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Association of SSR markers with primary branches in *Brassica juncea* L.

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Abstract: The study utilized $F_{2:5}$ lines derived from a cross between Kafiav N Zagora and Pusa Karishma cultivars to tag genomic regions controlling primary branches in *Brassica juncea*. One hundred and thirty $F_{2:5}$ plants were used to characterize primary branch numbers, resulting in two pools of 12 genotypes for high (HPB) and low (LPB) branches. The average number of primary branches for HPB and LPB were 12.16 and 4.50, respectively. A set of 148 SSR (Simple Sequence Repeats) markers was used for parental polymorphism screening from which 14 polymorphic SSRs were used for molecular characterization of HPB and LPB bulks, tagging genomic regions. The allelic data scored for 14 polymorphic lines was tested using Student's *t*-test analysis to understand relationships for primary branches with SSR markers and amplified alleles. Based on this, two B-genome markers (Ni2-C12 and Ni2-A11) were discovered to be strongly linked to the number of primary branches. Bioinformatic analysis located these two markers within a 9 Mb region on chromosome B5 of *B. juncea*. Utilising $F_{2:5}$ lines of an inter-gene pool genetic cross, the current study was able to locate the loci regulating the number of primary branches on *B. juncea*'s sub-genome chromosome B5. Before proceeding with fine-mapping investigations to dissect the genomic region (between 55.9 and 64.9 Mb) of sub-genome chromosome B5, it is imperative to emphasize the necessity of verifying these results across diverse genetic backgrounds.

Keywords: *Brassica juncea*, primary branch number, BSA, SSR.

Introduction

Brassica juncea L. (Indian Mustard) is an annual herb belonging to an economically important plant family of angiosperms called *Brassicaceae* (formerly Cruciferae). This annual plant is an amphidiploid (AABB, 2n=4x=36) as it is the product of spontaneous hybridization between *Brassica nigra* (BB, 2n=16) and *Brassica rapa* (AA, 2n=20), (Axelsson et al., 2000). Two major gene pools of *B. juncea*, the Indian and the East European gene pool, contrast significantly for major agronomic traits such as oil content, branch number, yield, plant height, and pod size. Therefore, the main goal of *B. juncea* breeders is to transmit desired inheritable traits from one gene pool to another (Pradhan et al., 1993). *B. juncea* is mainly cultivated for its seed oil, which is widely consumed in South Asian countries like India, Bangladesh, Nepal, and Pakistan. India is the third largest producer of rapeseed mustard in the world, with 9.98% of the total area under cultivation coming from *B. juncea, B. rapa, and B. napus*. India makes up roughly 11.27% and 19.29% of the world's total mustard-producing area and yield, respectively (Kumar, 2016). Rapeseed-mustard, the second most significant edible oilseed crop in India, contributes 27.8% to the economy, with over 80% production from *B. juncea*, making it an essential industry element (Singh et al., 2013). The Jammu division of J&K state farmed 28000 ha of rapeseed-mustard, yielding over 800 kg ha^{-1} and producing 3188.32 quintals in 2021-2022 (Digest of Statistics 2020–21, J&K).

India's oilseed production increased by 43% between 2015-16 and 2020-21, but production hasn't kept up with demand as there's a shift towards processed foods, leading to increased vegetable oil imports. India's per capita vegetable oil consumption is projected to rise by 2.6% annually by 2030, necessitating a 3.4% annual import growth (Economic Survey, 2021-22). Per capita consumption of edible oil is predicted to rise from 13.4 kg to 23-43 kg by 2030 (Singh et al., 2017). Therefore, in order to meet the growing demands, seed output must be increased.

Oil yield can be raised by increasing the seed output (Chen and Heneen, 1992). Hence, it is necessary to accelerate *B. juncea*'s seed yield potential to meet the current oil yield requirements. The final seed yield is influenced by various factors such as the number of branches, siliques, seeds, and seed size (Snowdon, 2007). Branching in *B. napus* significantly influences seed output, with a positive correlation between grain yield and primary branches bearing siliqua. Factors like branch number, siliqua length, and phenotypic and genotypic levels are positively associated. The most important direct factors influencing seed yield are plant height, the number of siliquae per plant, and the number of primary and secondary branches per plant (Tiwari, 2019).

The seed output of the Indian mustard is closely associated with the number of primary branches on the main axis as primary and secondary branches on the main stalk, bear seed-bearing silique (Ramanujam and Rai, 1963; Singh and Singh, 1972). Thus, it is ideal to have more primary branches in order to produce more seeds. Therefore, it would be beneficial to be able to genetically modify branching in *Brassica* species to increase seed yield. For this, we must have a sufficient understanding of the genetic control of primary branches in *Brassica* species. Therefore, in order to use genotypes in breeding programmes in an efficient manner, a greater comprehension of this topic is required. The identification and mapping of QTLs in several *Brassica* species has been the focus of research efforts in recent years. Prior research on markers linked to branching, more precisely, the number of primary branches, has been reported for *B. juncea*, although not as much as for other *Brassica* species, such as *B. napus*. It is necessary to have markers that are closely linked to the target locus in order to apply MAS (Marker Assisted Selection) to a large number of samples and diverse crossings in various breeding programmes. Therefore, using a gene-tagging method known as bulk segregant analysis, the current work seeks to identify markers/genomic areas related to the number of primary branches in *B. juncea*.

Bulked Segregant Analysis (BSA) is a method for identifying markers associated with specific genes or genomic regions linked to phenotypic responses. It involves comparing DNA samples from segregating populations and assaying the bulks contrasting for a specific trait. Polymorphic markers are linked genetically to the locus associated with the trait used to create the bulk (Michelmore et al., 1991). Polymorphic molecular markers, closely

linked to major QTLs regulating a trait, co-segregate with the QTL, causing significant variation in the frequency of marker alleles within extreme groups. Chromosome location of these markers can be determined without genotyping every individual in the segregating population. The concept behind BSA is to capture representative genotypes at a specific locus while generating a random genetic background at unlinked loci (Michelmore et al., 1991). Selective genotyping offers a significant advantage over traditional QTL analysis by reducing the number of individuals analyzed, saving time and money by focusing on severe traits, and enabling the monitoring of gene behavior at specific gene loci through advancements in molecular marker techniques.

