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Identification of SSR Markers Associated with Yield-Related Traits and Heterosis Effect in Winter Oilseed Rape (*Brassica Napus* L.)

Joanna Wolko [1], Agnieszka Łopatyńska [1,*], Łukasz Wolko [2], Jan Bocianowski [3], Katarzyna Mikołajczyk [1] and Alina Liersch [1]

[1] Plant Breeding and Acclimatization Institute-National Research Institute, Strzeszyńska 36, 60-479 Poznań, Poland; j.wolko@ihar.edu.pl (J.W.); k.mikolajczyk@ihar.edu.pl (K.M.); a.liersch@ihar.edu.pl (A.L.)
[2] Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, Dojazd 11, 60-632 Poznań, Poland; lukasz.wolko@up.poznan.pl
[3] Department of Mathematical and Statistical Methods, Poznań University of Life Sciences, Wojska Polskiego 28, 60-637 Poznań, Poland; jan.bocianowski@up.poznan.pl
* Correspondence: a.lopatynska@ihar.edu.pl

Abstract: The identification of markers responsible for regulating important agronomic traits in rapeseed supports breeding and increases the seed yield. Microsatellite (SSR) markers are mainly used as 'neutral' genetic markers but are also linked with many biological functions. The objective of this study was identification of microsatellite markers associated with important agronomic traits affecting the seed yield of winter oilseed rape and with the heterosis effect for these traits. The plant material consists of four parental lines, 60 doubled haploid (DH) lines, 60 single cross hybrids, and 60 three-way cross hybrids. The association between molecular markers and observed traits was estimated using regression analysis. Among 89 SSR markers, 43 were polymorphic, and 15 were selected for mapping because they demonstrated stability in both years of observation. These markers were physically mapped in the rapeseed reference genomes and their immediate vicinity was searched to identify candidate genes associated with the studied traits. Six markers (BrGMS3837, BnEMS1119, BrGMS2901, BnGMS0509, BrGMS3688, BrGMS4057), which showed a positive estimation effect in our association analysis, and thus increased the value of a given trait or heterosis effect, turned out to be linked with genes that could be responsible for the development and growth of plants.

Keywords: microsatellite markers; association analysis; physical mapping; rapeseed

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1. Introduction

Rapeseed is the most important oil crop in Poland and the European Union, and the third largest in the world, after oil palm and soybean. Due to the high demand and versatile use of rapeseed as a source of oil used for food, industrial purposes, and feed protein, the demand for seeds of this plant is constantly growing. Poland is the largest oilseed rape producer among the European Union countries after France and Germany. The rapeseed cultivation acreage in Poland in 2021 was record-breaking and amounted to approximately 980,000 hectares, producing over 3.2 million tons of seeds, while the European Union produced 17 million tons, and world production amounted to 70 million tons of seeds. In order to increase the yield without increasing the acreage, the heterosis effect has been successfully used in oilseed rape breeding for many years. Heterosis refers to the superior phenotypes observed in hybrids relative to their parents, who are selected as two genetically diverse lines. Heterosis occurs concerning traits such as yield, growth rate, and increased resistance to various biotic and abiotic stresses [1].

Rapeseed (*Brassica napus* L., genome *AACC*, $2n = 38$) is an allopolyploid plant formed as a result of spontaneous hybridization between *B. rapa* (genome *AA*, $2n = 20$) and *B. oleracea*

(genome *CC*, 2*n* = 18) about ~7500 years ago in Europe [2]. Its genome is large (1130 Mb) and is additionally complicated by a continuous process of homeologous exchanges between A and C subgenomes [3]. The genome size of oilseed rape and its complexity make genetic research difficult and limit progress in determining the genetic basis of major agronomic traits [4].

Agronomic traits of rapeseed, which influence its yield and quality, are usually controlled by QTLs (quantitative trait loci) and can be strongly modified by the environment. The heterosis effect is even more challenging to capture because it additionally depends on the interaction between parental genotypes [1]. Selection for these traits and heterosis effect is therefore quite difficult, but identification of markers associated with them could facilitate obtaining valuable breeding materials. The principal objective of such studies is to identify genes that could be used in breeding through marker-assisted selection (MAS). The application of molecular marker techniques for QTL has become a popular approach [5]. Candidate genes present within identified QTL region can be potentially involved in phenotype formation [6]. This effect could be clarified through examination of the gene arrangement and interaction of loci affecting trait variation [7].

