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Hybridity confirmation in F_1s of Indian mustard (*Brassica juncea* Czern & Coss) by integrating microsatellite markers and disease reaction

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Abstract
Confirmation of true F_1 hybridity in Indian Mustard is a crucial step in the selection of positive segregating individuals after the hybridization program in crop breeding. The use of molecular markers is the most reliable and rapid method rather than the grow-out test (GOT). The present study aimed at the assessment of genetic purity of the generated F_1 plants in Indian Mustard (*Brassica juncea)* using molecular markers as well as visual disease reaction as PDI. Out of 80 SSR markers used for the parental polymorphism study, 18 were found to be polymorphic between the parents Varuna and CAULC-2 (Locally collected from Kakching wairi, Manipur and highly tolerant o *Albugo candida*). Among polymorphic markers obtained from two parental line, the markers At2g36360 could identify the true F_1 plants. The plants that showed lower PDI were selected for further development of segregating or mapping population. This study showed that these SSR markers may be used as referral SSR markers for testing hybridity and genetic purity in Brassica breeding programs.

Keywords: *Albugo candida, Brassica juncea,* GOT, molecular marker, PDI

Introduction
Among nine oilseed crops grown in the country, seven are edible (Soybean, Groundnut, Rapeseed-Mustard, Sunflower, Sesame, Safflower, and Niger), and two are nonedible oils (Castor and Linseed). Brassica ranks second largest edible oilseed crop after Soybean in terms of area (22.2% of the total oilseed cultivated area) and production (32% of the country's total oilseed production). *Brassica juncea* (Czern & Coss.), commonly known as Indian Mustard, is one of the major oilseed crops in the Indian sub-continent and in China. It belongs to Brassicacae family and the family comprises three diploid species, *Brassica rapa* (2n=20, AA), *Brassica nigra* (2n=16, BB), and *Brassica oleracea* (2n=18, CC); and three allopolyploid species, *Brassica carinata* (2n=34, BBCC), *Brassica juncea* (2n=36, AABB), and *Brassica napus* (2n=38, AACC) (El-Esawi, 2015 and Labana and Gupta, 1993) [1, 2]. These six species represent the U triangle of Brassica. All the species are self-pollinated in nature except *B. nigra*, which is self-sterile, and *B. rapa* cv. Toria and *lotni* brown sarson are cross-pollinated due to self-incompatibility. The crop is being widely and economically cultivated over an acreage of 6.82 mha in India. Over 50% of the Indian population consumes Mustard oil as a cooking medium daily (Govt. of India, 2021) [3].

The development of segregating populations is one of the vital steps for the establishment of successful crop breeding programs. Initially, the F_1 plants are generated by crossing two diverse parents and the selection of a true F_1 hybrid are determining step to produce segregating progenies. The conventional method of identification of F_1s based on morphological traits limits whether a crossed/F_1 plant is a true hybrid or selfed by chance self-fertilization. This method is quite time-consuming and influenced by the environment, even though it was quite successful in the past. In the later years, molecular marker technology enables plant breeders to identify putative F_1 plants precisely as they are based on genotype rather than phenotype. The microsatellite markers are being commonly used due to their highly polymorphic, codominant, independent of the environment and stage of the plant (Sundaram *et al*., 2007) [4]. Identification of true to-type F_1 plants in a short period of time is important in mustard breeding programs because it speeds up the screening of progenies and there are high

chances of self-fertilization since the crop (Indian Mustard) is self-pollinating in nature. Therefore, the present study was undertaken with the primary objective of selecting of true F_1 plants derived from two diverse and utility of microsatellite markers in hybridity assessment.

Materials and methods

The present study involved the generation of F_1 plant progeny to produce subsequent progenies. The two parental materials of Indian Mustard, Varuna used as a female parent (highly susceptible to *Albugo candida*) and CAULC-2 used as a male parent (Locally collected from Kakching wairi, Manipur and highly tolerant o *Albugo candida*). The Varuna and CAULC-2 lines were provided by the Department of Genetics and Plant Breeding, College of Agriculture, Central Agricultural University, Imphal, Manipur. The seeds of the two parents were sown in pots and made their hybridization to develop F_1 plants (Figure 1). Both the male and female parent plants were sown in staggered manner to achieve synchronized flowering for hybridization. Emasculation was performed in the evening by selecting an unopen but matured bud (high chances of opening the bud next day) and followed by bagging it with butter bags to avoid pollen contamination. On the next day, pollens from male parents, CAULC-2 were dusted on the emasculated female parent, Varuna and bagged again. After, 4-5 days, pod development was noticed. The matured F_1 seeds were harvested separately and kept in a dry place. The healthy and filled seeds from the crossed plants were collected and raised during *Rabi*, 2020-21. Then, true hybrid seedlings will be confirmed through visual selection based on disease score and molecular marker.