The goal of the current study was to find genomic areas or markers related to *B. juncea*'s primary branch number. A combination of BSA and molecular analysis using PCR-based molecular markers, or SSRs, was used to identify markers linked to the number of primary branches in *B. juncea*. The current study's findings will be applied to fine mapping and then MAS. Main research questions for this study:

- What is the utility of the segregating mapping population ($F_{2:5}$) in examining the genetics of the number of main branches?
- Is the segregating population diverse enough to support genomics and genetics research?
- To what extent would bulks (derived from morphological data) be pertinent to the desired trait?

Considering the aforementioned, the current study was carried out in *B. juncea* with the following goals:

1. Morphological characterisation of $F_{2:5}$ lines for primary branches to develop pools with extreme phenotypes for Bulk Segregant Analysis (BSA)
2. Molecular marker-based BSA of $F_{2:5}$ lines for primary branches.

Materials and Methods

Plant material

130 $F_{2:5}$ plants (representing 83 progenies) of a cross between Kafiav N Zagora (east European type from Bulgaria) and Pusa Karishma (Indian type, developed by IARI, New Delhi), which were significantly contrasting for major agronomic traits (Table 1), were assessed for this study. These two genotypes were used as parents for making genetic cross. Single seed descent method was used to establish the $F_{2:5}$ population, with plants chosen randomly for the following year, with no selection from F_2 onward.

Phenotyping and genotyping of the parents

Based on the previous years' data provided, it was found that the number of primary branches vary significantly for both the parents. Both Kafiav N Zagora and Pusa Karishma were having average primary branch count of 18.67 and 7.20, respectively. Genotyping of the parents of the mapping population was carried out by employing 148 arbitrarily selected SSR primers; out of which 73 belong to A genome (*B. rapa*) while 75 markers were from B genome (*B. nigra*).

Table 1. Table showing contrasting characters of the parents of the cross.

Trait	Kafiav N Zagora	Pusa Karishma
Plant height (cm)	262.77	156.62
Main shoot length (cm)	14.03	49.14
Siliqua number	26.50	35.20
Number of primary branches	18.67	7.20
Number of secondary branches	46.67	18.80
Siliqua length (cm)	2.57	4.00

Sowing of experimental material

$F_{2:5}$ lines from a genetic cross of Kafia N Zagora x Pusa Karishma were planted in a single 2-meter row, in the School of Biotechnology's experimental field at SKUAST-Jammu.

Morphological characterization and bulk formation

The number of primary branches on the main stalk of the $F_{2:5}$ segregating population, which was derived from a genetic cross between Kafia N Zagora and Pusa Karishma, was counted. First, 83 plant progenies with a high or low number of primary branches were chosen. Out of the 83 progenies, at least one plant was chosen for further analysis (selective genotyping) . Two bulks with extreme phenotype (number of primary branches), were formed by grouping together the lines with almost identical numbers of branches. Only plants with 10 or more primary branches were chosen for high primary branches. In a similar vein, plants with five primary branches or fewer were chosen for low primary branches. It was made sure that no two plants were chosen for bulk creation from the same offspring.

DNA extraction

Genomic DNA was isolated from young leaves by CTAB method of plant DNA extraction.

Molecular analysis

The initial marker polymorphism was identified by screening the parental genotypes. For further analysis of the $F_{2:5}$ lines, the markers that were polymorphic on the parents were utilised. SSR PCR profile used is given in Supplementary Table 1.

Results

Morphological analysis for primary branches

130 plants, representing 83 $F_{2:5}$ plant progenies, were used to score the primary branch numbers in the $F_{2:5}$ population. These 130 plants have varying numbers of primary branches, ranging from 2 (Block 1, line 22) to fifteen (Block 2, line 6), as depicted in Figure 1. Most plant progenies appear to have an approximately similar number of primary branches among different plants within each progeny based on visual observation. However, as these progenies comprised plants with both low and high numbers of main branches, a total of 5 (6.02%) plant progenies were discovered to be segregating based on primary branch count.

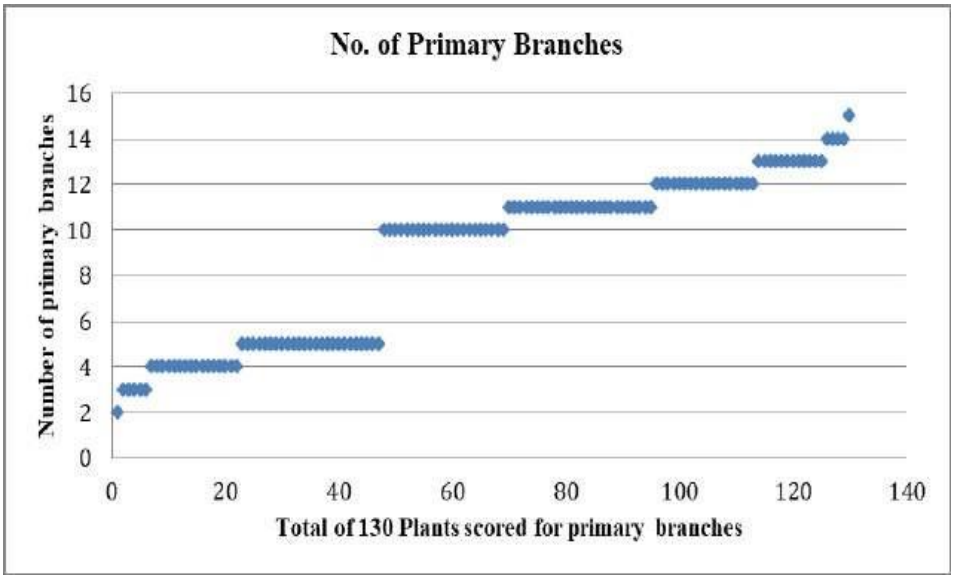

Figure 1. Scatter diagram showing distribution of primary branch number among 130 plants of F2:5 population.

Formation of bulks for primary branches

Plants with varying numbers of primary branches were selected to produce bulks for gene tagging using molecular markers (Table 2). Twelve plants each, were chosen from among the 130 plants for the higher primary branches (designated as "HPB") and lower primary branches (designated as "LPB"). There were 10 to 14 primary branches for HPB bulk, whereas there were about 4 to 5 primary branches for LPB bulk. For HPB and LPB bulks, the average number of primary branches was 12.16 and 4.50, respectively. Markers found to be polymorphic on parental genotypes were then utilised for gene tagging on these bulks.

