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Identification of the SSR marker linked to powdery mildew resistant gene(s) in *Brassica* spp.

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Abstract
The present investigation was carried out to obtain information on screening of molecular markers for powdery mildew resistance in Indian mustard. The molecular analysis for powdery mildew resistance in *Brassica* spp. was carried out with four females, three males, F_1S and F_2S generation. The experimental materials were evaluated in randomized block design with three replications. The SSR markers *viz*, OI10-B12 and OI10-C01 clearly distinguished between susceptible and resistant bulks of interspecific cross GM-3 x PusaSwarnim. However, these markers can also be utilized for identifying disease resistance in other brassica species also, since over the years the 'C' genome introgression to other *Brassica* genotypes may have occurred through natural out crossing. But disease resistant reaction of genotypes will greatly depend upon the congenial environment prevailing for diseases outbreak. Therefore the markers identified in this study may be influenced by the prevailing environmental and the eco-geographical factors which need to be taken into consideration while attending further phenotypic screening for disease resistance and validating with the molecular markers.

Keywords: Molecular marker, Powdery mildew, Simple Sequence Repeat (SSR), Bulk Segregant Analysis (BSA), *Brassica* spp.

Introduction
With the use of molecular markers it is now a routine to trace valuable linked gene/s in a segregating population and mapping them. These markers once mapped enable dissection of the complex traits into component genetic units more precisely, thus providing breeders with new tools to manage these complex units more efficiently in a breeding program. Once mapped, these markers are efficiently employed in tagging several individual traits that are extremely important for a breeding programme like yield, disease resistance, stress tolerance, seed quality etc. A large number of monogenic and polygenic loci for various traits have been identified in a number of plants which are currently being exploited by breeders and molecular biologists together, so as to make the dream of marker-assisted selection come true.

In India, mustard is ravaged by several major diseases *viz.*, *Alternaria* blight [*Alternaria brassicae* (Berk.) Sacc.], white rust [*Albugo candida* (Pers. Ex. Lev.) Kuntze], powdery mildew *(Erysiphe cruciferarum)* and *Sclerotinia* rot *Sclerotinia sclerotiorum* (Lib.) de Bary which influences the quantity and quality of yield. *Alternaria* blight disease has been reported from all the continents of the world and is one among the important diseases of Indian mustard causing up to 47 percent yield losses **(**Kolte *et al.*, 1985) [10]. No proven source of resistance against the disease has been reported till date in any of the hosts (Meena *et al.*, 2010) [17]. *Erysiphe cruciferarum* is the ascomycete causative agent of crucifer powdery mildew (Alkooranee *et al.*, 2015, Meena *et al.*, 2013 and Koch and Slusarenko, 1990) [2, 18, 9] infecting a wide range of crucifers, including Chinese cabbage (*Brassica rapa* ssp. *Pekinens* is; syn. *B. pekinensis*), garden cress (*Lepidium sativum* L.), garlic mustard (*Alliaria petiolata*), African mustard (*Malcolmia africana*) and *Arabidopsis thaliana.* It can infect any terrestrial part of crucifers and lead to important fluctuations in crop yields by reducing plant growth and the quality and quantity of the seeds (Enright *et al.*, 2007) [5]. *E. cruciferarum* has been identified for the first time on *B. napus* (AACC) in China (Alkooranee *et al.*, 2015a) [2]. *B. napus* (AACC, 2n = 38), an allopolyploid resulting from the natural hybridization between *B. rapa* (AA, 2n = 20) and *B. oleracea* (CC, 2n = 18) is the main oil seed crop in China (Leflon *et al.*, 2006) [13].

Cultivated *Brassica* varieties do not have resistance to *Erysiphe cruciferarum*. Therefore farmers are forced to use frequent application of hazardous chemical pesticides for getting economic yield. As no source is available for mustard powdery mildew till date the existing field screening techniques of *Brassica* germplasm often generate false resistance status of the test material.

Material and Methods

Collection of Plant Material: The experimental material comprised of four females *viz*, GM-1, GM-2, GM-3 and GDM-4 (*Brassica juncea* L.) and three males, *viz*, NRCY-05-02 (*B. rapa* L.), Neelam (*B. napus L.*) and PusaSwarnim (*B. carinata* L.). Were used to obtain the varieties from Department of Genetics & Plant Breeding, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat.