Inoculum Preparation

The disease inoculum was prepared by collecting fresh and naturally infected leaves with White rust, *Albugo candida* from the field. The leaves possessing sporangiospore was scratched to collect only white powder from the lower surface of leaves using a sterilized needle. The suspension of Zoospore in distilled water was made of concentration 2.5 X 10^5 /ml with the help of Haemocytometer slide (Superior1, Germany). Then, the suspension was kept under dark at 4^0 C for 2-4 hours to facilitate spore germination.

Inoculation of A. *candida* suspension

Each individual F_1 plant progenies were sprayed the suspension on to the leaves until complete wetness using a hand operated sprayer. The plants were covered with a transparent sheet polythene to create optimum relative humidity and then shifted to a shaded area to maintain favourable condition for pathogen sporulation. Diseased symptoms were observed just after appearance of disease symptom to know the degree of severity.

Disease assessment

All raised F_1 plants were numbered and tagged. Then disease intensity of each plants was observed and compared with the parental plants**.** The disease scoring 0-9 was done (Fox and William, 1984) [5]. The Percent Disease Intensity (PDI) was calculated with the following formula:

$$\text{Percent Disease Index (\%)} = \frac{\sum (n \times 0) + (n \times 1) + (n \times 3) + (n \times 5) + (n \times 7) + (n \times 9)}{\text{Total number of plants/leaves observed} \times \text{Maximum disease score (9)}} \times 100$$

where n-number of plants showing 0, 1, 3, 5, 7 and 9 disease scale.

Molecular characterization

The genomic DNA of the parents and F_1 plant progenies were isolated using HiPurA^R Plant genomic DNA miniprep purification Kit (MB507). The quality and quantity of DNA were estimated using gel electrophoresis and spectrometric method. The polymorphism between two parents was performed using molecular markers (Supplementary Table No.1). A total of 80 SSR and IP markers from different *Brassica species* and related Brassicaceae family covering the whole chromosomal regions were selected (Table No. 1) from Brassica Database *http://www.brassica.info.* The 10 µl reaction volume of PCR mixture containing 1 µl of 10 X Taq buffer, 1.0 µl of 50 mM $MgCl_2$ solution, .75 µl of 2.5 mM dNTPs mixture, and 0.5 µl of each of the forward and reverse primers at a concentration of 10 pmole/µl, 0.2µl of 3 U/µl T*aq* DNA polymerase, 1 µl of diluted genomic DNA (100 ng/ µl) and 5.05 µl of nuclease-free water was made. Then, the PCR was performed using Thermocycler (Applied Biosystems, 2720 Thermal Cycler) with the following cycle: initiation denaturation at 95 ºC for 3 minutes followed by 35 cycles of denaturation at 94 ºC for 1 minute, annealing at a respective temperatures of different primer pair for 1 minute and extension at 72 ºC for 1 minute and final extension at 72 ºC for 7 minutes. The PCR products were separated on 3% agarose gel stained with ethidium bromide, in 1X TAE buffer. Thereafter, gel documentation was followed.

Results and Discussions

Identification of polymorphic DNA bands between parental lines

The genomic DNA of parental lines was used for the identification of a true hybrid. Confirmation of hybridity of crossed material using molecular markers is a very precise and quick method for further developing segregating lines. It is commonly used by the public and private sectors for large-scale testing of commercial hybrids. In the present study, out of 80 molecular markers were used for screening of parental polymorphism between Varuna and CAULC-2 (Figure No.2, plate 1-8). The parental polymorphism study revealed that out of 80 primers used, 18 could differentiated between the two parents while the remaining 63 were found as monomorphic between the parents (Supplementary Table No. 2). There was cent percent amplification (100%) while polymorphic was 22.5%. The different percentage of polymorphism was reported, 9.5% out of 509 SSR markers in RIL population of Varuna X BIO-YSR (Behera C. 2020) [6], 13.6% (12 of 88 markers) in B. *carinata* to 32.1% (26 of 81 markers) in B. *oleracea* (Yadava *et al.*, 2009) [7].

Confirmation of true hybrid

Because identification of true to-type F_1 plants help to produce a subsequent generation/mapping population with an unaltered segregation ratio. Additionally, high efficiency of PCR-based molecular markers is cost effective and capable of replacing the high laborious GOT tests. And the crop (Indian Mustard) is being self-pollinated in nature, there are high

chances of self-fertilization. Therefore, confirmation of the artificially made crossed plants is necessary. From the listed polymorphic marker, At2g36360 was able to identify the hybrid plants (Figure No.3). The F_1 plants showing heterozygous nature (H) i.e., having alleles of both the parents were considered as the successful cross.