Table 2. List of plants selected for bulk formation based on number of primary branches and molecular analysis.

Block no.	Line no.	Plant No.	No. of primary branches	Code of bulked individual plant
B1	L2	P5	10	HPB 1
B1	L15	P4	12	HPB 2
B1	L31	P4	14	HPB 4
B1	L33	P3	12	HPB 5
B2	L4	P1	12	HPB 6
B2	L13	P2	12	HPB 10
B2	L23	P1	13	HPB 12
B3	L32	P1	12	HPB 20
B3	L35	P3	13	HPB 21
B2	L20	P1	12	HPB 23
B2	L31	P2	12	HPB 24
B3	L18	P1	12	HPB 27
B2	L28	P1	12	HPB 14
B3	L10	P1	12	HPB 26
B1	L4	P4	4	LPB 2
B1	L12	P1	5	LPB 4
B1	L8	P1	4	LPB 5
B1	L30	P2	4	LPB 9
B1	L34	P1	5	LPB 10
B1	L37	P1	4	LPB 11
B1	L40	P3	4	LPB 13
B2	L32	P1	5	LPB 16
B3	L28	P1	4	LPB 19
B1	L28	P1	5	LPB 22
B1	L10	P2	5	LPB 23
B1	L23	P1	5	LPB 24

Identification of polymorphism between parents

To check for initial polymorphism among parental genotypes, 148 SSR markers (73 from A genome & 75 from B-genome) in total were used. 40 markers were identified as polymorphic, since they exhibited distinct and observable bands with distinct alleles between the two parental genotypes (Table 3 & 4; Figures 2, 3 & 4). Of these 40 polymorphic markers, twenty-six came from the B-genome and fourteen from the A-genome. Replicating the parental polymorphism screening results revealed that 38 markers were confirmed to be polymorphic, whereas 2 markers (A03_3174449 & Ni2-B01) were found to be monomorphic.

Table 3. List of A-genome markers polymorphic on the parents.

SSR marker	Sequence (5'-3')	Sequence (5'-3')
A01_2688930	CAATGTAATGGGAAGAAAATG	GTACCTCTCCTGGTCCTGTAT
A03_3174449	AAAGAAGAGCTTTGAAGAGGA	TTGATTCACAACACACATACC
A04_11549954	CATTTTCCTCCTTGAGATCTAT	CTGGTGGAAAACTTGATTTTA
A04_13468345	CATCACAAGCCAAGAAGAAT	AGAGTCTGTGGTTCATCTCCT
A04_7703506	CCCGTGATACGGACTTTATAC	TCTCATGTTAAAAGTTAGAGTGG
A05_25290881	ATAAAGATTTGATGGGAGGAG	GGTGGAGGAGGATAGTTGTAG
A06_12596970	CCAAGTGTAGTTATACCGAGTT	TTACTAACTCAGTCGAATTTGG
A06_20161352	GCATTACAGAGAGTGAGCAAT	TCCTCCTTGAAGTTTAGTGTG
A06_25201785	CAGGTCTAATTGCCATCACTA	CATATGACAGGTCCACCCTA
A06_7568964	TGGACACCTAAATTCAACGTA	GTTTTAGATGCCAATTAACGA
A07_12938471	CAAGCTTCTTCAGCTCATAAC	AGCCAAAAAGACGAAGATAGT
A08_2087658	CAGCAGAGTCCTTCTTGTTTA	GCAAATTAGTAAATTCCACTCAG
A08_8336436	AAAATTATGATACGGGTACGG	GTTAGCGCGAGAATATGTTAC
A09_27227566	GAAAGCGAGTAAGAAGAGAGC	ACTCATTGTCCGTAAACACAC

Table 4. List of B-genome markers polymorphic on the parents.

SSR marker	Sequence (5'-3')	Sequence (5'-3')
Ni1-A04	TCCTCCTACTTTGATACTTGC	ACGTCAAATACTTCACTGCC
Ni2-A11	AACAAACAAGAGTCGAATACGG	AATGCCCTCTAACTGAGCCC
Ni2-B01	AAGGAGATTGTTTTTGGGGC	AAGACTAATAAACACACGGCG
Ni2-B03	ACTTCTTGCCCTCCTCACC	AAATACTCACTGCAATACCCAGG
Ni2-C03	CGTAGAAGATGAACTCGGGG	CTCTTTCAGCTACTGCTGCG
Ni2-C09	ACGGAAGAAATCCAACCTCG	TATGCTTGGAAATGGTTTGG
Ni2-C12	ACATTCTTGGATCTTGATTCG	AAAGGTCAAGTCCTTCCTTCG
Ni2-F02	TGCAACGAAAAAGGATCAGC	TGCTAATTGAGCAATAGTGATTCC
Ni2-H03	TTTGAAGAAACAAAAATGGCG	TCATCTTCCCCTCTCATTCC
Ni2-H06	CATCAGATCCGACGAAATCC	TCCTTTGGACTGTGAAAAACG
Ni3-B07	GGAGAAGAGGAAGAAGAAGCC	CGACTTCTAGAGGAACCCCC
Ni3-C05	TTTCGTGCTTTGGTGTGAAG	TCCCCAAATCGAACCATAAG
Ni3-C08	CCCTAACACGGTGTCAACAG	GGCAGAATCATCGAGAGGTC
Ni3-G05	AGGAAGCATTTGCGCTAGTC	TCTACAACCACAACGTCCAAG
Ni4-A02	AGGACCACTGGGATACAAGC	ATTTGGAGCTGCGTACTTCG
Ni4-A09	AAAGGGCGAAGAAGCAGC	TTTCTTCCATTTGACCGACC
Ni4-B10	GTCCTTGAGAAACTCCACCG	CCGATCCCATTTCTAATCCC
Ni4-C06	CAGAGGCGAAAACGAGAGAG	TTTATAGACTTCCCGTGGGC
Ni4-D10	ACATGCGAAAGGGATTTGAC	TGCAAGTGAACTCAAAACAAAAG
Ni4-F09	CTGTTATGCAAGGTCATCGC	TGTTCCAGGTGAAGAAACCG
Ni4-F11	CGTAAGTTTCAATTGTCAACGG	TCGTACGAAACAATCAACGG
Ni4-G02	TTGGTGTCAGAAACAACG	ACACACGACGGATCTCTGC
Ni4-G06	TGACGGCTGAAGAAAATCAG	GTTTAACCTAAACCGAAAATC
Ni4-G08	ATTTGACGGACTCCTCTTGC	CACTTGGTAACTCTATGGATGCC
Ni4-G10	AGACTGAAATATTTTGGGACC	CGTTCTTCAACTTGTTCATCATC
Ni4-H03	GATGAACAGCAACAGCTTGG	CAAAATGTCGTTTGTTAGTCTTGG