Generation of Hybrid Population: Twelve F_1s were developed by crossing four females with three males in an all possible crosses made (Excluding reciprocal) [Table 1]. The seeds of all F_1 hybrids were produced by hand emasculation and pollination while the parental seeds were obtained through selfing, during *Rabi* 2015-16. Subsequently, in the next year a part of the F_1 seeds were utilized for raising the F_1 plants. The F_1s were screened for powdery mildew incidence and selfed seeds of all the twelve hybrids were collected as F_2 seeds. However, only one cross *i.e.* GM-3 x PusaSwarnim was advanced to F2 generation due to its lowest disease score.

Table 1: Parents and interspecific crosses

S. No.	Genotypes	Species
1	GM-1	*B. juncea* L.
2	GM-2	
3	GM-3	
4	GDM-4	
5	NRCY-05-02	*B. rapa* L.
6	Neelam	*B. napus* L.
7	PusaSwarnim	*B. carinata* L.
8	GM-1 x NRCY-05-02	*B. juncea* L. x *B. rapa* L.
9	GM-2 x NRCY-05-02	
10	GM-3 x NRCY-05-02	
11	GDM-4 x NRCY-05-02	
12	GM-1 x Neelam	*B. juncea* L. x *B. napus* L.
13	GM-2 x Neelam	
14	GM-3 x Neelam	
15	GDM-4 x Neelam	
16	GM-1 x PusaSwarnim	*B. juncea* L. x *B. carinata* L.
17	GM-2 x PusaSwarnim	
18	GM-3 x PusaSwarnim	
19	GDM-4 x PusaSwarnim	

Development and Identification of Transgressive Segregants (F_2): The F_2 seeds of the cross GM-3 x PusaSwarnim were preserved for raising the F_2 generation. During the year 2017-18, F_2 evaluation trial for powdery mildew resistance was conducted along with the parents *viz.*, GM-3 and PusaSwarnim. The resistance plants were selected from the F2populationand the selected plants were used further for molecular analysis.

Assessment of Disease Infection: Observations of powdery mildew severity was recorded at fifteen days before maturity of randomly selected ten plants from each plot/row of F_2 population. In each F_2 individual plants were randomly selected and powdery mildew severity was recorded by referring the following 0-9 scale [Table 2] (Mayee and Datar, 1986) [15].

Table 2: Rating scale with infection/phenotypic class

S. No.	Disease intensity	Scale	Disease Intensity	Reaction
1	No symptoms on the leaf	0	0	Immune (I)
2	Small powdery specks on the leaves covering 1 % or less area	1	< 1 %	Highly resistant (HR)
3	Powdery lesions small, scattered covering 1-10 % of leaf area	3	1-10 %	Moderately resistant (MR)
4	Powdery patches big, scattered covering 11-25 % of the leaf area	5	11-25 %	Moderately susceptible (MS)
5	Powdery patches big, coalescing covering 26-50 % of leaf area	7	26-50 %	Susceptible (S)
6	Powdery growth covering 51 % or more of leaf area. Leaves turn yellow and dry up.	9	> 50 %	Highly susceptible (HS)

Identification of DNA Markers: A set of 165 A, B and C genome specific SSR primers including, functional SSR primer pairs were employed for the parental screening to detect the polymorphism between the resistant parent PusaSwarnim and susceptible parent, GM-3. The SSR primers were custom synthesized from Eurofins Bengaluru. The genomic DNA of the two parents was subjected to PCR amplification with individual DNA markers. The amplicons scored as codominant-differences in size of the amplicon corresponding the target loci was noted. The polymorphic markers were noted and later used in Bulk Segregant Analysis (BSA).

DNA Extraction: DNA from the F_1 of the cross GM-3 x PusaSwarnim along with the respective parents were extracted. The F_1 was advanced to F_2 generation and 210 plants were selected randomly for further molecular analysis (BSA). Leaves from 3-4 weeks old plants were collected from field, surface sterilized with 70 per cent alcohol and freshly used for DNA isolation. The genomic DNA was extracted following Doyle and Doyle (1990) [4] method with a few minor modifications**.**

Bulked Segregant Analysis (BSA): F_2 population of the cross GM-3 x PusaSwarnim was raised which had individuals segregating for disease resistance and susceptibility. Per cent Disease Index (PDI) was calculated with the above scales using the formula given by Wheeler (1969) [24]. The Per cent Disease Index (PDI) for individual plants were plotted each from two extremes *i.e.* resistant and susceptible were selected and their DNA were bulked. This was done with a view of gene interaction prevailing and thus resulting genotype-

phenotype deviation expectable as a deviation from simple Mendelian situation (Govindraj *et al.,* 2005) [7]. The SSR marker found polymorphic between parents were screened across all the bulks and markers resulting in expected amplicon patterns were further shortlisted. Shortlisted markers were tested in the respective constituents of the bulks and selected ones were genotyped across all the F2 individual plants of a cross.