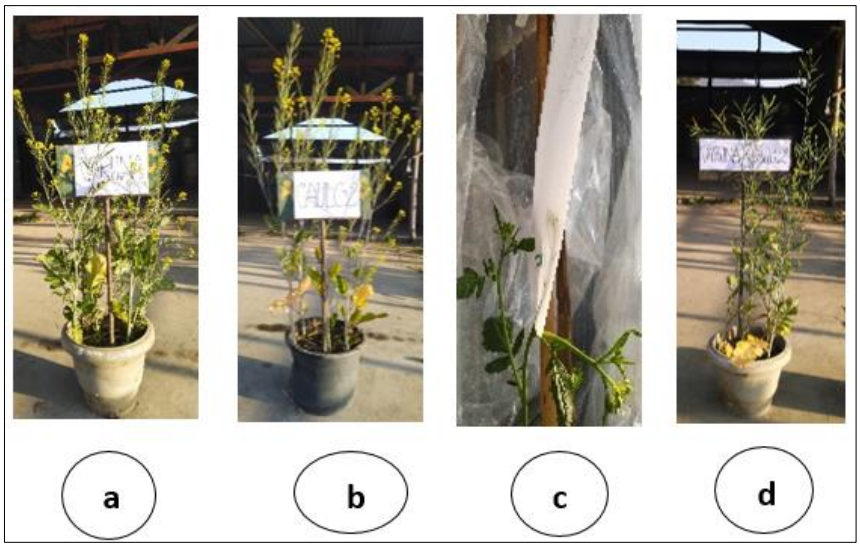

Fig 1. a) Parent Varuna, b) Parent CAULC-2, c) Bagging of hybridized plants d) Raising of F_1 plants

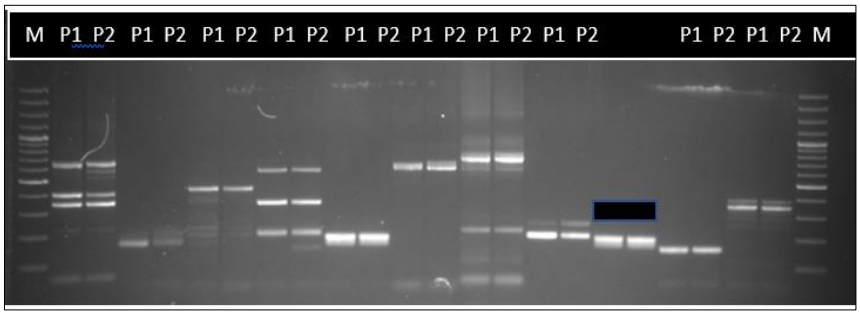

Plate 1: At5g40670, BRMS006, Ra2-B07, Na10-A08, BRMS017, At2g38130, At1g78560, Ni03-H07, BN25-1 & BrgMS339

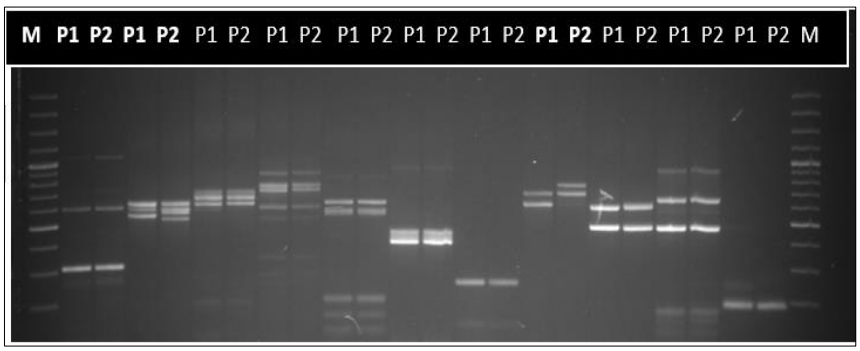

Plate 2: Ni2-A07, At3g23980, At2g28880, At4g39350, O110-H02, At5g41560, Ni2-A01, At4g33925, At4g34140, At3g59490 & Ni2D-10

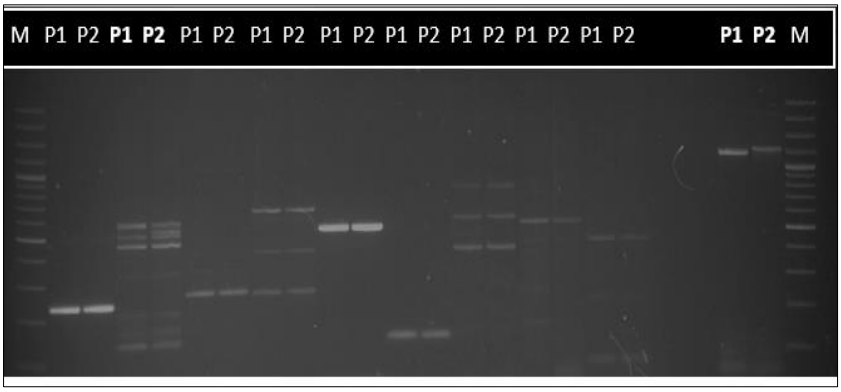

Plate 3: BRMS033, At5g40200, 59A1, Na10-C01c, At5g41940, BRMS034, Ra2-F11, Na12-D04, Ra2-E11 & At2g36360

