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Identification of SNP markers linked to *Rf* locus in carrot using GBS

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Abstract: Commercial carrot production is based on the use of hybrid seed varieties worldwide. Cytoplasmic genetic male sterility (CGMS) mechanism has been widely used as the main method for hybrid carrot seed production. There is a significant demand for a rapid method for identification of fertility restorer gene (*Rf)* carrying individual plants in a carrot breeding population. The objective of this research was to identify SNP markers linked to *Rf* locus and develop a genetic map of *Rf* gene in carrot. For this purpose, a carrot F2 population segregating for male sterility was produced and segregating 10,425 SNP markers were discovered by using genotyping by sequencing (GBS). Association mapping analysis demonstrated that *Rf* was located on chromosome 9 in the carrot genome. A genetic map of chromosome 9 was constructed by using GBS based SNP markers. In order to confirm GBS-generated SNP markers, three markers closely linked to *Rf* locus were converted to simple probe markers (DcSNP-ch9_3342931, DcSNP-ch9_4822988 and DcSNP-ch9_6233708). Simple probe assay was able to distinguish the individuals of F2 population as homozygous male fertile (*RfRf*), heterozygous male fertile *(Rfrf*) and male sterile (*rfrf*). Simple probe assay confirmed the phenotypic observation in the population and a genetic map of *Rf* was constructed by using these markers. In conclusion, mapping of *Rf* in this work would be an efficient tool for future fine mapping and cloning studies of *Rf* gene in carrot. Thus, this research can help to accelerate the identification of maintainer lines for the development the new hybrid carrot varieties.

Key words: *Daucus carota* L., GBS, male sterility, *Rf* genes, SNP, simple probe

1. Introduction

Carrot (*Daucus carota* L.) is one of the most important root vegetables in the *Apiaceae* which has been cultivated since the 10th century (Stolarczyk and Janick, 2011; Iorizzo et al. 2013). Today, carrots are among the top 10 most produced vegetable crops worldwide. In 2020, carrot was cultivated on 1.1 million ha (carrots and turnips' data together) with a production of 40.9 million t and the major producer countries were China, Uzbekistan, USA, Russia, and Ukraine (FAOSTAT, 2022). In recent years, carrot consumption has increased globally due to the fact that the carrot roots are potential sources of antioxidative compounds such as carotenoids, anthocyanins and other flavonoids (Nicolle et al., 2004; Shebaby et al., 2013; Silva Dias, 2014).

Carrots have been widely produced by using hybrid cultivars worldwide because using hybrid varieties in carrot production provides a homogeneous production in terms of color, shape, size and taste properties. (Havey, 2004; Singh et al., 2018). Carrot has hermaphroditic small flowers with five sepals, five petals, five stamens, and a pistil in the center. Carrot is a crosspollinated crop because stamens mature before pistils but self-pollination can occur between the different flowers on the same plant. Because artificial emasculation is difficult to implement, petaloid type of cytoplasmic genetic male sterility (CGMS) system has been employed in commercial hybrid carrot seed production (Welch and Grimball, 1947; Thompson, 1961; Mccollum, 1966; Morelock et al., 1996; Linke et al., 2003; Simon et al., 2008).

In CGMS mechanism, the determination of presence of the recessive allele of fertility restorer gene using the testcross methods can require two or more years and significant resources. Hence, molecular markers can be an effective tool to discriminate the maintainer lines at seedling stages of plant development. Up to date, several studies have been conducted on mitochondrial genes associated with carrot male sterility (Nakajima et al., 1999; Bach et al., 2002; Linke et al., 2003; Hanson and Bentolila, 2004; Robison and Wolyn 2006; Iorizzo et al., 2012; Szklarczyk et al., 2014; Tan et. al., 2018; Kalia et al., 2019) and several genes related with fertility restoration

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have been revealed (Wolyn and Chahal, 1998). Besides, the regions of *Rf* genes in the carrot genome have been genetically mapped at different loci in only a few studies. Alessandro et al. (2013) revealed the first linkage maps which control vernalization response (*Vrn1*) and fertility restoration (*Rf1*) in carrot genome by the use of new *SSR, RAPD, AFLP* and *SCAR* markers. Fuji and Shiroto (2019) found the quantitative trait locus (QTL) control the carrot petaloid phenotype. However, molecular aspects of male sterility caused by *Rf* genes are still need to be investigated more in order to differentiate cytoplasmic male sterile and male fertile carrots in breeding programs.

SNP discovery by GBS has been shown to be a rapid and cost-effective approach for mapping genes but the accuracy of the maps sometimes may be challenging and suppressed by genotyping errors and missing data. At this point, SNP validation assays based on real-time PCR technology can be utilized initially to obtain more accurate data (Mammadov et al., 2010). For this purpose, melting curve analysis established with simple probe format has been used for providing a rapid and homogeneous assay for SNP validation (Pryor and Wittwer, 2006; Ha et al., 2008). In recent studies, KASP assays have been well adopted for validation of SNP markers linked to restorer of fertility genes in various crops such as tobacco (Priya et al., 2019); pearl millet (Pucher et al., 2018); pakchoi (Lin et al., 2020) and cucumber (Han et al., 2018).

In carrot breeding, the use of marker-assisted selection (MAS) has still been an important demand for large-scale hybrid seed production even though CGMS system has been known as a valuable trait. Mapping of the gene responsible for CGMS with the development of new molecular markers can make a significant contribution to MAS. Particularly, these researches can accelerate the selection process of male sterile lines at the seedling stages of plant growth through eliminating time-consuming field observations. Although Alessandro et al. (2013) reported that *Rf1* gene was mapped to chromosome 9 Fuji and Shiroto (2019) found out the responsible locus for fertility restoration on chromosome 4. More researches need to be conducted by using modern techniques to reveal the molecular basis of fertility restoration in carrot for being used in MAS. Hence, the aims of this study were (i) to identify SNP markers linked with *Rf* locus using GBS method and (ii) to confirm SNP markers using simple probe assay in a F2 carrot population.