Validation of Molecular Markers: The 210 F_2 individuals plants of the cross GM-3 x PusaSwarnim and parents were genotyped with shortlisted SSR markers through BSA. Marker segregation pattern was scored and recorded. The SSRs were scored as co-dominant marker type. After scoring the individual progenies the data was entered in Microsoft Excel spreadsheet and tested for goodness of fit through *Chi-square* analysis. The calculated χ^2 values were compared with table values at one degree of freedom for each marker locus.

$$\chi^2 = \sum_{i=n}^{k} \frac{(O_i - E_i)^2}{E_i}$$

Where, $i = n = 1, 2, 3 \ldots 210$ and $k = 1, 2, 3 \ldots$ (Class of frequency), O = Observed frequency of k^{th} class and E= Expected frequency of k^{th} class. The data sheet was saved in a format suitable for Single Marker Analysis (SMA). A similar approach has also been adapted previously in mustard and other crops where gene interaction prevailed (Pushpa *et al.*, 2016, Govindaraj *et al.*, 2005, Zhang *et al*., 2009, Gemenet *et al.*, 2010 and Milad *et al.*, 2011) [21, 7, 25, 6, 20].

Results and Discussion

Segregation of Powdery Mildew Resistance in F_2 Generation: The incidence of powdery mildew differed significantly for various hybrids and parents. The entire twelve hybrids, four female parents and three male parents were screened for powdery mildew incidence and observation on Mean Diseases Score (MDS) and Per cent Diseases Intensity (PDI) were recorded. The parents and their respective crosses were grouped into five different categories of disease reaction *viz*, Immune (I), Resistant (R), Moderately Resistant (MR), Moderately Susceptible (MS), Susceptible (S) and Highly Susceptible (HS) to powdery mildew incidence.

The Mean Diseases Score (MDS) of parents ranged from 1.80±0.84 to 5.60 ± 3.71. Lowest MDS was recorded for PusaSwarnim (1.80±0.84) followed by NRCY-05-02 (3.20 ± 1.92) and Neelam (3.80 ± 1.92), whereas highest MDS was recorded for GM-1 (5.60 ± 3.71) followed by GM-2 and GDM-4 (5.40 ± 3.65), and GM-3 (4.00 ± 2.74). In case of hybrids the MDS ranged from 2.00 ± 1.22 to 6.80 ± 3.63. The lowest MDS were recorded for GM-3 x PusaSwarnim (2.00 ± 1.22) followed by GM-3 x Neelam (4.00 ± 2.55) and GM-3 x NRCY-05-02 (4.40 ± 2.07). All the other crosses showed Mean Diseases Score (MDS) up to highly susceptible level [Table 3]. From this observation it was found that the cross between GM-3 which was used as a susceptible parent and PusaSwarnim which was tolerant against powdery mildew incidence produced better hybrid having low Mean Diseases Score (MDS) suitable for development of powdery mildew resistant line (Kumar *et al.* 2016) [12]. Among male parents PusaSwarnim was found to have lower PDI (4.44 %), whereas NRCY-05-02 (35.56 %) and Neelam (42.22 %) belong to susceptible class while in case of female parents *viz.*, GM-1 (62.22 %) and GM-2 (60.00 %) were found to be highly susceptible while GM-3 recorded 53.33 % PDI and classified as susceptible. Meanwhile the reaction to powdery mildew was also recorded for the entire crosses and found that cross *viz.*, GM-3 x PusaSwarnim had (22.22 %) lower PDI as moderately resistant disease reaction. While the crosses GM-3 x Neelam (44.44 %) and GM-3 x NRCY-05-02 (48.89 %) exhibited susceptible disease reaction. Other crosses *viz.*, GM-1 x NRCY-05-02 (71.11 %), GM-2 x NRCY-05-02 (62.22 %), GDM-4 x NRCY-05-02 (66.67 %), GM-1 x Neelam (60.00 %), GM-2 x Neelam (66.67 %), GDM-4 x Neelam (62.22 %), GM-1 x PusaSwarnim (73.30 %), GM-2 x PusaSwarnim (75.56 %) and GDM-4 x PusaSwarnim (57.78 %) were found to show higher PDI and were classified as highly susceptible to powdery mildew reaction [Table 3]. The observation recorded in the field for powdery mildew incidence revealed that PusaSwarnim can be successfully used as a male parent for imparting powdery mildew resistance to *Brassica juncea* cultivars. The hybrid between GM-3 x PusaSwarnim showed better resistance against powdery mildew even in without fungicide application.