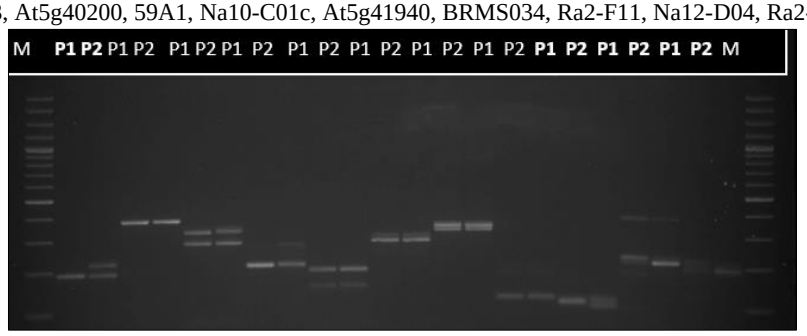

Plate 4: BrgMS4539, nia_m053a, BrgMS233, BrgMS841, BRMS007, BrgMS166, BrgMS36, BRMS-008, BrgMS139, BrgMS502 & BrgMS465

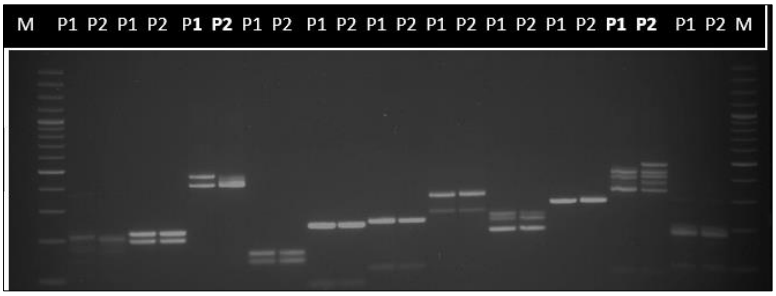

Plate 5: cnu_m584a, BrgMS3322, EJU1, BrgMS713, E039, SB0372, SJ1505, Ni4C09, SJ13133, ENA10 & BrgMS2766

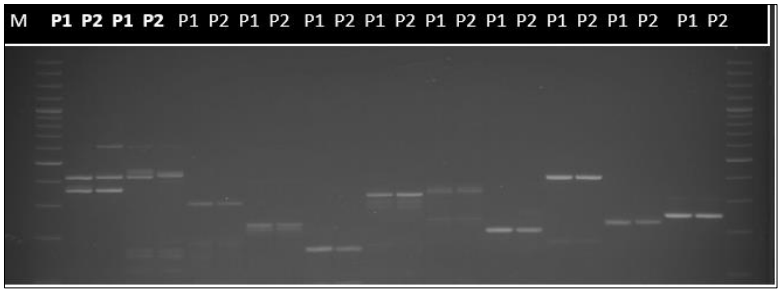

Plate 6: BrgMS643, SJ3302RI, BN6A3, BRMS096, At3g54130, BrgMS397, Ra2-C09, cnu_m585a, ENA17, BRMS-027 & BrgMS787

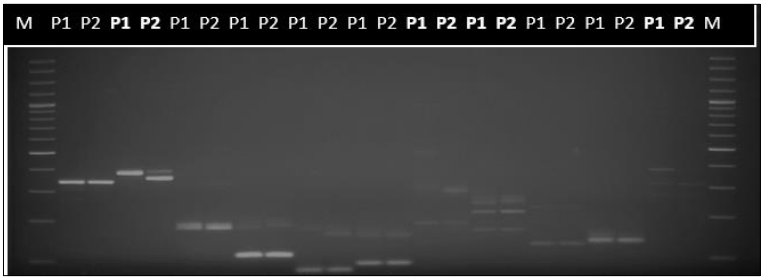

Plate 7: SJ4933, SA0306, SJ3874I, Ni2C12, Ni2A09, Ni4D10, BRMS-014, Ni2H03, MR52a, BRMS-018 & Nia_m141a

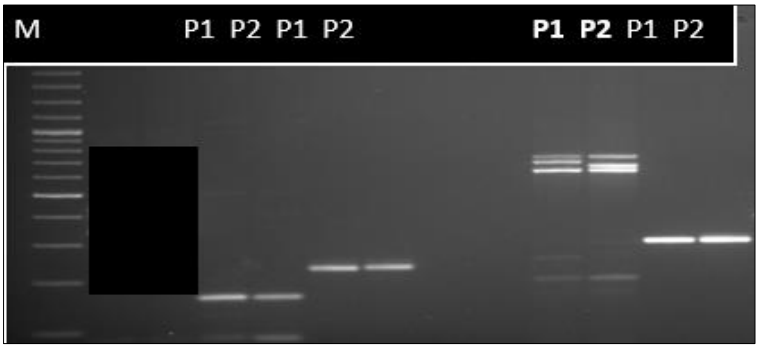

Plate 8: Ni2D03, Na10A09, At5g40390, At2g4510

Fig 2: PCR analysis of Parental lines for polymorphism using SSR/IP Markers, Where M- 100 bp Ladder (GCC), 1-Susceptible parent Varuna