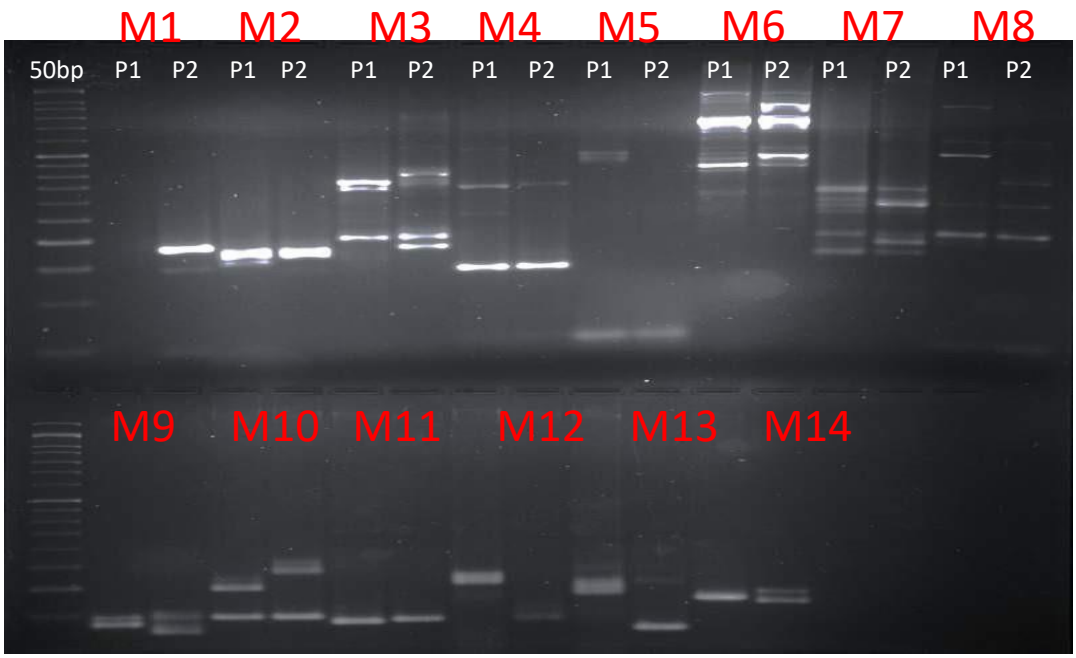

Figure 2. DNA polymorphism among two parental genotypes with SSR markers (markers labelled from M1 to M14; M1: A01_2688930, M2: A03_3174449, M3: A04_11549954, M4: A04_13468345, M5: A04_7703506, M6: A05_25290881, M7: A06_12596970, M8: A06_20161352, M9: A06_25201785, M10: A06_7568964, M11: A07_12938471, M12: A08_2087658, M13: A08_8336436, M14: A09_27227566). P1 and P2 refers to Parent 1 (Kafiav N Zagora) and Parent 2 (Pusa Karishma), respectively.

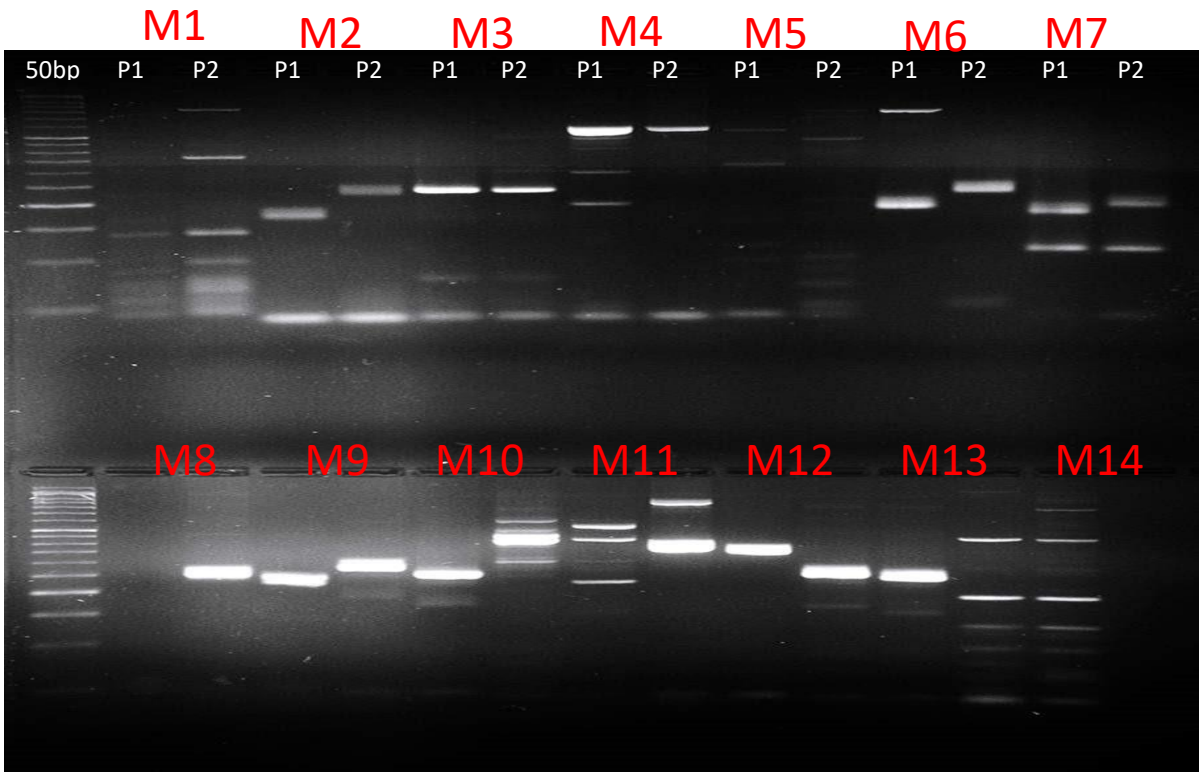

Figure 3. SSR Banding profile of the parents; M1: Ni1-A04, M2: Ni2-A11, M3: Ni2-B01, M4: Ni2-B03, M5: Ni2-C03, M6: Ni2-C09, M7: Ni2-C12, M8: Ni2-F02, M9: Ni2-H03, M10: Ni2-H06, M11: Ni3-B07, M12: Ni3-C08, M13: Ni3-G05, M14: Ni4-A02.