Table 3: Mean Diseases Score (MDS), disease reaction and per cent diseases intensity (PDI) of powdery mildew in parents and their F1 hybrids during *Rabi*- 2016-17.

S. No.	Genotypes	MDS		Disease reaction*	PDI (%)
1	GM-1	5.60	± 3.71	HS	62.22
2	GM-2	5.40	± 3.65	HS	60.00
3	GM-3	4.00	± 2.74	S	53.33
4	GDM-4	5.40	± 3.65	HS	60.00
5	NRCY-05-02	3.20	± 1.92	S	35.56
6	Neelam	3.80	± 1.92	S	42.22
7	PusaSwarnim	1.80	± 0.84	MR	4.44
8	GM-1 x NRCY-05-02	6.40	± 3.36	HS	71.11
9	GM-2 x NRCY-05-02	5.60	± 3.58	HS	62.22
10	GM-3 x NRCY-05-02	4.40	± 2.07	S	48.89
11	GDM-4 x NRCY-05-02	6.00	± 2.92	HS	66.67
12	GM-1 x Neelam	5.40	± 3.21	HS	60.00
13	GM-2 x Neelam	6.00	± 3.39	HS	66.67
14	GM-3 x Neelam	4.00	± 2.55	S	44.44
15	GDM-4 x Neelam	5.60	± 3.36	HS	62.22
16	GM-1 x PusaSwarnim	6.60	± 3.65	HS	73.33
17	GM-2 x PusaSwarnim	6.80	± 3.63	HS	75.56
18	GM-3 x PusaSwarnim	2.00	± 1.22	MS	22.22
19	GDM-4 x PusaSwarnim	5.20	± 3.11	HS	57.78

* R = Resistant, MR = Moderately Resistant, MS= Moderately Susceptible, S = Susceptible, HS = Highly Susceptible

Identification of Powdery Mildew Resistance Genes with SSR Markers: From the F_2 population of the cross GM-3 x PusaSwarnim cross, 210 plants were randomly selected and used for phenotyping for powdery mildew resistance. The powdery mildew Mean Diseases Score (MDS) and Per cent Diseases Intensity (PDI) were calculated as per protocol. Among parents GM-3 showed MDS and PDI of 4.80 ± 2.74 and 53.33 % respectively. Whereas PusaSwarnim showed MDS and PDI of 1.80 ± 0.84 and 4.44 % respectively. Among the 210 F2 plants, none of the plants were found highly resistant or immune, whereas 15 plants were moderately resistant, 10 plants moderately susceptible, 10 plants susceptible and 175 plants highly susceptible to powdery mildew reaction.

The polymorphic SSRs would enhance the density ofthe existing genetic maps which could also be a useful source of DNA markers suitable for high-throughput QTL mapping and marker-assisted selection, improvement and thus would be of value to breeders (Zhao, 2012) [26] and when such polymorphic markers are identified they can be used for bulk segregant analysis to identify putative marker for trait and to establish linkage with other trait of interest. Hence, total 165 molecular SSR markers spanning A, B and C genome of brassica were used for parental screening. Out of 165 SSR markers 10 SSR markers amplified in agarose gel and among the 10 SSR markers two SSR markers spanning C genome of *Brassica* were found to be polymorphic which clearly differentiated between susceptible and resistant parent. The primers with monomorphic banding patterns were excluded. A total of 4 alleles were detected at the loci of two microsatellite markers across GM-3 and PusaSwarnim genotypes.