and 2 - Resistant parent CAULC-2, Yellow colour indicates polymorphic marker

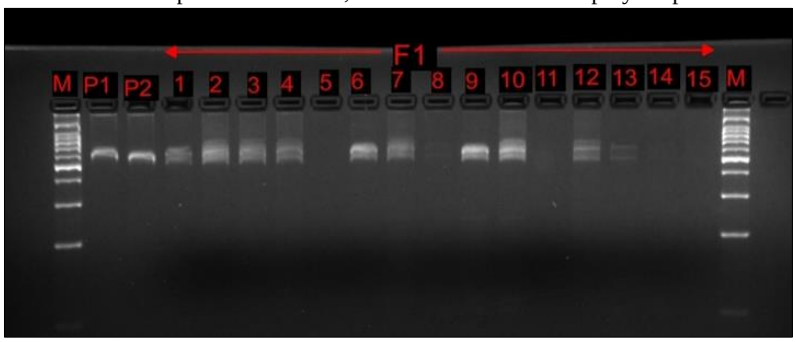

Fig 3: Banding pattern confirming hybridity using At2g36360 (M-200bp, P1-Varuna, P2-CAULC-2

A total of 26 F_1 seeds were harvested however 11 plants could not grow successfully till maturity. The remaining 15 F_1 plants were checked for the presence of heterozygous alleles that represents the true hybrid. The parent and F_1 plants with their appearance of disease which were used as phenotypic data were given in Table No.1 to select the heterozygous plant with minimum disease reaction. Among 15 F_1 plants, plant no. 1,2, 3, 4, 6, 7, 9, 10, 12 and 13 expressed both the alleles of the parents (heterozygous) and therefore confirmed the genuineness of hybrid plants. Thus, 10 plants from 15 were confirmed as a true hybrid. Earlier the evaluation of F_1 hybrids from its respective off type/selfed seed have been reported in Brassica, Tomato Rice, Lentil etc. Niemann *et al*., 2009 [8] demonstrated the use of cytological and molecular marker for identification F_1 progeny in Brassica. Wang *et al*., 2002 [9] demonstrated the genetic purity of hybrid in Maize using SSR markers, in Rice (Nandakumar *et al.*, 2004, Shanthala *et al*., 2013) [10, 11], in Tomato (Liu *et al*., 2008) [12] and in *Brassica juncea* (Sharma *et al.*, 2018) [13]. Suwabe *et al*., 2002 [14]; Ali *et al*., 2007 [15] and Sharma *et al*., 2018[13] study in local and exotic germplasm of *Brassica species* for confirmation hybrid based on the amplification pattern of SSR markers.

Table 1: Evaluation of F_1 progenies of Varuna X CAULC-2

Plant no.	Phenotype (PDI)	Molecular banding	Plant no.	Phenotype (PDI)	Molecular banding
F_1 Plant No 1	ND (0.00)	H	F_1 Plant No 9	D (10.00)	H
F_1 Plant No 2	ND (0.00)	H	F_1 Plant No 10	D (11.67)	H
F_1 Plant No 3	ND (0.00)	H	F_1 Plant No 11	D (3.33)	-
F_1 Plant No 4	D (3.33)	H	F_1 Plant No 12	D (8.89)	H
F_1 Plant No 5	ND (0.00)	-	F_1 Plant No 13	ND (0.00)	H
F_1 Plant No 6	D (7.78)	H	F_1 Plant No 14	D (1.67)	-
F_1 Plant No 7	D (11.67)	H	F_1 Plant No 15	ND (0.00)	-
F_1 Plant No 8	D (5.00)	-			
VARUNA	22.31		CAULC-2	3.73	

D- Diseased plant, ND-Non-diseased plant, H-Heterozygous, - No amplification

In addition to molecular characterization, phenotypic evaluation of White rust disease was performed. A significantly lower PDI than the disease tolerant parent (CAULC-2) was observed in plant F_1 progenies (F_1 Plant No. 1- 0.00, F_1 Plant No 2-0.00, F_1 Plant No.3-0.00, F_1 Plant No.4-3.33, F_1 Plant No 5-0.00, F_1 Plant No. 11-3.33, F_1 Plant No. 13-0.00, F_1 Plant No. 14-1.67, F_1 Plant No. 14-0.00) while the remaining F_1 showed higher PDI. The observed variations on hybrids in response to white rust indicate the role of environmental factors. All 15 hybrids obtained were found to be lower PDI than the female parent, Varuna (Table No. 1) and the plants showing no disease reaction (plant no. 1, 2, 3 and 13) and showed true hybrid were selected for development of segregating population.