2. Materials and methods

2.1. Plant materials and DNA extraction

In this study, a F2 carrot population composed of 96 plants segregating for male sterility with genetic ratio (3 male fertile: 1 male sterile) was developed. For this purpose, F1 progeny was derived from a cross between a male sterile and a male fertile carrot plant. One of several male fertile F1 plants was identified and an F2 population was generated by self-pollination. Phenotypic observation data was obtained by evaluating the flower types of the carrots individually during the growing season in order to determine the number of male sterile and male fertile plants in a F2 carrot population (Figure 1). DNA samples were extracted from 25 mg lyophilized and powdered leaf samples using the Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol. DNA concentrations were measured using a Qubit Fluorometer (Invitrogen, USA), adjusted to 50 ng/µL for analysis and kept at –80 °C until use.

2.2. SNP discovery

GBS analysis was carried out following the protocol described by Elshire et al. (2011). DNA samples from 96 plants of F2 population were digested individually with the *ApeKI* restriction enzyme (Elshire et al., 2011). Illumina sequencing adaptors with unique barcode nucleotides were ligated to DNA fragments digested with *ApeKI* to prepare GBS sequencing libraries. GBS libraries of 96 samples were pooled together and sequenced using 1 lane of the Illumina HiSeq 2500 sequencing system. GBS data files (FASTQ) were transformed to SNP calls and aligned to carrot reference genome (Accession code: LNRQ00000000; Iorizzo et al., 2016) using Bowtie2 program (Langmead and Salzberg, 2012) with "very-sensitive-local" option. Identification of segregating SNP markers and SNP genotyping were performed using the subprograms in TASSEL version 5.0 software (Bradbury et al., 2007).

2.3. Association map construction

Association mapping was performed using a GLM (general linear model) function in TASSEL version 5.0 (Bradbury et al., 2007) software in order to determine candidate SNP markers linked to *Rf* locus in carrot.

2.4. Genetic map construction

Genetic maps were constructed using JoinMap 4.0 (Van Ooijen and Voorrips, 2006) software. All SNP markers segregating in F_2 population were converted into genotype codes compatible with JoinMap 4.0 program. SNP markers with more than 10% of missing data were excluded from the analysis. The linkage groups (LGs) were obtained at a LOD threshold value 7.0 and maximum recombination frequency of 0.30. The distance of the integrated map was estimated using the Kosambi's mapping function in centimorgans (cM) (Kosambi, 1944). Maps were viewed using MapChart 2.3 software (Voorrips, 2002).

2.5. Confirmation of SNP markers with simple probe assay

According to the results of association and linkage mapping analysis, real-time PCR based simple probe assay was conducted to confirm GBS generated SNP markers

surrounding *Rf* locus. Simple probe assay was performed using Roche Light Cycler 480 system and Roche LightCycler DNA Master HybProbe mix. PCR primers and probes were designed with the Light Cycler Probe Design 2.0 software (Roche Applied Science, Indianapolis, IN, USA) (Table 1). All Simple Probe PCR reactions were performed with a total volume of 10 µL consisting of 2.5 µL of DNA (20–40 ng/µL) as template; 0.8 µL of 25mM $MgCl_2$; 0.2 µL simple probes and 1.0 µL Roche LightCycler DNA Master HybProbe mix. The total reaction contained 0.1 µL each of forward primers and 0.5 µL of each reverse primers. The thermal cycling condition was as follows: one cycle at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 55 °C for 15 s and 72 °C for 20 s.

2.6. SNP genotyping with melting curve analysis

SNP markers linked to *Rf* locus in carrot genome were genotyped by melting curve analysis with simple probe assay after real-time PCR amplifications with Roche Light Cycler 480 system. Melting curve analysis was performed at the end of the amplification cycles by increasing the temperature to 95 °C for 2 min, lowering the temperature to 40 °C for 2 min, and raising the temperature to 85 °C with continuous fluorescent acquisition followed by a cool down to 40 °C. The LC 480 software Tm calling and genotyping analysis modules were used to analyze the simple probe melting curve data. The software groups similar melting profiles together and automatically identifies each groups as a genotype. According to the results of this analysis, genotypes were determined as homozygous fertile (*RfRf*); heterozygous fertile (*Rfrf)* or heterozygous sterile (*rfrf*).

3. Results

3.1. Segregation analysis of male fertility and sterility

Male sterility in the F2 population was observed with a hypothesis of single gene maintaining petaloid type of CGMS. A segregating F2 population was composed of 96 plants of which 77 were male fertile and 19 were male sterile (Supplementary Table 1). The segregation of male sterility fits the 3:1 Mendelian segregation ratio for a single gene with chi-square test p-value of 0.24.

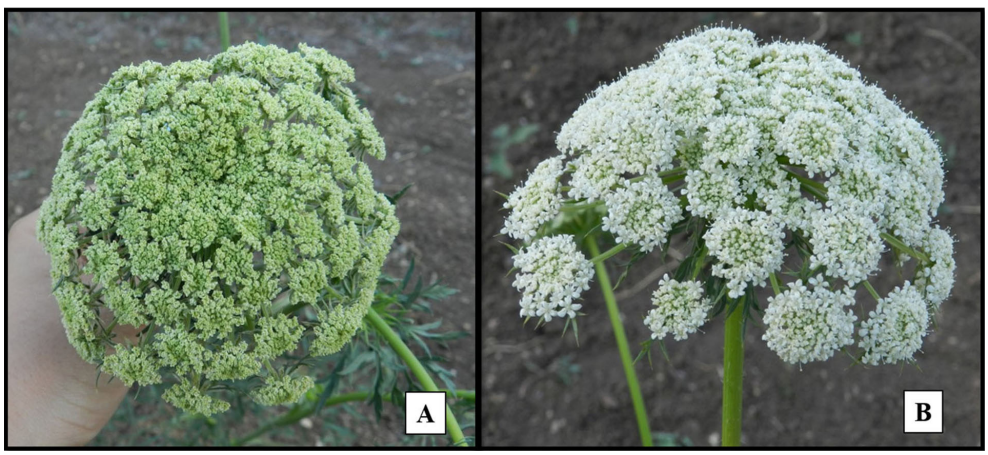


Figure 1. Petaloid type of cytoplasmic genetic male sterile carrot flower (A); male fertile carrot flower (B).