Bulked Segregant Analysis (BSA): BSA was used for the identification of markers linked with disease resistance in which resistance was mostly governed by major genes, usually qualitative in nature. This method is a most time and effort saving approach where DNA of progenies corresponding to the two phenotypic extremes *i.e*. Susceptible and resistant were used. Therefore, only two pools of extreme ends, susceptible and resistant and along with the parents were genotyped for the identification of markers linked with the trait of interest (Michelmoore *et al.* 1991) [19]. Recently, Ren *et al*. (2017) [22] used BSA for identification of SSR markers closely linked to the yellow seed coat color gene in *B. rapa.* The performed BSA with a total of 292 evenly distributed SSR markers from previous genetic linkage maps and identified molecular markers linked to the dwarf gene ds-3 in F2 population of *B. napus* and proved to be a great approach where large number of population need to be screened. In present investigation, two bulks having distinct and often contrasting phenotypes for disease powdery mildew, susceptible and resistant are generated from a segregating F_2 population from a single cross *i.e*. GM-3 x PusaSwarnim. Two bulks containing F_2 plant DNA along with those of parents were analyzed through the two polymorphic markers obtained in parental survey. Each bulk consisted of DNA of thirteen resistant plants and nine susceptible plants selected according to highest to lowest mean diseases score (MDS) and per cent disease intensity (PDI). Two SSR markers *viz*, SSR OI10-B12 and SSR OI10-C01 clearly distinguished susceptible bulks from resistant bulks. With marker SSR OI10-B12, which amplified 135 bp and 110 bp band respectively, from GM-3 and PusaSwarnim, it showed clear differences among resistant and susceptible. The band present in PusaSwarnim the resistant parent was amplified in resistant bulk also while the band present in GM-3 the susceptible parent was amplified in susceptible bulk. The same result was obtained by SSR-OI10-C01 which amplified 120 bp of band corresponding to resistant parent and resistant bulk and 90 bp of band corresponding to susceptible bulk [Figuer a & b]**.** These markers associated with disease resistant trait can be further used for validation of disease resistance.

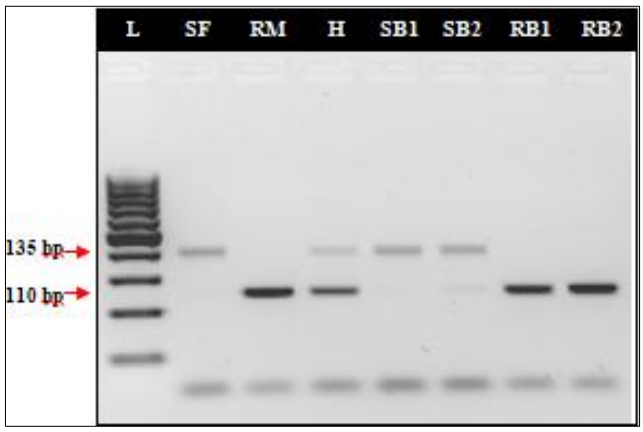
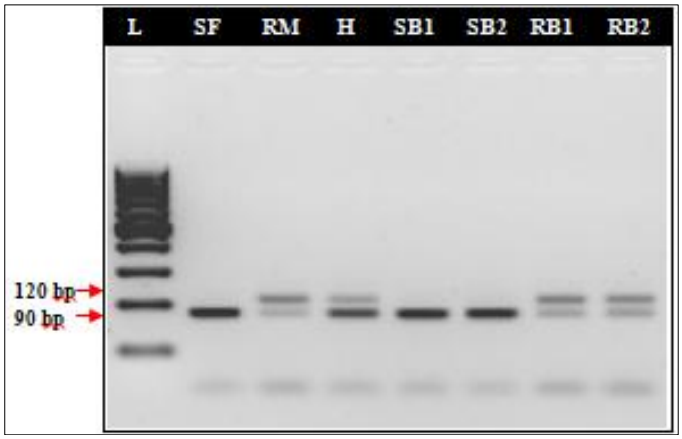


Fig 1: Bulk Segregant analysis with SSR-OI10-B12



L: 50 bp ladder, SF: Susceptible Female, RM: Resistant Male, H: Hybrid, SB1/2: Susceptible Bulk1/2, RB1/2: Resistant Bulk1/2