Conclusions

This study showed that confirmation of such true hybrid progeny with the help of molecular markers is a very precise and quick method for the development of segregating lines. The positive marker found in the present investigation may be used as referral SSR markers for testing hybridity and genetic purity in Brassica breeding programs. The true hybrid progeny could be used in future experiments related to the development of a mapping populations with a definite segregation ratio for studying genetics and tagging of the white rust resistance gene in Indian Mustard.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Table No. 1: List of molecular makers used for prenatal study

	Marker ID	F/R	Sequence	Tm	Species
1.	At5g41560	F	CCTCACAATTTCAGTCAACATCGT	60ºC	*A. thaliana*
		R	GAGGTGGAAGAGTACGGTTGTG		
2.	At5g41940	F	CATGGCATATATCAGGAGACTGAG	58ºC	*A. thaliana*
		R	GCCTCCATTGAGTTCCATC		
3.	At2g36360	F	AAACTTCGCCGGTCGAAGAC	63ºC	*A. thaliana*
		R	GAGTCTCGAAGTCGCCGTTAAC		
4.	Ni2-A01	F	TGCTGCTACAGACAGTGTTGG	60 ºC	*B. nigra*
		R	AAAGGCTACACACTCATGAAACC		
5.	At5g40670	F	GTGTTAGCCATTGGAACTGAATG	53ºC	*A. thaliana*
		R	GACCACCAGAGTTCCCAGA		
6.	At5g40390	F	CGTTCGTCAACGTGGCACTAAG	56 ºC	*A. thaliana*
		R	TCTGTAACTGTTGGGATTCTCTGG		
7.	At5g40200	F	GCTGCTGTTTCCGTTGGTCT	54 ºC	*A. thaliana*
		R	GATCGTGAATCCATCACCACC		
8.	At2g34510	F	TGATTACCAAGGAGCAAGAGATGC	56 ºC	*A. thaliana*
		R	CACAGATGCACTAGGCTCAGATT		
9.	BRMS006	F	TGGTGGCTTGAGATTAGTTC	54 ºC	*B. rapa*
		R	ACTCGAAGCCTAATGAAAAG		
10.	Ni2A07	F	GGAACCCAACAAGTGAGTCC	50 ºC	*B. nigra*
		R	AGAGCTTGAGACACATAACACC		
11.	Ni2D03	F	CGTATGTGAAAAATAAATGG	45 ºC	*B. nigra*
		R	TTGAGCTTGAGATCATCCCC		
12.	Ra2B07	F	TTTAACTGCTGCAGGTCGC	53 ºC	*B. rapa*
		R	GGGCAAATGTGATAAATCCG		
13.	Na10A08	F	CATGGTTAAAACAATGGCCC	53 ºC	*B. napus*
		R	CAAGAAACACCATCATTTCTCA		
14.	BRMS017	F	GGAAAGGGAAGCTTCATATC	53 ºC	*B. rapa*
		R	CTGGAAAGCATACACTTTGG		
15.	BRMS034	F	GATCAAATAACGAACGGAGAGA	63 ºC	*B. rapa*
		R	GAGCCAAGAAAGGACCTAAGAT		
16.	BRMS096	F	AGTCGAGATCTCGTTCGTGTCTCCC	55 ºC	*B. rapa*
		R	TGAAGAAGGATTGAAGCTGTTGTTG		
17.	At3g23980	F	CAAAGGGATCTTGATGCTTCA	50 ºC	*A. thaliana*
		R	TCCATATCATCTTTTAGTTGGTTGAC		