Molecular characterization of bulks using polymorphic markers

Twenty-six of the 38 polymorphic SSR markers were utilised to amplify the DNA of 24 lines, or two bulks. However, only 26 markers that were polymorphic on the parents could be tested for PCR amplification of the genotypes included in the two bulks. Moreover, no leaf samples were available at that time of the season for DNA isolation). Out of these 26 polymorphic markers tested on the bulks, 12 did not amplify any scorable polymorphism patterns on bulk genotypes, but 14 markers amplified segregating patterns for genotypes forming two bulks. Among the genotypes of two bulks, a total of 54 alleles were amplified by 14 polymorphic SSR markers (Table 6). Table 7 provides a comprehensive molecular profile of both bulk lines using 14 polymorphic primers.

Identification of markers associated with primary branches on B-genome

The relationship between the number of primary branches and genotypes that comprised two bulks was identified using both molecular and morphological data. To determine the relationship between marker alleles and primary branch number, the Student's *t*-test was utilised as none of the polymorphic markers produced distinct segregating patterns between the genotypes of two bulks. Based on the allele amplified in each genotype, the genotypes involved in the two bulks were divided into distinct classes, and each allele was assigned an alphabetic code for *t*-Test analysis.

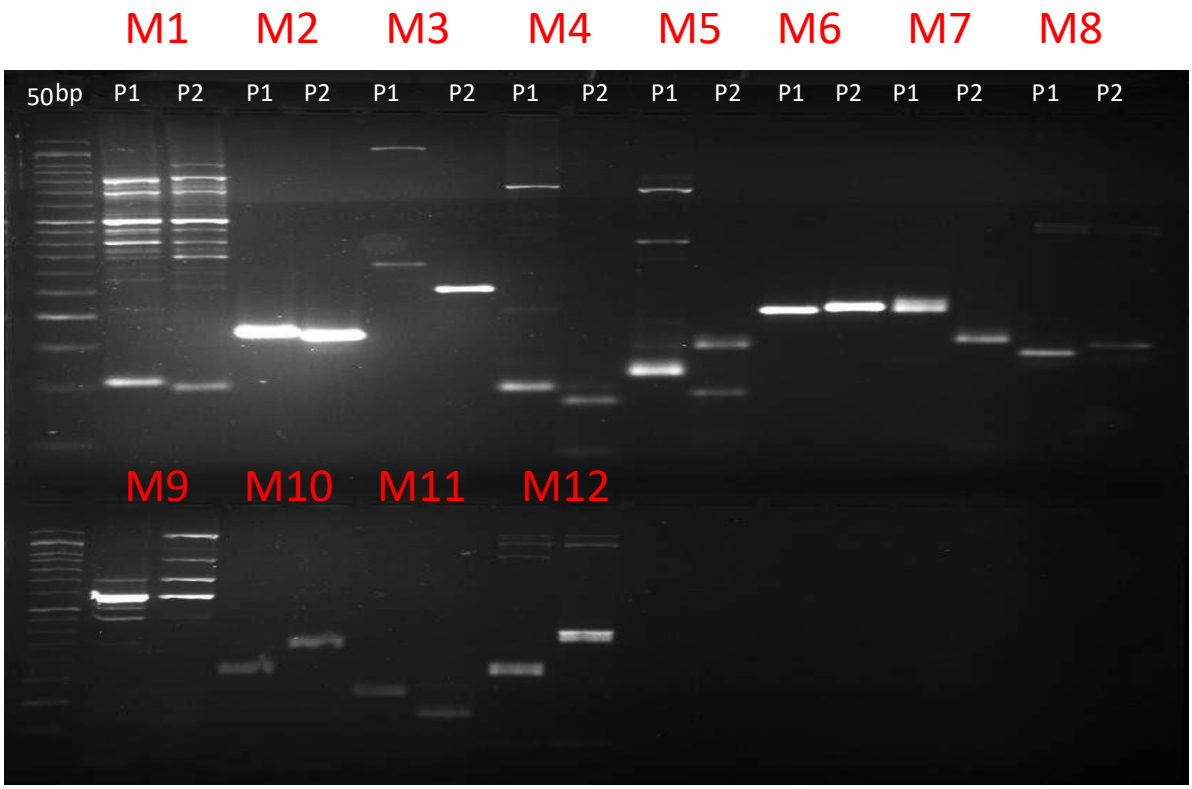

Figure 4. SSR Banding profile of the parents; M1: Ni4-A09, M2: Ni4-B10, M3: Ni3-C05, M4: Ni4-C06, M5: Ni4-D10, M6: Ni4-F09, M7: Ni4-F11, M8: Ni4-G02, M9: Ni4-G06, M10: Ni4-G08, M11: Ni4-G10, M12: Ni4-H03.

Table 6. List of SSR markers polymorphic on bulk lines and number of alleles amplified for each.