Table 1. Sequences of the primers and simple probes for melting curve analysis.

SNP name		SNP position	Sequence (5'–3')
DcSNP-ch9_3342931	Forward primer	A/G	gtacaatgttctgcaaagcccac
	Reverse primer		tgaaacagcacacctctttccac
	SimpleProbe		ggACAXITggTgggggAAAgCg--PH
DcSNP-ch9_4822988	Forward primer	A/C	accatttacagccagatattactgc
	Reverse primer		gcagctactccagaaatatgcg
	SimpleProbe		gAgTCTCgTCATgCAAXITCCCCg--PH
DcSNP-ch9_6233708	Forward primer	A/G	tagcacctggtgtgagcattaa
	Reverse primer		ggtgattagaagacggtgaacgt
	SimpleProbe		gCCTAgTAgTTgCAXITATATTTACATggACA--PH

3.2. GBS analysis and SNP discovery

A total of 252.972.251 clean GBS reads were obtained by assigning the 96 genotypes after quality trimming process. Reactions for two genotypes (DC-46 and DC-66) did not work and two genotypes (DC-59 and DC-78) had less than five hundred thousand good reads. Among the other 92 high-quality samples, the number of clean GBS reads per individual genotypes varied from 527,758 (DC-20) to 9,554,285 (DC-67) with an average of 2,802,325 (Figure 2).

GBS reads from each carrot genotypes were aligned to the carrot reference genome (Iorizzo et al. 2016). The fraction of clean GBS reads aligned to the carrot genome differed from 75 to 90% (Figure 3). A total of 21,216 raw SNP markers were discovered and the number of high qualified SNP markers reduced to 10,425 after eliminating markers with missing data more than 20%.

3.3. Association mapping analysis

Using 10,425 good quality SNP markers, association mapping analysis was conducted to identify the chromosomal location of *Rf* locus. Chromosomal position of SNP markers was shown in Table 2. The number of SNP markers per chromosomes ranged from 874 (chromosome 9) to 1679 (chromosome 1) and SNP markers were distributed from the beginning of the chromosomes to the end of chromosomes, demonstrating well coverage of chromosomes by GBS generated SNP markers. Manhattan plot based on p-values [– log10(P)] showed significant association of SNP markers with fertility restorer gene on chromosome 9 (Figure 4).

3.4. SNP-based genetic linkage map construction

A SNP-based genetic linkage map of chromosome 9 was constructed using 874 SNP markers because association mapping analysis demonstrated that significant association between SNP markers on chromosome 9 and *Rf* locus. After removal of SNP markers which did not fit to Mendelian segregation ratio of 1:2:1, we were able to map *Rf* locus with 119 SNP markers. The total map length was 66 cM with an average marker distance of 0.55 cM and *Rf* was mapped to the distal end of chromosome 9 (Supplementary Figure).

After evaluating the results of both association and genetic mapping analyses together, the most closely linked SNP markers to *Rf* were selected from the both sides of *Rf* locus. These three flanking SNP markers to *Rf* locus were selected from the region of 3.342.931 and 6.379.153 bp on chromosome 9. These markers were named according to their physical positions of SNP markers on chromosome 9 (DcSNP-ch9_3342931, DcSNP-ch9_4822988, DcSNP-ch9_6233708).

3.5. Confirmation of SNP markers linked to *Rf* locus using simple probe assay

In order to confirm GBS-based SNP data, real-time PCR based simple probe assay with melting curve analysis was employed. For this purpose, three SNP markers were converted to simple probe markers (DcSNP-ch9_3342931, DcSNP-ch9_4822988, DcSNP-ch9_6233708). The melting curve analysis of simple probe markers was able to discriminate three genotypic classes of homozygous fertile (*RfRf*), heterozygous fertile (*Rfrf*) and male sterile (*rfrf*) in this F2 segregating population (Figure 5).

Our results showed that simple probe assay confirmed the phenotypic data for all SNP markers by 100%. However, in GBS analysis, DcSNP-ch9_3342931 and DcSNP-ch9_4822988 markers gave consistent results with the phenotypic observation data by 95.7% and the results for DcSNP-ch9_6233708 were compatible with the phenotypic observation data by 94.6%. For instance, DC-74 and DC-80 were determined as male sterile in phenotypic evaluation even though they were genotyped as male fertile in GBS analysis (Supplementary Table 2).

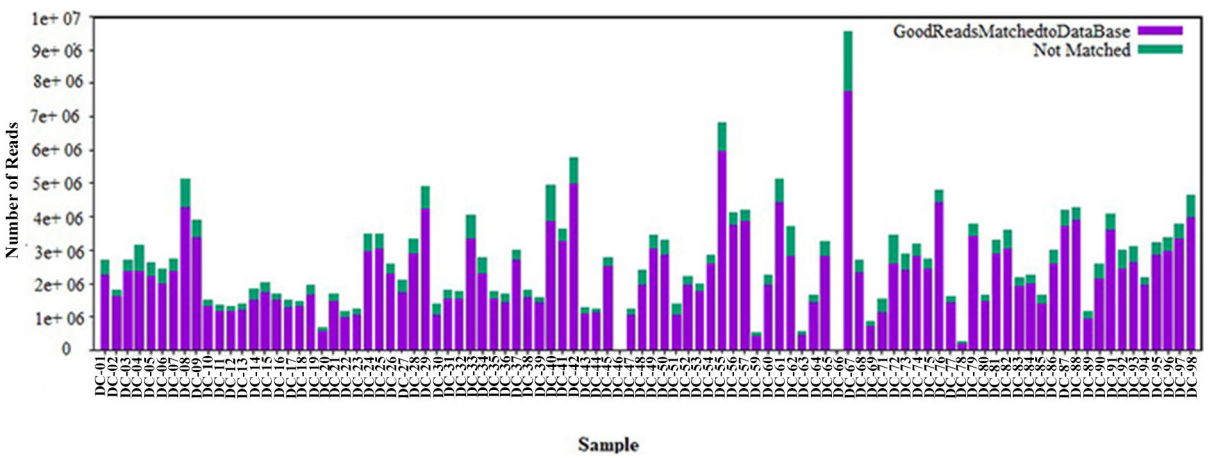


Figure 2. Number of clean GBS reads per sample.

