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Molecular Marker Assisted Confirmation of Hybridity in Indian mustard (*Brassica juncea* L.)

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A B S T R A C T

The present study was undertaken to confirm the hybridity of *Brassica juncea* L. F_1 plants based on the amplification pattern of SSR markers. The complementary banding pattern of the male and female parents helped to confirm the genuineness of F_1 plants developed. The seeds of two genotypes of *Brassica juncea*, RSPR-01 used as female parent and Donskaja-IV used as male parent were raised in pots under field conditions and crossed to develop F_1 seeds. Out of 20 random SSR primers used for the screening of parental genotypes for polymorphism, 5 primers were found polymorphic. Based on the complementary banding patterns between the hybrid plants and parents, the polymorphic SSR markers BR_A04_9627743 and BR_A01_13393871 were identified as the specific markers which enable to distinguish and identify hybrid form their parental lines. The expression of both the parental alleles in six out of the seven plants confirmed their true hybrid nature.

Introduction

Brassica juncea L. (2n = 36) is a self-pollinated natural allopolyploid (AABB) evolved from interspecific hybridization between diploid *B. rapa* (AA, n=10) and *B. nigra* (BB, n=8). It is cultivated as an oilseed, condiment as well as a vegetable in some parts of the world. It occupies major area in India contributing more than 80 percent of the total rapeseed mustard production. In order to increase the efficiency of a hybrid breeding to achieve a breakthrough in the productivity of Indian mustard, careful evaluation of hybrid combinations between suitable parental lines is important. Conventional screening methods have limited applicability to confirm whether a crossed/F_1 plant is actually a hybrid or selfed by chance self-fertilization. Therefore, selection of promising hybrids based on phenotypic observations is many times doubtful due to morphological similarity between F_1 and one of the parental lines. In seed industry, distinguishing the hybrid seeds from the non-hybrid seeds is very important as F_1 plants growing from hybrid seeds have higher yield potential, resistance to biotic and abiotic stresses as well as genetically and physically more uniform (homogenous) due to phenomenon of heterosis and similar (heterozygous) genetic composition. In

comparison to morphological and biochemical characterization, DNA based molecular characterization is more useful for ascertaining true genetic relationships among the genotypes.

Molecular markers, such as RAPDs, RFLPs, AFLPs, SSRs and ISSRs have been used in cultivar fingerprinting, seed purity testing and germplasm identification in many crops because they are highly polymorphic, devoid of environmental interactions and represent the genomic constitution of a plant (Rabbani *et al.,* 2010; Zeb *et al.,* 2011; Azam *et al.,* 2013 and Ahmad *et al.,* 2014). Among the various molecular markers systems available, simple sequence repeats (SSRs) or microsatellites are widely accepted and reliable as they are abundant, co-dominant, robust, detect high levels of allelic diversity, easy scoring of the alleles, reproducibility and accessibility to laboratories and can be analysed by a convenient PCR-based method, which makes it easy to screen a large number of individuals (Morgante and Olivieri, 1993; Hancock, 1995 and Paniego *et al.*, 2002).

F_1 plants contain one copy of genome from both the parents and SSR markers detect alleles of both male and female parents used in cross, thus allowing differentiation of true hybrids from selfed individuals and outcrossed individuals with foreign pollen. Thus, the current study was planned to confirm the hybridity of F_1 plants using polymorphic SSR markers in *Brassica juncea* L.

Materials and Methods

Seeds of two cultivars of *Brassica juncea* L., namely RSPR-01 (P1) of Indian gene pool, used as female parent and Donskaja-IV (P2) of European gene pool, selected as male parent were raised in pots under field conditions and crossed to develop F_1 seeds during Rabi 2015-16. F_1 plants (RSPR-01 x Donskaja-IV) were raised during Rabi 2016-17 (Fig. 1, 2 and 3). They were subjected to molecular characterization in the Genomics Laboratory, School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, Chatha.

