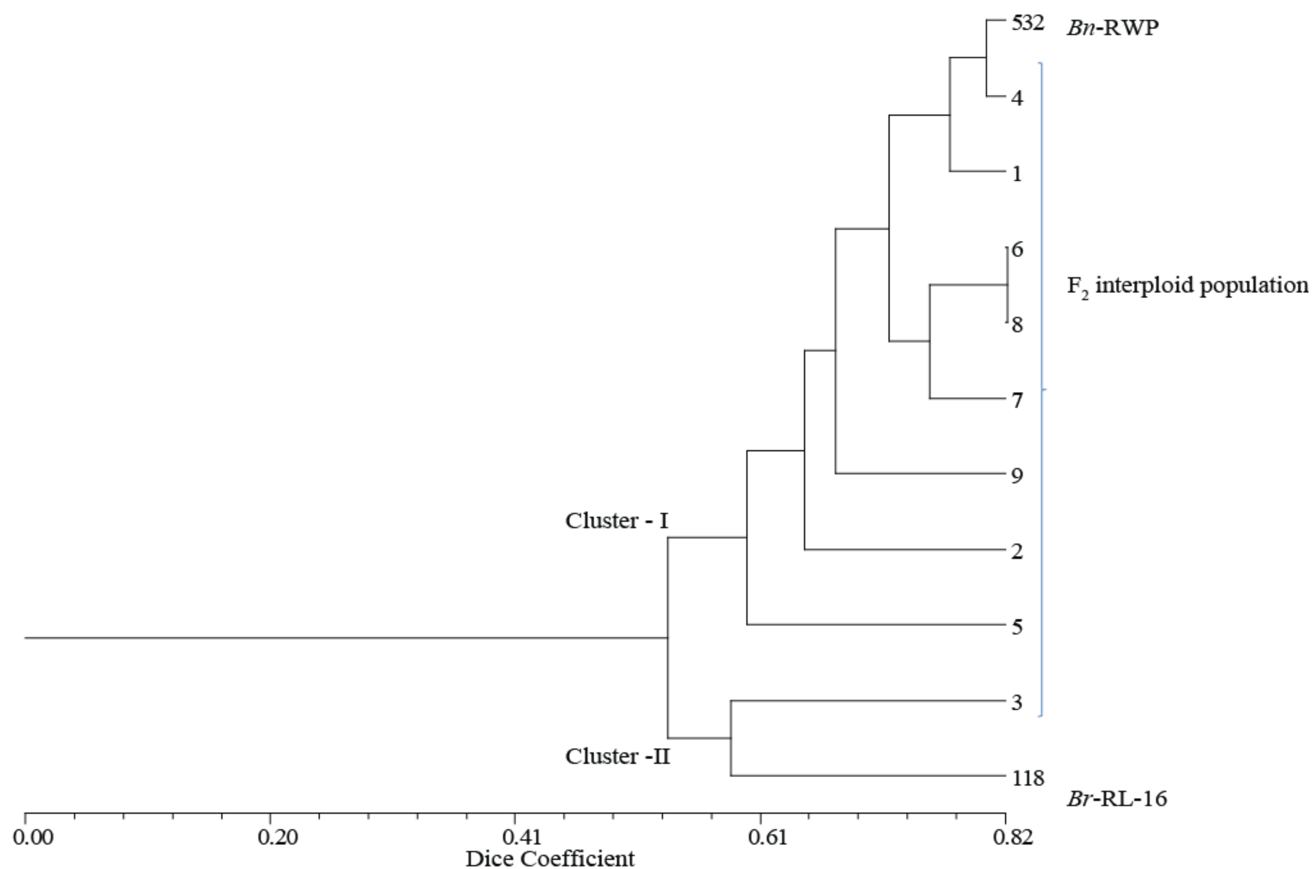

Fig. 4. Relationship among parental lines and F_2introgressed hybrids (*Bn*-RWP x *Br*-RL-16) based on SSR marker scoring.

Table 2. PIC values, band size and annealing temperatures of SSR primers.

SSR primer	Polymorphism information content (PIC)	Band size (bp)	Annealing temp.(°C)
BRMS-008	0.50	150-170	56
BRMS-019	0.53	200-250	55
BRMS-027	0.48	220-260	56
BRMS-037	0.34	250-260	54
BRMS-040	0.62	180-240	52
Na10-D03	0.63	160-200	56
Na10-D09	0.65	270-300	55
Na10-D11	0.57	180-200	55
Na10-E02	0.75	130-200	56
Na10-F06	0.48	100-120	55
Na12-A02	0.63	160-200	54
Na12-A07	0.42	150-180	56
Na12-E02	0.60	100-140	56
Na14-D07	0.56	120-140	54
Ni2-B03	0.55	145	54
Ni4-D09	0.65	170-240	56
Ol09-A06	0.46	95-110	56
Ol10-F11	0.42	145	54
Ol10-F12	0.26	110-140	54
Ol10-H02	0.49	200-220	55
Ra2-A11	0.46	330	52
Ra2-D04	0.65	160-200	55
Ra2-E03	0.24	280-300+	54
Ra2-E11	0.44	180-210	52
Ra2-E12	0.65	120-200	56
Ra2-F11	0.68	190-260	55
Ra2-G09	0.43	24-260	55
Ra3-H10	0.66	130-160	56
PBCESSRJU9	0.66	200	55
PBCESSRJU10	0.74	140	55
BRMS-036	0.47	250-260	52

Microsatellite markers are proved to be the best choice to investigate the divergence pattern in closely related species as they share highly conserved pattern of arrangement of genetic material in terms of sequence homology (Kalia *et al*., 2011). This gives an edge to SSR markers as if developed in one species, can be utilized in other related species to detect the presence of conserved flanking regions within them. Same primer set was used in both crosses to see that with which parents shares more sequence homology with the progeny, which in turn can confirm the genome that has taken active part in introgression. The species-specific markers were used to screen plants from three different genome background i.e. AA, AACC, AABB. It is already reported that most of the SSR's match up to A and C genome loci in oilseed *Brassica* (Saal *et al*., 2001). *B. napus* being a relatively younger species genetically closes to its ancestral species *B. rapa*. We found more alleles in *B. napus* (AACC), as compared to one or few alleles in *B. juncea* (AABB). *Brassica rapa* (AA) and *B. napus* had maximum common alleles explaining why *B. juncea*was isolated from remaining group of plants in the cluster for the cross *Bn*-RWP x *Bj*-UCD-40.

The deviance towards female parent in both hybrid populations may be due to the reason that *B. napus* carries both (A and C) genomes. Genome "A" is also carried by *B. juncea*, but it can be seen that no hybrid shared the cluster with male juncea parents. From the results, it can be concluded that C genome has an active role in the process of introgression.

Conclusions

Microsatellite markers helped us in understanding the process of introgression in the studied species. Although introgressed hybrids have combined phenotypic traits of both parents, yet female parent shared more similarity at molecular level by sharing same cluster with the progeny. These introgressed hybrids may serve as base populations for breeding programs intended to widen the narrow genetic base of the existing resources. It is further suggested that introgressive hybridization may be practiced in other members of Brassicaceae family as well. Hence it can be concluded that SSR markers can serve as a reliable tool for the genomic assessment in brassica species.

Acknowledgements

The authors highly acknowledge HEC project "Collection and Characterization of Indigenous Brassica Species of Pakistan" for the provision of research material and financial support. Special thanks are extended to the Institute of Agri-Biotechnology & Genetic Resources, National Agricultural Research Center, Islamabad for providing laboratory facilities.

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(Received for publication 16 December 2016)