Fig 2: Bulk Segregant analysis with SSR-OI10-C01

Marker-phenotypic Association Analysis: The testing of statistical data to verify whether there is an association between a phenotype and a molecular marker is very important for identification of markers in relation to disease resistance and susceptibility. The phenotype of interest is selected based on quantitative data, the data obtained from a random sample of population and their phenotype association with marker is obtained by single factor ANOVA or linear regression method (Zhu *et al.*, 2012) [27]. In order to identify the SSR marker association with disease resistance, the F_2 progenies were genotyped with two primers SSR OI10-B12 and SSR-OI10-C01 which clearly distinguished between resistant and susceptible bulk. These two markers were genotyped in all 210 F_2 progenies. Segregation pattern with marker SSR OI10-B12 recorded a resistant allele of donor in 40 plants; susceptible allele of recipient was amplified in 47 plants while 120 plants exhibited both the alleles (heterozygous). While segregation pattern with marker SSR-OI10-C01 recorded a resistant allele of donor in 71 plants, susceptible allele of recipient was amplified in 42 plants while 95 plants exhibited both the alleles (heterozygous)**.**

For single marker analysis for both the markers, entire F2 population was grouped into three categories. The clearly resolved amplicons of SSR were scored manually as homozygote for the allele for susceptible parent (A), homozygote for the allele for resistant parent (B) and heterozygote carrying the alleles from both parents (H) in the data she *et al* on with their Mean Diseases Score (MDS) which was used as a phenotypic data [Table 4 & 5]. For marker OI10-B12 the counts for A, B and H were found to be 40, 47 and 120 with variance of 3.09, 7.40 and 0.76 respectively. *P-value* of 3.64E-13 was found greater than *F-critical* value (33.06) which indicated that the means of both the marker and phenotypic values were different. This indicated significant association of markers with the phenotypic trait. In case of marker OI10-C01 the counts for A, B and H were 71, 42 and 98 with variance of 2.34, 7.26 and 0.55 respectively, in this case also the P-value of 1.67E-16 were found to be lower than F-critical value (3.04) which indicated non-significant association of marker with the phenotypic trait at 5 % level of significance [Table 6 & 7].

Table 4: Summary of results of powdery mildew in parents and their F2 plants in relation to Mean Diseases Score (MDS), disease reaction and Per cent Diseases Intensity (PDI) during *Rabi*- 2017-18.

Particulars	*MDS		PDI	Disease reaction	SSR-OI10-B12	SSR- OI10-C01
✓ Minimum	0.40	± 0.00	4.44		-	-
✓ Maximum	7.60	± 4.27	84.44		-	-
➢ Immune (I)				0	-	-
➢ Highly Resistant (HR)				0	-	-
➢ Moderately Resistant (MR)				15	-	-
➢ Moderately Susceptible (MS)				10	-	-
➢ Susceptible (S)				10	-	-
➢ Highly Susceptible (HS)				175	-	-
▪ Total A bands (allele of recessive parent)					41	72
▪ Total B bands (allele of donor parent)					48	43
▪ Total H bands (allele of both the parents heterozygote)					120	95

Table 5: Mean Diseases Score (MDS), disease reaction and per cent diseases intensity (PDI) of parents and F2 population of resistance and susceptible genotypes for bulk segregant analysis (BSA).

Sr. No.	Resistance genotypes	*MDS		Disease reaction	PDI
1	PusaSwarnim	1.80	±0.84	MR	4.44
2	GM-3 x PusaSwarnim -F_2-17-15	0.40	±0.55	MR	3.30
3	GM-3 x PusaSwarnim -F_2-17-37	0.40	±0.55	MR	3.30
4	GM-3 x PusaSwarnim -F_2-17-107	0.60	±0.55	MR	5.00
5	GM-3 x PusaSwarnim -F_2-17-108	0.80	±0.45	MR	6.67
6	GM-3 x PusaSwarnim -F_2-17-137	0.60	±0.55	MR	5.00
7	GM-3 x PusaSwarnim -F_2-17-154	0.80	±0.45	MR	6.67
8	GM-3 x PusaSwarnim -F_2-17-155	0.80	±0.84	MR	6.67
9	GM-3 x PusaSwarnim -F_2-17-156	0.80	±0.84	MR	6.67
10	GM-3 x PusaSwarnim -F_2-17-157	0.80	±0.45	MR	6.67
11	GM-3 x PusaSwarnim -F_2-17-164	0.60	±0.55	MR	5.00
12	GM-3 x PusaSwarnim -F_2-17-201	0.80	±0.84	MR	6.67
13	GM-3 x PusaSwarnim -F_2-17-210	0.80	±0.84	MR	6.67
Sr. No.	**Susceptible genotypes**	***MDS**		**Disease reaction**	**PDI**
1	GM-3	4.00	±2.74	S	53.33
2	GM-3 x PusaSwarnim -F_2-17-1	7.60	±3.05	HS	63.33
3	GM-3 x PusaSwarnim -F_2-17-10	7.20	±3.70	HS	60.00
4	GM-3 x PusaSwarnim -F_2-17-13	7.20	±4.15	HS	60.00
5	GM-3 x PusaSwarnim -F_2-17-30	7.20	±3.70	HS	60.00
6	GM-3 x PusaSwarnim -F_2-17-38	7.60	±3.05	HS	63.33
7	GM-3 x PusaSwarnim -F_2-17-49	7.20	±3.70	HS	60.00
8	GM-3 x PusaSwarnim -F_2-17-72	7.20	±3.70	HS	60.00
9	GM-3 x PusaSwarnim -F_2-17-88	7.20	±4.27	HS	60.00