No	SSR Marker	No. of alleles amplified
1.	A04_13468345	3
2.	A06_12596970	5
3.	A08_8336436	2
4.	A06_7568964	5
5.	Ni2-H03	2
6.	Ni2-A11	3
7.	Ni2-B03	3
8.	Ni2-C09	8
9.	Ni2-C12	2
10.	Ni2-H06	3
11.	Ni3-C05	4
12.	Ni3-C08	3
13.	Ni4-G08	5
14.	Ni4-G10	6
	Grand Total	**54**

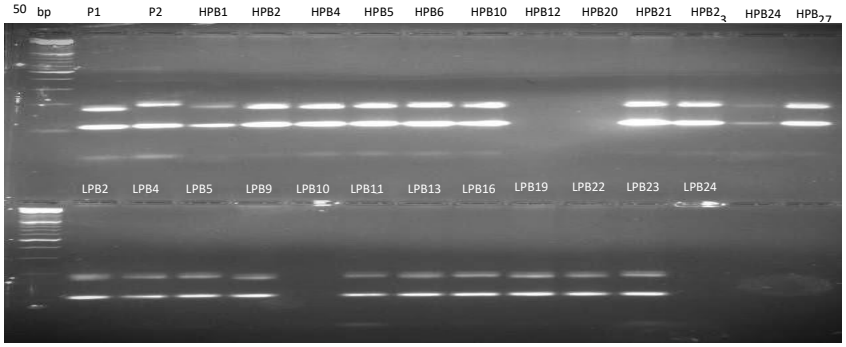

Figure 5. SSR banding profile of the bulk genotypes with the marker Ni2-C12. P1 as well as plants of the bulk 1 have common allele i.e., 190 bp whereas P2 and the plants of bulk 2, both have same (200 bp) allele amplified in them.

Two SSR markers, Ni2-C12 and Ni2-A11, were found to be significantly linked with the number of major branches based on *t*-test analysis. The estimation of association was based on the P-value for the *t*-test between alleles. The marker Ni2-C12 amplified two alleles of 190 bp and 200 bp among 20 genotypes out of the 24 genotypes of the two bulks. Scoreable bands were not amplified by the other four genotypes (Figure 5). The average number of primary branches for genotypes amplifying the 190 bp allele was 11.55, whereas the number for the 200 bp allele was 4.45 (Table 8). The *P*-value between the two alleles (i.e., 190 bp and 200 bp) was calculated using the data to be($P<0.001$) . Likewise, two alleles, 180 bp and 230 bp, were amplified by the Ni2-A11 alleles among the bulk genotypes. Four genotypes were heterozygous for Ni2-A11, as these four genotypes amplified these two alleles in heterozygous state. For alleles 180 bp, 7.75 was the average number of primary branches; for alleles 230 bp, it was 6.00; and for genotypes with heterozygous status, it was 12.67. For the *t*-Test, the *P*-value between the alleles 180 bp and 230 bp and 180 bp and the heterozygous condition (180 bp/230 bp) was 0.02. The heterozygous condition (180 bp/230 bp) and the 230 bp *P*-value for the *t*-test were found to be non-significant (Table 8).

Table 7. Results of molecular analysis of bulk lines.

Genotype	No. of primary branches	Ni2-A11	Ni2-C09	Ni2-H03	Ni2-H06	Ni2-C12	Ni2-B03	Ni3-C08	A0883364 36	A06_12596 970	A06_7568964	A04_13468 345	Ni4-G08	Ni4-G10	Ni3-C05
P1-Kafiav N Zagora	18.67	180	190	260	280	190	400	380	190	250	200	140	280	215	180
P2-Pusa Karish ma	7.20	230	230	250	250	200	null	360	130	300	240	140	360	180	150
HPB1	12	230	190/ 220	260	-	190	null		130/ 190	300	240	150	280	160	190
HPB2	12	180	218	250	250	190	400	380	130	300	240	140/ 150	220	215	180
HPB4	14	180	190/ 218	260	250	190	400	380	130/ 190	300	240	140/ 150	-	215	-
HPB5	12	230	230	250	250	190	390	380	130	250	220	140	280	200	160
HPB6	12	230	230	260	250	190	400	390	130	300	240	140/ 150	-	180	190
HPB10	12	180/ 230	190/ 220	260	250	190	400	380	130/ 190	300	245	150	280/ 360	215	180
HPB12	13	-	-	-	-	-									
HPB14	12						-	-	130	250	240	140	-	-	-
HPB20	12	-	190		-										
HPB21	13	230	190/ 220	260	250	190	400	360	130	300	240	140	-	-	180
HPB23	12	230	190	260	250	190	400	380	130		-	140/ 150	-	-	-
HPB24	12	-	190/ 230	260	-	190	null	380	-		null	140	-	-	-
HPB26	12						null	380	130	300	240	140/ 150	-	160/ 200	180
HPB27	12	180	190/ 230	260	250/ 280	190	400	380	130	300	-	150	-	-	180
LPB2	4	180/ 230	190/ 230	250	280	200	400	380	-	250	240	140/ 150	280/ 360	180	180
LPB4	5	230	218	260		200	400	380	130	300	-	150	360	180	180
LPB5	4	230	190	250	250	200	400	380	130/ 190	300	240	140	280/ 360	180	210
LPB9	4	180/ 230	190	260	250	190	-	380	130	300	245	140/ 150	-	215	180
LPB10	5	-	230	-	-	-	400	-	-	300	220	140	360	180	180
LPB11	4	230	190	260	250	200	null	380	130/ 190	250	240	140	360	180	190
LPB13	4	180/ 230	220	260	250/ 280	200	400	380	-	300	240	150	-	-	180
LPB16	5	230	190/ 220	260	250/ 280	200	400	380	130	300	200	150	260	180/ 215	180
LPB19	4	230	220/ 230	260	250/ 280	200	400	380	130	250	240	140	280		180
LPB22	5	230	190/ 220	250	250/ 280	200	400	380	-	300	240	140	-	-	180
LPB23	5	230	220	250	250	200	400	380	130	300	240	140/ 150	280	-	210
LPB24	5	-	190/ 220	250	250	-		380	130	300	240	150	280	215	180

Table 8. Details of polymorphic primers, alleles amplified and the significance among alleles for primary branches.