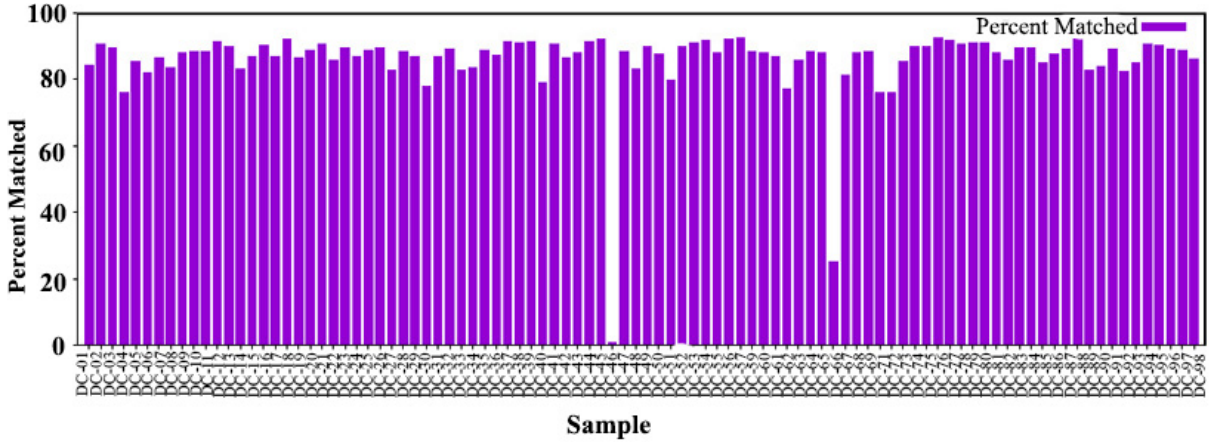


Figure 3. Percent of GBS reads aligned to the carrot reference genome for each genotype.

Table 2. Distribution of the number of SNP markers across the nine chromosomes (bp: base pair).

Chromosomes	Size of the chromosomes (Mb)	The number of SNP markers	Position of SNP markers* (bp)
Chr 1	51.47	1679	3.515.146–51.352.107
Chr 2	43.91	1206	4.937.223–42 819.468
Chr 3	50.31	1260	77.431–50.146.117
Chr 4	35.92	908	823.834–35.910.203
Chr 5	41.96	1243	386.020–40.767.829
Chr 6	36.61	992	114.523–36.512.814
Chr 7	36.36	1099	65.856–36.264.097
Chr 8	31.75	1164	103.954–31.697.488
Chr 9	33.68	874	579.378–32.894.779

*Position of SNP markers in accordance with the carrot reference genome.

In this carrot population, GBS analysis could successfully discriminate 92 plants in F2 population as homozygous fertile, heterozygous fertile and male sterile for all SNP markers. Out of these 92 plants, 27 were homozygous fertile, 48 were heterozygous fertile and 17 were male sterile for DcSNP-ch9_3342931; 25 were homozygous fertile, 50 were heterozygous fertile and 17 were male sterile for DcSNP-ch9_4822988; and 25 were homozygous fertile, 49 were heterozygous fertile and 18 were male sterile for DcSNP-ch9_6233708. In simple probe assay, all SNP markers were able to clearly differentiate homozygous fertile and heterozygous fertile genotypes from sterile ones. Ninety-one members of F_2 population were genotyped for DcSNP-ch9_3342931 and DcSNP-ch9_6233708. Out of 91 samples, 31 were homozygous fertile, 43 were heterozygous fertile and 17 were male sterile for DcSNP-ch9_3342931 while 26 were homozygous fertile, 47 were heterozygous fertile and 18 were male sterile for DcSNP-ch9_6233708. DcSNP-ch9_4822988 was successful to genotype 93 plants of F2 population and 27 were homozygous fertile, 48 were heterozygous fertile and 18 were male sterile (Supplementary Table 2; Table 3).

Based on the GBS analysis and simple probe assay results, the chi-square tests were also performed. According to the GBS analysis, the segregation of the homozygous fertile, heterozygous fertile and male sterile individuals in the F2 population fit at 1:2:1 ratio with the probabilities of 0.30, 0.39, and 0.47 for DcSNP-ch9_3342931, DcSNP-ch9_4822988, and DcSNP-ch9_6233708, respectively. In simple probe assay, segregation ratio was fit at 1:2:1 for DcSNP-ch9_3342931, DcSNP-ch9_4822988, DcSNP-ch9_6233708 with the probabilities of 0.31, 0.88 and 0.88 (Table 3). The chi-square tests demonstrated that the probability values of two simple probe markers (DcSNP-ch9_4822988, DcSNP-ch9_6233708) are higher than GBS based SNP markers.