18.	At2g28880	F	GATGAGTGGACGTGGGAAGAA	50 ºC	*A. thaliana*
		R	AAATCAGAGTTTCTCCCGGATG		
19.	At2g38130	F	TGGAATCTGGCTGTGAAGAG	54 ºC	*A. thaliana*
		R	TGAACCCGAGTCTCCCATATAG		
20.	At4g33925	F	TAACCGAAGAACAGCTCTCAATGTC	60 ºC	*A. thaliana*
		R	GAAAGCTTCAGATCT TTG AGAGCA		
21.	At4g34140	F	AGGATGGTCGTTACTACAAGCATG	60 ºC	*A. thaliana*
		R	GGA ATC CAT TCT CCC TCT TCA AG		
22.	At3g59490	F	CTCTCTCAGATCAAATGGCGACT	60 ºC	*A. thaliana*
		R	AGCTTCTGAATCGACCTAAGCTG		
23.	At3g54130	F	CCGATCTCGACGGGAAGGAGCG	50 ºC	*A. thaliana*
		R	GGGTCTATCTGCGCAGGCTCTGCA		
24.	At1g78560	F	GCTGGCTTTGTGTTGCAG	53 ºC	A. *thaliana*
		R	CCTGGACAGCAACCAACC		
25.	At4g39350	F	ACTGGTGGTCGACTCATTGC	50 ºC	*A. thaliana*
		R	TTTGCACTGAGGACAAGCTTG		
26.	BN59A1	F	TGGCTCGAATCAACGGAC	60 ºC	B. *napus*
		R	TTGCACCAACAAGTCACTAAAGTT		
27.	Ra2-F11	F	TGAAACTAGGGTTTCCAGCC	50 ºC	*B. rapa*
		R	CTTCACCATGGTTTTGTCCC		
28.	Na12-D04	F	ACGGAGTGATGATGGGTCTC	50 ºC	*B. napus*
		R	CCTCAATGAAACTGAAATATGTGTG		
29.	Ra2-E11	F	GGAGCCAGGAGAGAAGAAGG	45 ºC	*B. rapa*
		R	CCCAAAACTTCCAAGAAAAGC		
30.	O110-H02	F	AACAGGAAGAAACGACGAGG	50 ºC	*B. oleracea*
		R	AGAGAGCCATGAGAAGCACC		
31.	Na10-A09	F	AGAGAGCCATGAGAAGCACC	54 ºC	*B. napus*
		R	TCTTGAGCAAAGAAACTTGG		
32.	ENA10	F	ATCGTCTCCTCTCATCTCAA	50 ºC	*B. rapa*
		R	ATTACATCCTCCACCTTCTTC		
33.	nia_m141a	F	CAGCGGCTGCAATAGAAT	45 ºC	B. *rapa*
		R	TTGCATTTTGTTTCACTGGA		
34.	BrgMS397	F	CAAAACCCATCTGTGTGTGAGT	55 ºC	B. *rapa*
		R	GTGTGGCCGTTGTATTTTATTG		
35.	BrgMS4539	F	ATGCTTGAAGCTATGTGTGCTT	53 ºC	*B. rapa*
		R	TAACCAGATTTCCCGACAAAGT		
36.	nia_m053a	F	AAAATCTCGCTTCTGCGCTT	53 ºC	*B. rapa*
		R	TCCCTCACCCTGTGCAATAG		
37.	BrgMS2766	F	GCATTGGATTTGGATTGGAT	50 ºC	*B. rapa*
		R	AATCCATGTCTGCAACACAAAC		
38.	Ra2-C09	F	ATCCCCTTCATCATCCTCG	55 ºC	*B. rapa*
		R	TCTGGACTGATCAGAACTCGG		
39.	BrgMS233	F	TGGAGATAGTGAAGCAGTGGAA	53 ºC	*B. rapa*
		R	GTGCTGGAAGTGGAACCTTTAC		
40.	BrgMS841	F	CCGGTTTAGTTAGATGGATGCT	53 ºC	*B. rapa*
		R	GGTTTTGCCTTCGTATTTGGT		
41.	cnu_m585a	F	TTTATCAGTCCGGTTTTGCC	55 ºC	*B. rapa*
		R	GATGCTCTGAGACACCCAAA		
42.	BRMS007	F	AAATTGTTTCTCTTCCCCAT	53 ºC	*B. rapa*
		R	GTGTTAGGGAGCTGGAGAAT		
43.	BrgMS643	F	CGTAGGAGAAACATAGGGCACC	50 ºC	*B. rapa*
		R	CCCACTCCCGTATAATTTGCTT		
44.	BrgMS166	F	CGTCAAGAAGAGGAGCAGAGTT	53 ºC	*B. rapa*
		R	GCTACTGAAGGATTCGCATTTT		
45.	ENA17	F	CAGTTATTTCGCCTCGTCT	55 ºC	*B. rapa*
		R	TATTTGTGGTCTGTTATTGGA		
46.	BRMS-014	F	CCGTAAGGAATATTGAGGCA	53 ºC	*B. rapa*
		R	TTCCCAATTCTCAAACGGTA		
47.	BRMS-027	F	GCAGGCGTTGCCTTTATGTA	55 ºC	*B. rapa*
		R	TCGTTGGTCGGTCACTCCTT		
48.	BRMS-008	F	AGGACACCAGGCACCATATA	53 ºC	*B. rapa*
		R	CATTGTTGTCTTGGGAGAGC		
49.	BrgMS787	F	CCATCTCAGCTCTATCTACCAAAA	53 ºC	*B. rapa*
		R	TCAAAACACCGAGTAAACTGGA		
50.	BrgMS36	F	AGCACTTGAGTTTCTCCCTGAA	53 ºC	*B. rapa*
		R	GACTTCTTGCATCATCTTGTCG		
51.	BRMS-018	F	TCCCACGCCTTCTAGCCTTC	58 ºC	*B. rapa*