Marker name	Alleles	No. of genotypes allele amplified	Average number of primary branches/allele class	P-value (*t*-test)
A04_13468345	140	9	7.89	Non-significant
	150	7	7.86	
	140/150	8	9.34	
A06_12596970	200	1	15.00	Non-significant
	220	2	8.50	
	240	15	8.13	
	245	2	8.00	
A08_8336436	130	14	8.93	Non-significant
	130/190	5	9.20	
Ni2-A11	180	3	12.67	Significant 0.029 between 180bp and 230 bp, 0.02 between 180bp and heterozygous state
	230	12	7.75	
	180/230 (heterozygous)	4	6.00	
Ni2-B03	390	1	12.00	Non-significant
	400	16	8.00	
	Null allele	4	10.00	
Ni2-C09	190	5	7.20	Non-significant
	218	2	8.50	
	220	2	8.50	
	230	3	9.67	
	190/218	1	14.00	
	190/220	6	8.67	
	190/230	3	9.34	
	220/230	1	4.00	
Ni2-C12	190	11	11.55	Significant (P<0.001) (1.007 E-10
	200	9	4.45	
	250	12	9.08	
Ni2-H06	280	1	4.00	Non-significant
	250/280	6	6.00	
Ni3-C05	160	1	12.00	Non-significant
	180	14	7.29	
	190	3	9.34	
	210	2	4.50	
Ni3-C08	360	18	1.00	Non-significant
	380	1	7.50	
	390	1	1.00	
Ni4-G08	220	1	12.00	Non-significant
	260	1	5.00	
	280	5	7.60	
	360	3	4.67	
	280/360	3	6.67	
Ni4-G10	160	1	12.00	Non-significant
	180	7	5.58	
	200	1	12.00	
	215	5	9.40	
	160/200	1	12.00	
	180/215	1	5.00	
A06_7568964	200	1	5.00	Non-significant
	220	2	8.50	
	240	15	8.13	
	245	2	8.00	
Ni2-H03	Null	1	12.00	Non-significant
	250	7	6.71	
	260	14	8.92	

Tagging of 9 Mb region on chromosome B5 of B-genome for primary branches

The *B. nigra* genome assembly at http://brassicadb.cn/#/BLAST/ was utilised to do a sequence similarity search using the clone sequences of two highly linked markers, Ni2-C12 and Ni2-A11 (Supplementary Figure 1 and 2). Both markers' sequence similarity searches matched them to a 9 Mb range on *B. nigra*'s chromosome B5. B5 was mapped to the markers Ni2-A11 and Ni2-C12 at 55.9 Mb and 64.9 Mb, respectively. The sequence similarity e-values for markers Ni2-A11

and Ni2-C12 were 4.19 x 10-121 and 6.28 x 10-80, respectively (Supplementary Figure 3 and 4).

Discussion

The present research focuses on identifying informative markers using BSA to identify markers related to primary branches in *B. juncea*. There have been some prior reports of markers associated with branching, specifically about the number of primary branches, in *B. juncea*, but not as much as in other *Brassica* species, such as *B. napus*.

A QTL analysis conducted on *B. juncea* (AABB) revealed 65 significant QTLs spread across 13 linkage groups, of which 8 were linked to branching habit, 4 to primary branch number, and 4 to secondary branch number (Ramchiary et al., 2007). Twelve genes that affect branch number, plant height, and seed output in *B. juncea* have been linked with yield, agronomic, and morphological features in the Brassica database (Yadava et al., 2012). The mustard gene for plant growth habit was mapped using molecular markers, with the Sdt1 gene found on the linkage group 15 of *B. juncea*. Ni4-A10 and SJ6842 primer pairs were found to be linked to the Sdt1 gene, with marker SB3140 (present Just above the marker Ni4-A10 in the linkage group) specifically targeting chromosome 5 of the B-genome, which corresponds to chromosome 15 of *B. juncea* (Kaur and Banga, 2015). Several markers for traits related to yield were found in *B. juncea* employing genome-wide association mapping. Chromosomes 2 and 3 of the B-genome displayed QTLs for grain yields. A QTL for secondary branch number was found on chromosome 7, which was also the site of a prior identification by Ramchiary et al. (2007)Ramchiary et al. (2007)Ramchiary et al. (2007)Ramchiary et al. (2007). Additionally, a QTL for seed size was discovered on chromosome A6 of the A-genome, confirming previous theories that a similar QTL existed on the corresponding chromosome of *B. juncea* (Ramchiary et al., 2007). The marker SB1822-1, which was found at 17.5 cM on chromosome B3, also had an effect on grain yield. On the other hand, SB3872-3, which was found at 60.9 cM on chromosome B5, had the most effect on seed size (Akhatar and Banga, 2015). The Pi gene, which creates pink leaves in ornamental kale (*B. oleracea*), was located between 0.6 and 2.4 cM on either side of the SSR marker Ni2-C12 and the co-dominant SCAR marker Boac04. Based on the *B. oleracea* reference genome sequence, Pi was located near the top of chromosome C3, syntenic to B5 of *B. nigra* (Zhu et al., 2016). A total of 57 MQTLs, of which 31 are located in sub-genome A and 26 in subgenome B, were determined by the scientists' consensus map, which effectively projected 798 QTLs linked to yield-related attributes, including primary branch number. The number of MQTLs found for traits associated to yield varied from one on chromosome B5 to a maximum of six on chromosomes A3 and B3 (Kumar et al., 2022).

Above findings provide a basis for additional genetic studies related to *B. juncea*'s branching process. Therefore, using BSA, an attempt was made in the current study to identify genetic markers linked to the number of primary branches. MAS for the target trait will eventually benefit from the current research problem's findings and fine mapping.

Morphological diversity for primary branches in $F_{2:5}$ population

Genetically fixed (almost) $F_{2:5}$ lines from a hybrid between East European and Indian genotypes were used in the current investigation. It was determined that there are differences between the two parents for a number of features, including the number of primary branches, based on the data from prior years for significant morphological traits. With no selection from F_2 onward, the $F_{2:5}$ population was created by a single seed descent approach, with plants being randomly picked for the following year. Based on this, morphological study of 130 plants was performed for the primary branches. Since primary branches in different plants ranged from 2 to 15, there was heterogeneity in the $F_{2:5}$ mapping population for primary branches. This suggested that there was sufficient variance in the population for genetic analysis to likely identify the gene or QTL causing the distribution of primary branches among F_5 plant progenies.

Use of F2:5 population for BSA

Common genetic variations governing a given trait are probably found by analysis of genotypes that are comparable to each other for that trait. Morphological data of 130 plants was analysed. As a result, a total of 24 genotypes were found and

divided into two pools (the HPB and LPB), each including 12 members. The primary number branch count in each pool varied significantly. The number of primary branches in the two bulk pools that were created differed significantly, with 12.16 primary branches in the HPB pool and 4.50 primary branches in the LPB pool. Similar primary branches (higher or lower) among genotypes would probably indicate that the alleles within each genotype pool are similar. Based on this, the significant marker allele-traits association was found using BSA and the Student's *t*-test.