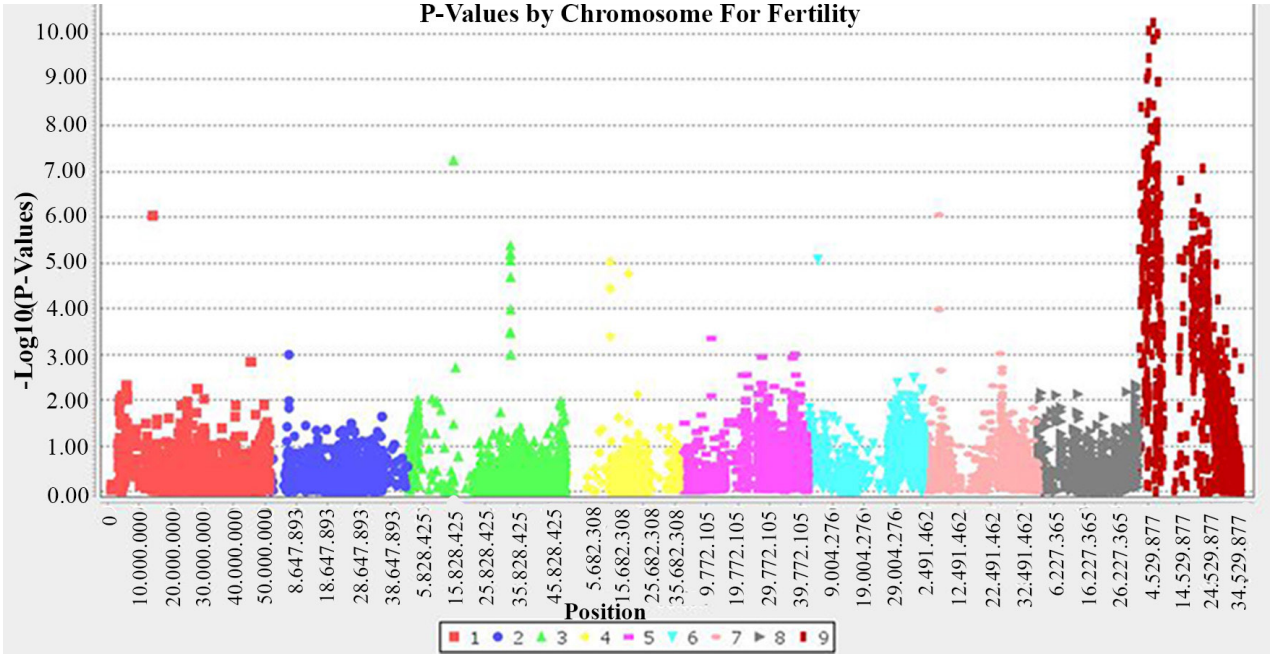


Figure 4. Manhattan plot showing association of SNP markers with *Rf* locus.

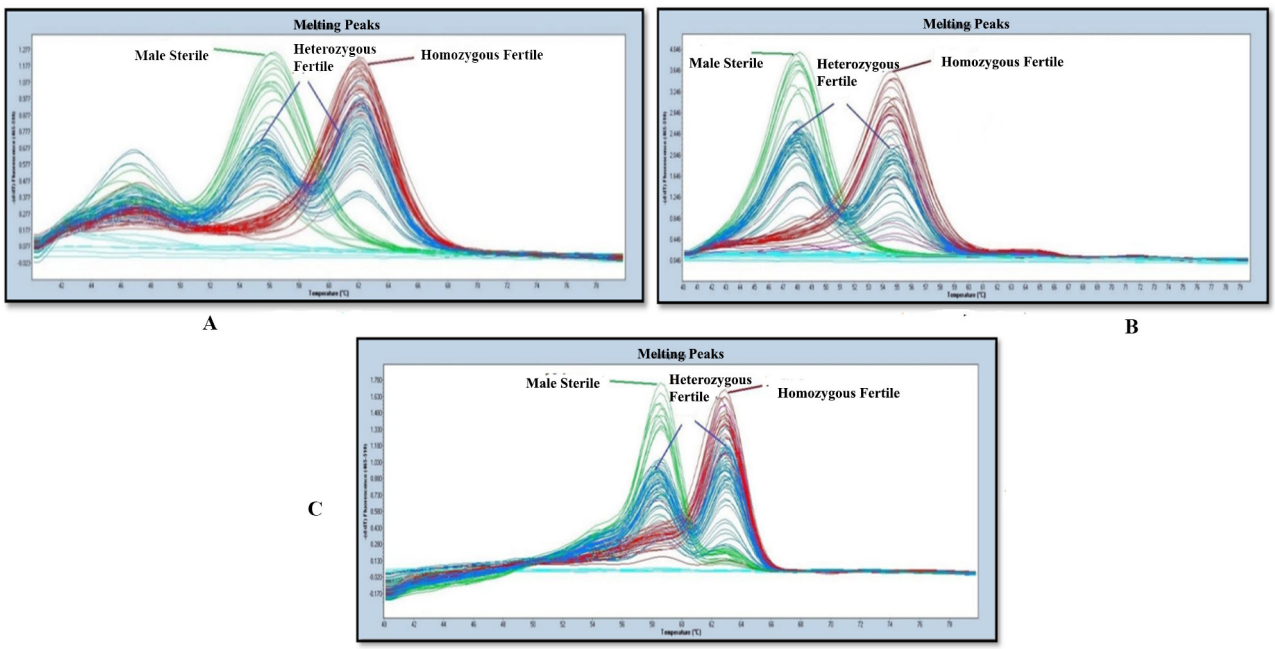


Figure 5. The real-time PCR melting curve peaks of DcSNP-ch9_3342931 (A), DcSNP-ch9_4822988 (B) and DcSNP-ch9_6233708 (C) SNP markers. Red, blue, and green lines indicated homozygous fertile, heterozygous fertile and male sterile genotypes, respectively.

3.6. Genetic mapping of *Rf* locus

Using the genotypic data of SNP markers, DcSNP-ch9_6233708, DcSNP-ch9_4822988 and DcSNP-ch9_3342931, from simple probe analysis, *Rf* locus was mapped. The length of mapped chromosomal region was 7.3 cM with an average marker distance of 2.43 cM. DcSNP-ch9_6233708 was on the distal end of the linkage group and 2.4 cM away from *Rf*. Other DcSNP-ch9_4822988 and DcSNP-ch9_3342931 were on the other side of *Rf* locus with the distance of and 1.7 and 4.9 cM, respectively (Figure 6).