*MDS= Mean Diseases Score and PDI = Per cent Diseases Intensity

Table 6: Single Marker Analysis for marker SSR OI10-B12

Data Summary Table					
Groups	**No. of F2 plants**	**Sum of disease score**		**Average of disease score**	**Variance**
A	40	228.80		5.72	3.09
B	47	186.20		3.96	7.40
H	120	751.20		6.26	0.76
A=allele of recessive parent, B=allele of donor parent and H = allele of both the parents (Heterozygote).					
Anova: Single Factor Analysis					
Source of variation	**d. f.**	**SS**	**MS**	**F**	***P-value***	***F crit***
Between Groups	2	178.76	89.38	33.06*	3.64E-13	3.04
Within Groups	204	551.44	2.70	-	-	-
Total	206	730.20	-	-	-	-

The f-ratio value is 33.06514. The p-value is <.00001. The result is significant at p<.05.

Table 7: Single Marker Analysis for marker SSR OI10-C01

Data Summary Table						
Groups	**No. of F2 plants**	**Sum of disease score**		**Average of disease score**		**Variance**
A	71	418.80		5.90		2.34
B	42	152.80		3.64		7.26
H	95	601.40		6.33		0.55
A=allele of recessive parent, **B**=allele of donor parent and **H** = allele of both the parents (Heterozygote).						
Anova: Single Factor Analysis						
Source of variation	**d. f.**	**SS**	**MS**	**F**	***P-value***	***F crit***
Between Groups	2	218.37	109.18	43.60*	1.67E-16	3.04
Within Groups	205	513.35	2.50	-	-	-
Total	207	731.72	-	-	-	-

The *f-ratio* value is 43.60092. The *p-value* is <.00001. The result is significant at *p*<.05.

These results indicated that the marker OI10-B12 and OI10-C01 may be linked to disease resistant trait. Chi-square (χ^2) test was performed to test the goodness of fit of the F_2 population for the SSR marker data by comparing an observed frequency distribution with an expected one to test the deviation from the theoretically expected Mendelian segregation ratios of 1:2:1 for co-dominant marker. It was observed that χ^2 value for the marker OI10-B12 was found to be 5.73 whereas deviation from the expected values were -11.75, -4.75 and 16.50 for genotype A, B and H respectively with P-value of 0.60. When it was compared with the χ^2 table value at 2 degrees of freedom it was found to be non-significant and the test indicated that, goodness of fit for co-dominant ratio (1:2:1) was confirmed [Table 8]. Similarly the χ^2 value for the marker OI10-C01 was 9.64 where deviation from the expected values were 19, -10 and-9 for genotype A, B and H respectively. At 0.01 level of significance it was found non-significant again confirming the co-dominance of the marker [Table 9]. Pushpa *et al.* (2016) [21] validated molecular markers linked to low glucosinolate QTLs for marker assisted selection in Indian mustard by utilizing single factor ANOVA and χ^2 test and found that markers namely GER1 and GER5 are together responsible for 71 per cent variation in glucosinolate content. Jin *et al.* (2014) [8] identified and mapped a novel dominant resistance gene, TuRB07 to Turnip mosaic virus in *Brassica rapa*.

Table 8: Chi-square ($\chi 2$) test of goodness of fit for marker SSR OI10-B12

Groups	Observed (O)	Expected (E)	Deviation (O-E)	$(O-E)^2$	$(O-E)^2/E$
A	40	51.75	-11.75	138.0625	2.67
B	47	51.75	-4.75	22.5625	0.44
H	120	103.5	16.50	272.25	2.63
$\sum\chi^2$					5.74

Table 9: Chi-square ($\chi 2$) test of goodness of fit for marker SSR OI10-C01

Groups	Observed (O)	Expected (E)	Deviation (O-E)	$(O-E)^2$	$(O-E)^2/E$
A	71	52	19	361	6.94
B	42	52	-10	100	1.92
H	95	104	-9	81	0.78
$\sum\chi^2$					9.64*

A=allele of recessive parent, B=allele of donor parent and H = allele of both the parents (Heterozygote). Significant at P= 0.05 level of probability.