The genomic DNA was extracted from young seedlings of both parents and progenies using CTAB method (Doyle and Doyle, 1990) with slight modifications; quality of DNA for each sample was assessed on 0.8% of agarose gel and then stored at 4^0C for further use. DNA amplification was carried out using twenty SSR primer pairs (Table 1) in polymerase chain reaction (PCR) tubes containing 12 µL reaction mixture. The reaction mixture contained 1.5 µl of template DNA (50ng/µl), 1.2 µl of 2.5 mM/ µl dNTP (dTTPs, dGTPs, dCTPs, dATPs), 0.5 µl of each forward and reverse primers, 5 U of Taq polymerase, 1.2 µL of 10X PCR buffer with $MgCl_2$ (Sigma-Aldrich). Amplification cycle comprised of initial denaturation for 5 min at 94 ^{0}C; 30 cycles of 94 ^{0}C for 1min, annealing at 58 ^{0}C for 1 min and extension at 72 ^{0}C for 2 min.; followed by a final extension at 72 ^{0}C for 7 min. in Master Cycler Gradient (Eppendorf, Germany). The products of amplification were stored at 4 ^{0}C and resolved by electrophoresis in horizontal agarose gel system at 110 V for 1 h 30 min. on 2% agarose gel stained with ethidium bromide (10mg/ml) using 1X TBE buffer. The amplified products were visualized under gel documentation system and the size of amplicons was estimated with the help of 50bp ladder (Fermentas). Genetic polymorphism of SSR markers used in study was recorded on the basis of relative size of bands with 50bp ladder and hybridity confirmation was done by using 100bp ladder.

For analyzing the hybridity of F_1s, the banding patterns of SSR markers were compared and markers showing polymorphism between parents were identified (Fig. 4). The hybridity

of F_1 plants was confirmed when they showed presence of both male and female parent alleles.

Results and Discussion

Identification of suitable SSR primers

The good quality genomic DNAs of two *Brassica juncea* L. cultivars used as parental lines and seven F_1 plants without shearing were obtained by modified CTAB extraction method and amplified by a panel of 20 SSR primers. Out of the 20 SSR primers used for screening of parental genotypes, 15 primers were found monomorphic while 5 primers namely BR_A01_13393871, BR_ A03_22221630, BR_A04_9627743, BR_A04_15440685 and BR_A05_25290881 were observed to be polymorphic (Fig. 4) as there was difference in the size of the amplicons obtained after PCR between two parental lines. Suwabe *et al.,* (2002) studied characterization of microsatellites in *Brassica rapa* L. while Ali *et al.,* (2007) have done molecular characterization of some local and exotic *Brassica juncea* germplasm for establishing their true genetic status. The markers found polymorphic in this study were used to confirm the hybridity of F_1 plants. Besides genetic variation, molecular makers have been used for evaluating the genetic stability of crops (Koshy *et al.,* 2013). Pallavi *et al.,* (2011) in sunflower, Liu *et al.,* (2008) in tomato, Dunja *et al.,* (2014) in cabbage identified SSR markers associated with hybridity and genetic purity testing of hybrids. Hipi *et al.,* (2013) showed that SSR markers were more reliable for assessing genetic purity as compared to morphological markers

Fig.1 Potted seedlings of P1- RSPR-01

Fig.2 Potted seedlings of P2- DONSKAJA-IV

Fig.3 Potted seedlings of Hybrid Plants obtained by crossing RSPR-01 x Donskaja-IV (P1xP2)

Fig.4 Banding pattern of SSR primers with parental genotypes (Primers in red indicate polymorphic primers)