Table 3. Comparison of the chi-square p-values of phenotypic data, GBS analysis and simple probe assay of F2 segregating population.

Analysis	SNP names	The number of homozygous fertile plants	The number of heterozygous fertile plants	The number of male sterile plants	The number of total fertile plants	The number of male sterile plants	χ^2 test p-value (3:1)	2 test p-value (1:2:1)
Phenotypic observation	-	-	-	19	77	19	0.24	-
GBS analysis	DcSNP-ch9_3342931	27	48	17	75	17	-	0.30
	DcSNP-ch9_4822988	25	50	17	75	17	-	0.39
	DcSNP-ch9_6233708	25	49	18	74	18	-	0.47
Simple probe assay	DcSNP-ch9_3342931	31	43	17	74	17	-	0.31
	DcSNP-ch9_4822988	27	48	18	75	18	-	0.88
	DcSNP-ch9_6233708	26	47	18	73	18	-	0.88

4. Discussion

CGMS has been primarily utilized for hybrid carrot seed production worldwide. Initially, rapid detection of male sterile lines and their maintainers at an early stage of plant development has become a significant demand in commercial hybrid carrot breeding programs. With this respect, using molecular markers would be a cost-effective tool to facilitate expensive breeding methods in development of hybrid carrot varieties.

Up to date, several SNP-based genetic mapping studies for important traits of carrots have been reported (Just et al., 2007; Cavagnaro et al., 2014; Parsons et al., 2015). Particularly, with the high throughout sequencing technologies and availability of carrot reference genome (Iorizzo et al., 2016), GBS-based linkage maps have been also published. There have been various studies focused on the mechanism of CGMS in carrot (Chahal et al., 1998; Nakajima et al., 1999; Nothnagel et al., 2000; Bach et al., 2002; Linke et al., 2003). Besides, the location of fertility restorer gene with the associated molecular markers was previously reported in some researches (Alessandro et al., 2013; Fuji and Shiroto, 2019). However, molecular basis of the fertility restorer gene and the identification of high-throughput SNP markers associated with *Rf* locus have still need to be more investigated.

The first published study on linkage mapping of *Rf* genes in carrot found out that the fertility restoration gene mapped to chromosome 9 (Alessandro et al., 2013). However, in the other recently developed research, the QTL locus related to petaloid type of male sterility identified on chromosome 4 (Fuji and Shiroto, 2019). This present research demonstrated that *Rf* gene located to chromosome 9 by supporting to the findings of previous research by Alessandro et al (2013). In this study, the genetic map of chromosome 9 consisted of 119 good quality SNP markers

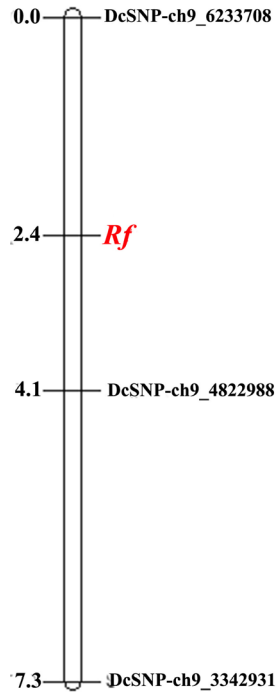


Figure 6. Genetic mapping of *Rf* gene with tightly linked SNP markers on chromosome 9. Markers' names are presented on the right of chromosome, and their positions (in cM) are on the left. *Rf* denoted in red.

in one linkage group and the total map length was 66 cM with an average marker distance of 0.55 cM/SNP. The previous genetic map included 25 SSR, AFLP and SCAR markers in one linkage group on chromosome 9 with the total map coverage of 73 cM and the mean marker spacing was 1.88 cM. On the other hand, in this study, GBS which is known as an effective technique for developing thousands

of SNP markers was employed to identify new SNP markers associated with *Rf* locus. With this aspect, the method utilized in this study for the identification of novel SNP markers related to *Rf* was distinct from the techniques used in previously published studies.

In recent years, fine mapping studies of male sterility traits have brought a rapid approach to hybrid breeding programs in various crops. For instance; ms_1 was mapped on chromosome 5 with the identification of twelve flanking HRM markers in pepper (Jeong et al., 2018; Naresh et al., 2018); *Pis1* was located in a 4.1-cM region of wheat genome with description of surrounding two SNP markers by using GBS (Yang et al., 2017); fine mapping of ms-3 gene with the revealing of the closest SNP markers were conducted in cucumber (Han et al., 2018); three SNP markers linked with male sterility were published in Chinese cabbage (Lin et al., 2020); the first SNP-based fine mapping of *ms1* by using GBS was reported in chicory (Palumbo et al., 2019). In carrot, two flanking AFLP markers which were 4.38 and 1.12 cM away from *Rf1* have already been reported (Alesandro et al., 2013). On the other hand, the genetic map of *Rf* gene was developed including SNP markers by using GBS analysis in this study. In addition, a genetic map of *Rf* gene was successfully constructed by using three closest SNP markers which were 1.7, 2.4, and 4.9 cM from *Rf* locus.

GBS approach has been widely used as an effective method for the determination of the approximate locations of the genes in the chromosomes. However, some genotyping errors can occur depending on the limited power of GBS for discriminating the heterozygous genotypes from homozygous genotypes. Nevertheless, it is crucial to confirm GBS-based SNP markers for increasing the accuracy of the genotyping analysis. In this study, real-time PCR based simple probe technology was performed to confirm genotypic segregation of SNP markers linked to *Rf* gene. Simple probe assay has been one of the recent technology for SNP genotyping and it is a faster, timesaving and sensitive method for validation for SNP genotyping. Heterozygous fertile, homozygous female and male sterile genotypes could be successfully differentiated by this recent technology.