		R	ACCGGAGCTTTTCTGTTGCC		
52.	BrgMS139	F	GTGAGGGCTTTTAGGGTTTCTT	53 ºC	*B. rapa*
		R	GGACACGACTTGGTAGGTTTCT		
53.	BrgMS502	F	GACTTCACTCCATACAGGCACA	53 ºC	*B. rapa*
		R	CAACCATTGACGAGATGCAATA		
54.	cnu_m584a	F	CGGAAAGAACACATTGAAAGAA	53 ºC	*B. rapa*
		R	AGCATCAAAGAAAAAGGCGA		
55.	BrgMS3322	F	CACAACAGAAACAGGTGGAAGA	53 ºC	*B. rapa*
		R	GGTGAAGAGCATCCTCCATAAA		
56.	EJU1	F	GGTGAAAGAGGAAGATTGGT	53 ºC	*B. rapa*
		R	AGGAGATACAGTTGAAGGGTC		
57.	BrgMS713	F	TCCTCATCATCATCACCTTCTG	53 ºC	*B. rapa*
		R	GCGAGGTAAAACTTATCCACCA		
58.	E039	F	CTTGAGTGCTCAGGTCAAAGC	53 ºC	*B. rapa*
		R	GAACCCTTACCCCCAAGACTAC		
59.	Ni2H03	F	TTTGAAGAAACAAAAATGGCG	63 ºC	*B. nigra*
		R	TCATCTTCCCCTCTCATTCC		
60.	SJ4933	F	CTTGCAAATATTCAGGCCGT	55 ºC	*B. nigra*
		R	TGCAATCAGATCCGACTCAG		
61.	SJ3302RI	F	GGGGCAAAAGGAACTTGAG	50 ºC	*B. nigra*
		R	AAATAACCTGCGACGGTGAC		
62.	SA0306	F	CCGTTTTAAGCCATGGAAGA	55 ºC	*B. nigra*
		R	TGGGGTGGGTCTTAAATGAA		
63.	SB0372	F	ACGCCGGTTGATATTAGCAC	53 ºC	*B. nigra*
		R	CACACTGCCATCTCTCTCCA		
64.	Ni2C12	F	ACATTCTTGGATCTTGATTCG	53 ºC	*B. nigra*
		R	AAAGGTCAAGTCCTTCCTTCG		
65.	SJ3874I	F	ATGAGGTTTCATCCCGACAG	55 ºC	*B. nigra*
		R	ACAACATTTTCCATTCCCCA		
66.	Ni2A09	F	CGCGAGTAAATCAATGTGAATC	53 ºC	*B. nigra*
		R	CGACCCACCAACTCACTAAC		
67.	SJ1505	F	TACGATGCGTATGAACCACG	53 ºC	*B. nigra*
		R	CTTTTTGCATTCCATCCACTT		
68.	Ni4C09	F	AGCATCAATCTTTTGCTCTGC	53 ºC	*B. nigra*
		R	TGCACACAAACTCCTTCTCC		
69.	SJ13133	F	GGCATCGATCAAGTGACCTT	53 ºC	*B. nigra*
		R	AACCAAACCAAACCAAGTCG		
70.	Ni4D10	F	ACATGCGAAAGGGATTTGAC	49 ºC	*B. nigra*
		R	TGCAAGTGAACTCAAAACAAAAG		
71.	Ni03-H07	F	GCTGTGATTTTAGTGCACCG	53 ºC	*B. nigra*
		R	AGCCGTTGATGGAATTTTTG		
72.	Na10-C01c	F	TTTTGTCCCACTGGGTTTTC	54 ºC	*B. napus*
		R	GGAAACTAGGGTTTTCCCTTC		
73.	BN25A	F	CACGTGGTATGTTGGTATTGGG	54 ºC	*B. napus*
		R	TGATTCTCCTCCGACGCATGC		
74.	Ol10B11	F	AAAATGTGAGGCTGTTTGGG	53 ºC	*B. oleracea*
		R	TTTCGCAGCAGTAAACATGG		
75.	Ni2D-10	F	GATGCCCCAAATCTGTTACG	53 ºC	*B. nigra*
		R	CAATTCGTGAAAAATAGCCG		
76.	BRMS-011	F	GAACGCGCAACAACAAATAGTG	54 ºC	*B. rapa*
		R	CGCGTCACAATCGTAGAGAATÃ		
77.	BRMS-033	F	GCGGAAACGAACACTCCTCCCATGT	54 ºC	*B. rapa*
		R	CCTCCTTGTGCTTTCCCTGGAGACG		
78.	MR-25a	F	TCGACATGGATTCTACCAAA	55 ºC	*B. napus*
		R	GAACTTGCAAGCTGCAATTA		
79.	BrgMS339	F	CTACCTGAAGATGACCCAGACG	53 ºC	*B. rapa*
		R	GCATACAAACCTCGTCCTAAGC		
80.	BN6A3	F	GCTACCCACTCATGTCCTCTG	53 ºC	*B. napus*
		R	CCAAGCTTATCGAATCTCAGCTA		

Supplementary Table 2: List of polymorphic markers

Sl. No.	List of Marker	Amplicon size (bp)			
		No. of amplicons	P1 (Varuna)	P2 (CAULC-2)	M/P
1.	At3g23980	4	550, 600 & 630	525, 590 & 630	P
2.	At4g33925	2	620 & 710	710 & 790	P
3.	At5g40200	4	485, 500 & 685	485, 500, 550 & 685	P
4.	At2g36360	2	1080	1150	P
5.	BrgMS4539	2	190	190 & 220	P
6.	BrgMS139	2	135	110	P
7.	BrgMS502	4	200, 250 & 400	225 & 400	P
8.	BrgMS465	2	210 & 235	210	P
9.	cnu_m584a	2	225	210	P
10.	EJU1	2	410 & 480	410	P
11.	ENA10	5	390, 465, 495	390, 420, 465, 495 & 520	P
12.	SJ3302R1	4	410, 430, 600	420, 600	P
13.	SA0306	2	380	350 & 380	P
14.	Ni2A09	2	190	170	P
15.	BRMS-014	4	190, 315, 490	190, 300	P
16.	NiH03	4	180, 220 & 360	180, 220, 360 & 380	P
17.	Nia_m141a	2	320 & 395	320	P
18.	At5g40390	4	620, 700 & 730	620, 680 & 730	P

bp-basepair, M-Monomorphic, P-Polymorphic