Note: Band excluded when was not observed

In their investigation they used 238 SSR markers in association with segregation data of resistant and susceptible responses for the F_2, backcross and doubled haploid generations to analyze expected segregation ratio by Chi-square for goodness of fit and single marker analysis for detection of possible linkage between marker and phenotypic data. Sharma *et al.* (2016) [23] also used same strategy to map black rot resistance locus XCA1BC on Chromosome B-7 in Ethiopian Mustard (*B. carinata*) where they found significant association of marker with the disease resistant trait and identified monogenic dominant control of the locus which segregated in 3:1 ratio. Map of OI10-F04 and OI10-F06 conferring resistance to mustard aphid Extensive efforts have been made to develop SSR markers in *B. napus* and its two diploid progenitors, *B. rapa* and *B. oleracea*, through genomic library screening using probes containing repeated motifs and followed by DNA sequencing (Kresovich *et al.* 1995 and Lowe *et al*. 2004) [11, 14]. However, as indicated at the *Brassica* Microsatellite Information Exchange (BMIE) the total number of currently mapped SSR markers in *Brassica* is still too limited. Experimental methods to develop SSR markers are laborious, time-consuming and expensive. With the ever-increasing number of sequences in public databases, development of SSR markers using *in silico* approaches has become a practicable and inexpensive alternative for many crop species (Chen *et al.* 2007 and McCouch *et al.* 2002) [3, 16].

Conclusion

Single marker analysis for both the markers, entire F_2 population were grouped into three categories. The clearly resolved amplicons of SSR were scored manually as homozygote for the allele for susceptible parent (A), homozygote for the allele for resistant parent (B) and heterozygote carrying the alleles from both parents (H) in the data she *et al* on with their mean DI which was used as a phenotypic data. For marker OI10-B12 the counts for A, B and H were found to be 40, 47 and 120 with variance of 3.09, 7.40 and 0.76 respectively. *P-value* of 3.64E-13 was found greater than F-critical value (33.06) which indicate that the means of both the marker and phenotypic values were

different. Hence found significant results of marker with phenotypic value could be inferred. In case of marker OI10-C01 the counts for A, B and H were 71, 42 and 98 with variance of 2.34, 7.26 and 0.55 respectively, in this case also the P-value of 1.67E-16 were found to be lower than F-critical value (3.04) which indicate non-significant association of marker with the phenotypic trait at 5 % level of significance. These results indicated that the marker OI10-B12 and OI10-C01 are may linked disease resistant trait. *Chi-square* (χ^2) test was performed to test the goodness of fit of the F_2 population for the SSR marker data by comparing an observed frequency distribution with an expected one to test the deviation from the theoretically expected Mendelian segregation ratios of 1:2:1 for co-dominant marker. χ^2 value for the marker OI10-B12 was found to be 5.73 where deviation from the expected values were -11.75, -4.75 and 16.50 for genotype A, B and H respectively with P- value of 3.64E-13. When it is compared with the χ^2 table value at 2 degrees of freedom it is found to be non-significant and the test indicated that, goodness of fit for co-dominant ratio (1:2:1) inferred. Similarly the χ^2 value for the marker OI10-C01 was 9.64 where deviation from the expected values were 19, -10 and -9 for genotype A, B and H respectively. At 0.01 level of significance it was found non-significant again confirming the co-dominance of the markers.

The two SSR markers *viz,* OI10-B12 and OI10-C01 clearly distinguish between susceptible and resistant bulks of interspecific cross GM-3 x PusaSwarnim. However, these markers can also be utilized for identifying Disease resistance in other *Brassica* species also, since over the years the 'C' genome introgression to other *Brassica* genotypes may have occurred through natural out crossing. But disease resistant reaction of genotypes will greatly depend upon the congenial environment prevailing for diseases outbreak. Therefore the markers identified in this study may be influenced by the prevailing environmental and the eco-geographical factors which need to be taken into consideration while attending further phenotypic screening for disease resistance and validating with the molecular markers.

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