In conclusion, this present study located *Rf* locus to chromosome 9 and three SNP markers tightly linked to *Rf* were identified by using GBS method. To confirm our GBS generated SNP data, three closely linked SNP markers were converted to simple probe markers. *Rf* was mapped using the data from three simple probe SNP markers. The findings of present study would be useful information for future fine mapping and cloning studies of *Rf* in carrot. Fine mapping or cloning *Rf* in carrot can help to reach 100% genotyping accuracy. Availability of three SNP markers tightly linked *Rf* can provide a rapid and accurate method for early identification of *Rf* carrying plants in MAS during the development new carrot hybrid varieties.

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Supplementary Table 1. Phenotypic observation data of flower types in a F2 carrot population.

Sample no.	Sample name	Flower type
1	DC-01	Male fertile
2	DC-02	Male fertile
3	DC-03	Male fertile
4	DC-04	Male sterile
5	DC-05	Male fertile
6	DC-06	Male sterile
7	DC-07	Male fertile
8	DC-08	Male fertile
9	DC-09	Male fertile
10	DC-10	Male fertile
11	DC-11	Male sterile
12	DC-12	Male fertile
13	DC-13	Male fertile
14	DC-14	Male fertile
15	DC-15	Male fertile
16	DC-16	Male fertile
17	DC-17	Male fertile
18	DC-18	Male sterile
19	DC-19	Male fertile
20	DC-20	Male fertile
21	DC-21	Male fertile
22	DC-22	Male sterile
23	DC-23	Male sterile
24	DC-24	Male fertile
25	DC-25	Male fertile
26	DC-26	Male sterile
27	DC-27	Male fertile
28	DC-28	Male sterile
29	DC-29	Male fertile
30	DC-30	Male fertile
31	DC-31	Male fertile
32	DC-32	Male fertile
33	DC-33	Male fertile
34	DC-34	Male fertile
35	DC-35	Male fertile
36	DC-36	Male sterile
37	DC-37	Male fertile
38	DC-38	Male fertile
39	DC-39	Male fertile
40	DC-40	Male fertile
41	DC-41	Male fertile
42	DC-42	Male fertile
43	DC-43	Male fertile
44	DC-44	Male fertile
45	DC-45	Male sterile
46	DC-46	Male sterile
47	DC-47	Male fertile
48	DC-48	Male sterile
49	DC-49	Male fertile
50	DC-50	Male sterile
51	DC-51	Male fertile
52	DC-52	Male sterile
53	DC-53	Male fertile
54	DC-54	Male fertile
55	DC-55	Male sterile
56	DC-56	Male fertile
57	DC-57	Male fertile
58	DC-59	Male fertile
59	DC-60	Male fertile
60	DC-61	Male fertile
61	DC-62	Male fertile
62	DC-63	Male fertile
63	DC-64	Male fertile
64	DC-65	Male fertile
65	DC-66	Male sterile
66	DC-67	Male fertile
67	DC-68	Male fertile
68	DC-69	Male fertile
69	DC-71	Male fertile
70	DC-72	Male fertile
71	DC-73	Male fertile
72	DC-74	Male sterile
73	DC-75	Male fertile
74	DC-76	Male fertile
75	DC-77	Male fertile
76	DC-78	Male fertile
77	DC-79	Male fertile
78	DC-80	Male sterile
79	DC-81	Male fertile
80	DC-82	Male fertile
81	DC-83	Male fertile
82	DC-84	Male fertile
83	DC-85	Male fertile
84	DC-86	Male fertile
85	DC-87	Male fertile
86	DC-88	Male fertile
87	DC-89	Male fertile
88	DC-90	Male fertile
89	DC-91	Male fertile
90	DC-92	Male fertile
91	DC-93	Male fertile
92	DC-94	Male fertile
93	DC-95	Male fertile
94	DC-96	Male fertile
95	DC-97	Male sterile
96	DC-98	Male fertile

Supplementary Table 2. Comparison of the results of GBS and simple probe analysis with phenotypic observation data (Hef: heterozygous male fertile; Hof: homozygous male fertile; S: male sterile; -: Missing data).