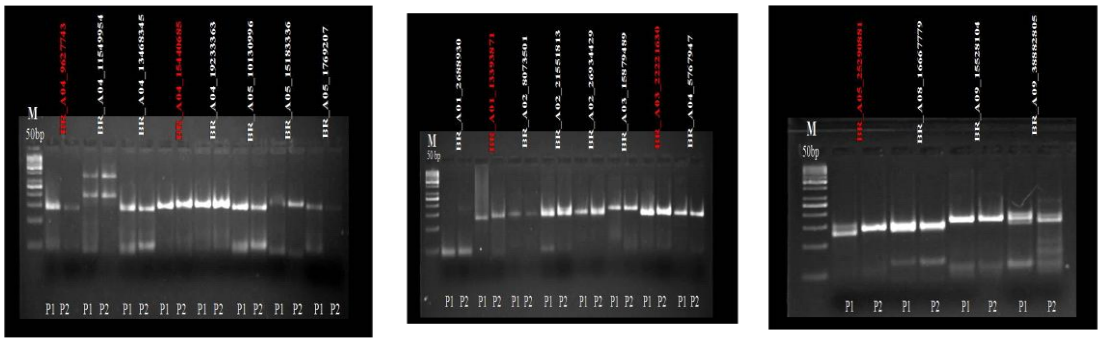

Fig.5 Banding pattern confirming hybridity obtained by primer BR_A01_13393871

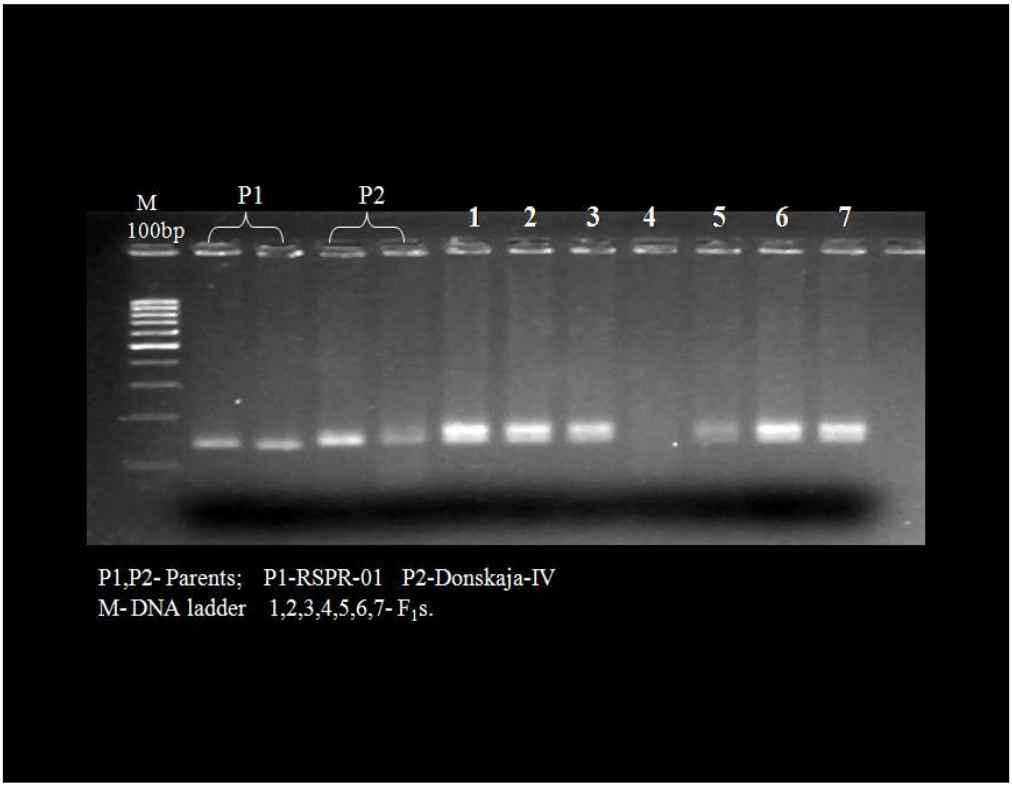

Table.1 List of primers used in the experiment:

PRIMERS	SEQUENCE 5'-3'
1. BR_A01_2688930	Forward: CAATGTAATGGGAAGAAAATG Reverse: GTACCTCTCCTGGTCCTGTAT
2. BR_A01_13393871	Forward: CCGTTTTTATGTCACAAATCT Reverse: AAACAAAACGAACTTTGTCAG
3. BR_A02_8073501	Forward: CCACCTTTACCAGCACTAAAT Reverse: TTCTTCAGAGAAGAGAAGAAATG
4. BR_A02_21551813	Forward: GATCACACTTTTGAACCGTTA Reverse: TGAGAATGAAGGAGAAGAACA
5. BR_A02_26934429	Forward: TTGTAGACCTTCTGCTACCAA Reverse: AAAGACCATACCCTACGAAAT
6. BR_A03_15879489	Forward: AGTTCAAGGTATTCGCCTAAG Reverse: TACATCCTCATAGCACTCCTC
7. BR_ A03_22221630	Forward: ATCGTCTCTTTCGTCTTGTCT Reverse: CGTAAAACTGAAACCATTCAC
8. BR_A04_5767947	Forward: GACAATGTTCTTGCTATCACC Reverse: ATAGTTCCTTCGCAACCTATT
9. BR_A04_9627743	Forward: ATGGAATCTGCTCATCTCAC Reverse: TAAGCTGCAATGATCAAAGAT
10. BR_ A04_11549954	Forward: CATTTTCCTCCTTGAGATCTAT Reverse: CTGGTGGAAAACTTGATTTTA
11. BR_ A04_13468345	Forward: CATCACAAGCCAAGAAGAAT Reverse: AGAGTCTGTGGTTCATCTCCT
12. BR_A04_15440685	Forward: TTTGAACGATACACAACAACA Reverse: GTTGGTCCACGAGTAAAAGAT
13. BR_A04_19233363	Forward: AAAGAAGGGGAAAGTAAACCT Reverse: GCAACTCTCTTCATTTTCAGA
14. BR_ A05_10130996	Forward: CCTTGTGGTATCGTATTGACT Reverse: AAAGAATACAACCGCACTGTA
15. BR_A05_15183336	Forward: GTTGAGCTCTCCTTCACCTAT Reverse: CGTGCGGGTATTTATTTTTAT
16. BR_A05_1769207	Forward: ACCCAAATATAGCATCAAGGT Reverse: ATGTTTGGTATCTGGGTTTGT
17. BR_A05_25290881	Forward: ATAAAGATTTGATGGGAGGAG Reverse: GGTGGAGGAGGATAGTTGTAG
18. BR_ A08_16667779	Forward: GAGAGCTTCTTCTGGTTGATAC Reverse: ACAAAACAGCGAGATCTCTTA
19. BR_A09_15528104	Forward: GAACAATCTACTGCTGAGTGG Reverse: CCAAGCTTGCTCCATAGTTA
20. BR_A09_38882805	Forward: AGTCAGTTTGCAAAGGTATGA Reverse: ATCTAAGAGAAATCGGGAAAA

Confirmation of hybridity of F_1s

Based on the complementary banding patterns between the hybrid and its parents, the SSR markers BR_A04_9627743 and BR_A01_13393871 were identified as the specific markers which enabled to distinguish and identify hybrid form their parental lines.

In hybrid plants, the SSR marker BR_A04_9627743 amplified two alleles of size 135 and 150 bp. The allele of 135 bp was expressed in its female parent (RSPR 01) and allele of size 150 bp was expressed in its male parent (Donskaja-IV). Similarly, in F_1 plants the SSR marker BR_A01_13393871 amplified two alleles of size 150 and 140 bp. The alleles of 150 bp were expressed in its female parent (RSPR 01) and allele of size 140 bp was expressed in its male parent (Donskaja-IV).

The expression of both the parental alleles in hybrid plants 1, 2, 3, 5, 6 and 7 confirmed their origin from the two parents used in the present study, as well as genuineness of hybrid plants (Fig. 5). Thus, out of seven F_1 plants, six plants were confirmed as true hybrids on the basis of amplification pattern of SSR marker. Results of the present study are in agreement with the conclusions of Hipi *et al.*, (2013) in maize. The use of SSR markers for genetic purity testing has also been demonstrated in maize (Wang *et al.*, 2002) and in rice (Nandakumar *et al.*, 2004).

This study showed that SSR markers are more reliable and robust for assessing genetic purity as compared to morphological marker. The results of study are expected to be useful in the verification of genetic purity of hybrid seeds in *Brassica juncea* L. accurately. The study suggests that identification and use of SSR markers can effectively reduce the cost and simplify the procedure of hybridity and purity testing.

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