Identification of 9 Mb genomic region for primary branches

According to sequence similarity e-values, both markers mapped to a high degree of certainty within a 9 Mb region of chromosome B5, suggesting that the genetic regulation of the primary branches in *B. juncea* is most likely located on chromosome B5. However, there are a lot of additional genomic areas or loci that could be involved in *B. juncea's* number of major branches.

Validation and fine mapping of 9 Mb region for primary branches

The number of primary branches was mapped to the 9 Mb region on chromosome B5 of B-genome of *Brassica juncea*. However, this finding needs to be confirmed using the same set of markers in genetically diverse populations**.** If verified, the 9 Mb region would be an excellent candidate for creating a detailed map of the area, which would enable the discovery of important genes and markers linked to the (number of) main branches. In the current study, the genomic region determining the number of primary branches in *B. juncea* was tagged using $F_{2:5}$ lines of a genetic hybrid between Kafiav N Zagora (east European type) and Pusa Karishma (Indian type). The following summarises the study's main findings:

- The primary branch numbers of the $F_{2:5}$ population were scored using a total of 130 plants, which came from 83 plant progenies.
- Of the plant progenies, 5 (6.02%) had plants with both low and high numbers of primary branches, indicating that these progenies were segregating based on primary branch count.
- Of the 130 plants, a total of 12 plants each were chosen to make HPB and lower primary branches LPB bulks.
- For HPB and LPB bulks, the average number of primary branches was 4.50 and 12.16, respectively.
- To check for initial polymorphism between parental genotypes, 148 SSR markers in total were employed.
- It was found that 38 SSR markers were polymorphic because they produced distinct and scoreable bands with distinct alleles between the two parental genotypes.
- Among the genotypes that make up two bulks, a total of 14 markers amplified 54 alleles.
- Two SSR markers, Ni2-A11 and Ni2-C12, were found to be significantly linked with the number of primary branches by Student's *t*-test analysis.
- On *B. nigra* chromosome B5, two highly associated markers (Ni2-A11 and Ni2-C12) were mapped to a 9 Mb genomic area spanning from 55.9 Mb to 64.9 Mb.

Conclusion

The study created two pools of genotypes with high and low numbers of primary branches from an $F_{2:5}$ population derived from a genetic cross between genotypes from the European and Indian gene pools. The molecular and morphological data of the parental genotypes and the pools was recorded. When the molecular and morphological data was subjected to bioinformatic (sequence similarity using BLAST) and statistical (Student's *t*-test) analysis, a 9 Mb genomic area between 55.9 Mb and 64.9 Mb on chromosome B5 of the B-genome of *B. juncea* was located.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_712349.html.

Supplementary Table 1: Thermal Profile used for PCR.

Supplementary Figure 1. Screenshot of NCBI page showing clone sequence of Ni2-A11**.**

Supplementary Figure 2. Screenshot of NCBI page showing clone sequence of Ni2-C12**.**

Supplementary Figure 3. Blast hit of Ni2-C12 to chromosome B5 of *B. nigra* at 64.9 Mb.

Supplementary Figure 4. Blast hit of Ni2-A11 to chromosome B5 of *B. nigra* at 55.9 Mb.

Author Contributions

Conceptualization: M.K.; Methodology: M.K.; Software: S.S.; Validation: L.K. and S.S.; Formal analysis: M.K.; Investigation: M.K.; Data curation: M.K.; Original draft preparation: M.K.; Review and editing: L.K.; Visualization: M.K.; Supervision: M.K.; Project Administration: M.K.; and L.K,; All authors have read and agreed to the published version of the manuscript.

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Abbreviations

BSA, Bulked Segregant Analysis; SSR, Simple Sequence Repeats; HPB, Higher Primary Branches; LPB, Lower Primary Branches; Mb, Megabytes; QTL, Quantitative Trait Loci.

Conflicts of Interest

The authors declare no conflict of interest.

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مقاله پژوهشی

ارتباط نشانگرهای SSR با شاخه‌های اولیه در خردل هندی (*Brassica juncea* L.)

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چکیده: در این مطالعه از لاین‌های F2:5 حاصل از تلاقی بین ارقام Kafiav N Zagora و Pusa Karishma برای نشان‌دار کردن مناطق ژنومی کنترل‌کننده شاخه‌های اولیه در *Brassica juncea* استفاده شد. ۱۳۰ گیاه F2:5 برای مشخص کردن تعداد شاخه‌های اولیه استفاده شد که منجر به دو خزانه از ۱۲ ژنوتیپ برای شاخه‌های زیاد (HPB) و کم (LPB) گردید. میانگین تعداد شاخه‌های اولیه برای HPB و LPB به‌ترتیب ۱۲.۱۶ و ۴.۵۰ بود. مجموعه‌ای از ۱۴۸ نشانگر SSR (توالی‌های تکراری ساده) برای غربالگری چندشکلی والدین استفاده شد که از آن ۱۴ نشانگر SSR چندشکل برای شناسایی مولکولی و نشان‌دار کردن مناطق ژنومی توده‌های HPB و LPB استفاده شد. داده‌های آللی امتیازدهی شده برای ۱۴ لاین چندشکل با استفاده از آزمون t– استیودنت (t-test) برای درک روابط شاخه‌های اولیه با نشانگرهای SSR و آلل‌های تقویت‌شده مورد آزمون قرار گرفتند. بر این اساس، دو نشانگر ژنوم B (Ni2-C12) و (Ni2-A11) شناسایی شدند که از همبستگی بالایی با تعداد شاخه‌های اولیه برخوردار بودند. تجزیه و تحلیل بیوانفورماتیک نشان داد این دو نشانگر در ناحیه ۹ Mb، بر روی کروموزوم B5 *B. juncea* قرار دارند. مطالعه حاضر با استفاده از لاین F2:5 حاصل از تلاقی خزانه بین ژنی، توانست مکان‌های تنظیم‌کننده تعداد شاخه‌های اولیه را در کروموزوم B5 زیر مجموعه ژنوم *B. juncea* شناسایی نماید. تایید این نتایج در زمینه‌های مختلف ژنتیکی پیش از مطالعات مکان‌یابی دقیق در ناحیه ژنومی کروموزوم B5 (بین ۵۵.۹ و ۶۴.۹ مگابایت) ضروری بنظر می‌رسد.

کلمات کلیدی: *Brassica juncea* ، تعداد شاخه اولیه، BSA، SSR.

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