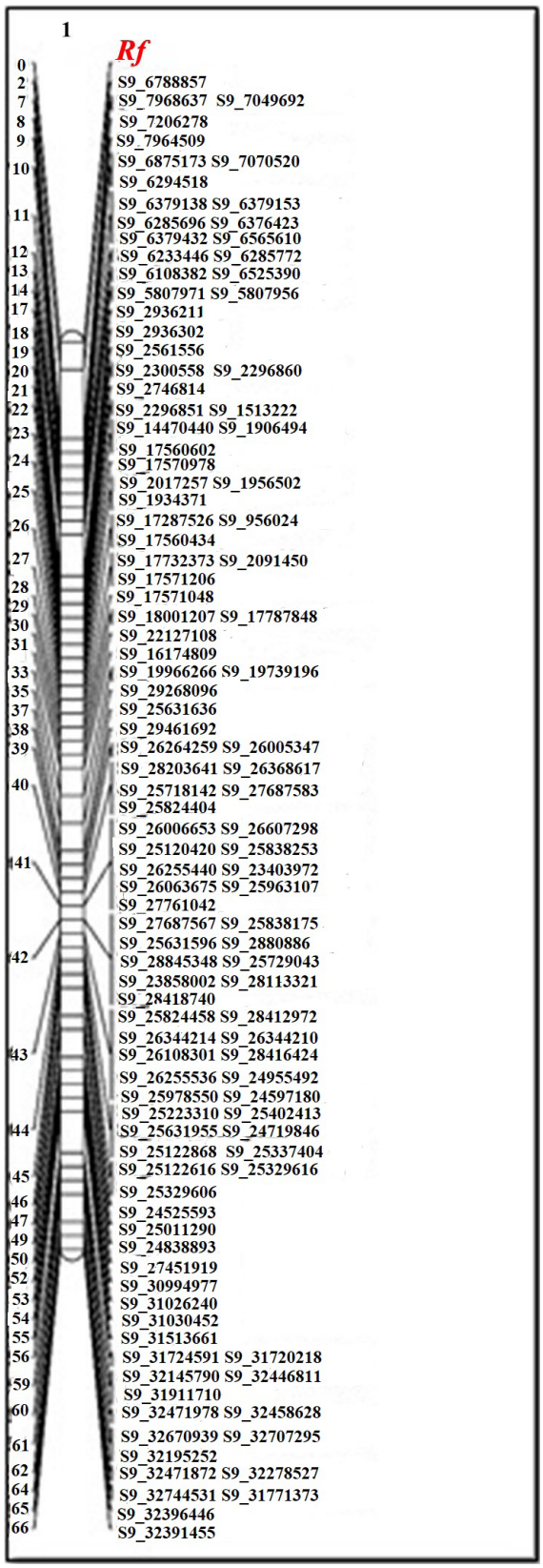
Sample name	Phenotypic observation data	GBS results			Simple probe analysis results		
		DcSNP-ch9_3342931	DcSNP-ch9_4822988	DcSNP-ch9_6233708	DcSNP-ch9_3342931	DcSNP-ch9_4822988	DcSNP-ch9_6233708
DC-01	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-02	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-03	Male fertile	HoF	HeF	HeF	HoF	HeF	HeF
DC-04	Male sterile	S	S	S	S	S	S
DC-05	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-06	Male sterile	S	S	S	S	S	S
DC-07	Male fertile	HeF	HeF	HoF	HeF	HeF	HeF
DC-08	Male fertile	HoF	HoF	HoF	HoF	-	-
DC-09	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-10	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-11	Male sterile	S	S	S	S	S	S
DC-12	Male fertile	HoF	HoF	HoF	HeF	HoF	HoF
DC-13	Male fertile	HeF	HeF	HeF	HoF	HeF	HeF
DC-14	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-15	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-16	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-17	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-18	Male sterile	S	S	S	S	S	S
DC-19	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-20	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-21	Male fertile	HeF	HeF	HeF	-	HeF	-
DC-22	Male sterile	S	S	S	-	-	-
DC-23	Male sterile	S	S	S	S	S	S
DC-24	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-25	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-26	Male sterile	S	S	S	S	S	S
DC-27	Male fertile	HoF	HoF	S	HoF	HoF	HoF
DC-28	Male sterile	S	S	S	S	S	S
DC-29	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-30	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-31	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-32	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-33	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-34	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-35	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-36	Male sterile	S	S	S	S	S	S
DC-37	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-38	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-39	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF

Supplementary Table 2. (Continued).

Sample name	Phenotypic observation data	GBS results			Simple probe analysis results		
		DcSNP-ch9_3342931	DcSNP-ch9_4822988	DcSNP-ch9_6233708	DcSNP-ch9_3342931	DcSNP-ch9_4822988	DcSNP-ch9_6233708
DC-40	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-41	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-42	Male fertile	HeF	HeF	HoF	HeF	HeF	HeF
DC-43	Male fertile	HoF	HeF	HeF	HoF	HeF	HeF
DC-44	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-45	Male sterile	S	S	S	S	S	S
DC-46	Male sterile	-	-	-	S	S	S
DC-47	Male fertile	HeF	HeF	S	HoF	HoF	HoF
DC-48	Male sterile	S	S	S	S	S	S
DC-49	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-50	Male sterile	S	S	S	S	S	S
DC-51	Male fertile	HoF	HoF	HoF	HoF	HeF	HeF
DC-52	Male sterile	S	S	S	S	S	S
DC-53	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-54	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-55	Male sterile	S	S	S	S	S	S
DC-56	Male fertile	HeF	HeF	HeF	HoF	HoF	HeF
DC-57	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-59	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-60	Male fertile	HeF	HeF	HoF	HeF	HeF	HoF
DC-61	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-62	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-63	Male fertile	-	-	-	-	HoF	-
DC-64	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-65	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-66	Male sterile	-	-	-	S	S	S
DC-67	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-68	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-69	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-71	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-72	Male fertile	HeF	S	HeF	HeF	HeF	HeF
DC-73	Male fertile	HeF	HeF	HeF	HoF	HoF	HoF
DC-74	Male sterile	HoF	HoF	HoF	S	S	S
DC-75	Male fertile	S	S	S	HeF	HeF	HeF
DC-76	Male fertile	HeF	HeF	HeF	HoF	HoF	HoF
DC-77	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-78	Male fertile	-	-	-	HoF	HoF	HoF
DC-79	Male fertile	HoF	HoF	HoF	HeF	HeF	HoF
DC-80	Male sterile	HeF	HeF	HeF	-	S	S
DC-81	Male fertile	S	HeF	HeF	HeF	HeF	HoF

Supplementary Table 2. (Continued).

Sample name	Phenotypic observation data	GBS results			Simple probe analysis results		
		DcSNP-ch9_3342931	DcSNP-ch9_4822988	DcSNP-ch9_6233708	DcSNP-ch9_3342931	DcSNP-ch9_4822988	DcSNP-ch9_6233708
DC-83	Male fertile	HoF	HoF	HoF	HeF	HeF	HeF
DC-84	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-85	Male fertile	HoF	HeF	HeF	HoF	HoF	HeF
DC-86	Male fertile	HoF	HoF	HeF	HeF	HeF	HeF
DC-87	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-88	Male fertile	HeF	HeF	HeF	HoF	HoF	HoF
DC-89	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-90	Male fertile	HeF	HeF	HeF	HoF	HeF	HeF
DC-91	Male fertile	HoF	HoF	HoF	HoF	HoF	HoF
DC-92	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-93	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-94	Male fertile	HoF	HoF	HeF	HoF	HoF	HeF
DC-95	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-96	Male fertile	HeF	HeF	HeF	HeF	HeF	HeF
DC-97	Male sterile	S	S	S	S	S	S
DC-98	Male fertile	HeF	HoF	HoF	-	-	-



Supplementary Figure. Genetic mapping of *Rf* locus with SNP markers on chromosome 9. Markers' names are presented on the right of chromosome, and their positions (in cM) are on the left. *Rf* denoted in red.