Genetic study has advanced significantly in recent years due to the development of molecular methods. The molecular markers are widely used in various areas of research such as map construction, genetic mapping, marker-assisted selection for plant breeding, analysis of genetic diversity, etc. [8–11]. Even though modern and high-throughput molecular techniques are becoming more common, microsatellite markers are still widely used for various purposes. Microsatellites, also known as SSR markers, are tandem repeat motifs of 1–6 nucleotides commonly occurring throughout the entire genome in all organisms. SSRs are mainly used as 'neutral' genetic markers, but it was proved that they are linked with many biological functions such as regulation of chromatin organization, DNA metabolic processes, gene activity, and RNA structure [12]. Microsatellite markers have a lot of significant advantages: they are co-dominant, multi-allelic (variable), reproducible, and abundantly and evenly distributed throughout the genome. SSR markers are also easy to detect, analyses can be automated, require only a small amount of DNA, and results are simple to interpret without specialized programs or bioinformatics [13,14]. All these advantages make microsatellites a common choice for various research. Unfortunately, in *B. napus* the use of SSR markers runs into complications because of its allotetraploid genome—some of them turned out to be multi-locus and amplify multiple alleles from homoeologous loci. Therefore, the evaluation of microsatellites and the assignment of alleles to specific loci can be difficult in *Brassica napus* [15]. Nevertheless, these markers are frequently chosen for various rapeseed research, e.g., genetic diversity [16–19], map construction, and identification of quantitative trait loci (QTL) [20–22], or investigating the heterosis effect [23,24]. Hence, we decided that these markers are suitable for our research.

Molecular studies of oilseed rape have developed more intensively in recent years due to the sequencing of its genome. Genome assemblies for four cultivars of *B. napus* have been published to date: Darmor-bzh [3], Tapidor [25], ZS11 [26], and Express 617 [27]. The ZS11 cultivar genome assembly is accessed in NCBI Genome database as reference Bra_napus_v2.0. The genomic sequences of *B. napus* confirm that the structural rearrangements frequently occur between the chromosomes of the alloploid genomes [27]. It was described that the allopolyploid genomes are recombined due to the pairing of non-homologous chromosomes during meiosis, which encourages homoeologous exchange (HE) events [28,29], resulting from the replacement of chromosomal segments of one subgenome with another. These genotype specific HEs have been shown to give rise to novel genetic diversities related to important agronomic traits such as flowering time [3,30,31] and seed composition [32,33].

The objective of this study was an attempt to identify SSR markers associated with important agronomic traits affecting the seed yield, as well as with the heterosis effect for these traits. Our interest in markers for the heterosis effect stems from the fact that most of the modern oilseed rape breeding programs are based on hybrid varieties charac-

terized by increased yield. Additionally, the identified markers for phenotypic traits and heterosis were physically mapped in the rapeseed reference genomes. A genome search was also performed around these markers to identify candidate genes associated with the studied traits.

2. Materials and Methods

2.1. Plant Material

The plant material used in this study (184 genotypes of winter oilseed rape) consists of four parental lines (RIL 324/2, RIL 622/3, CMS line, *Rfo* line) and three populations: (1) 60 doubled haploid (DH) lines developed from a F_1 cross between female parent RIL 324/2 (high oleic acid content 77.9%) and male parent RIL 622/3 (high oil content 51.9%) using isolated microspore cultures method, (2) 60 single cross hybrids (CMS × DH) produced through crossing all DH lines with one CMS *ogura* line, (3) 60 three-way cross hybrids (CMS/DH × *Rfo*) obtained by crossing all-male sterile single cross hybrids with one restorer (*Rfo*) line. The specific CMS and *Rfo* lines were chosen after analyzing the genetic distance in relation to DH lines. All plant material was developed in Poznań Research Center of Plant Breeding and Acclimatization Institute—National Research Institute. DH population was developed using the isolate microspores culture method according to Cegielska-Taras et al. [34]. CMS *ogura* and *Rfo* lines were produced in the Laboratory of Heterosis and described by Bartkowiak-Broda [35] and Liersch et al. [36]. In the same Laboratory, single and three-way cross hybrids were obtained and described by Dobrzycka et al. [37].

2.2. Phenotypic Evaluation

DH lines, two groups of hybrids, CMS *ogura* line, and *Rfo* line were evaluated under field conditions in the two-year experiment (2014/15 and 2015/16) in Borowo (52°70′ N, 16°46′ E), Plant Breeding Strzelce Ltd., Co. (Strzelce Opolskie, Poland)—PBAI-NRI Group. Plants were grown in four rows (2 m long, 50 seeds per row), spaced 30 cm apart in a randomized complete block design with three replications. Trials were conducted according to standard agronomic practice. Detailed information about soil and weather conditions was described by Wolko et al. [38]. The following quantitative traits were evaluated: beginning of flowering (days), length of flowering (days), plant height (cm), the number of branches per plant, the number of siliques per plant, silique length (mm), the number of seeds per silique, and thousand seed weight (g). Phenotypic evaluation of measured agronomic traits and their variation was described in detail by Łopatyńska et al. [39].

The heterosis effect was assessed for the previously examined traits, except for traits related to the flowering. Detailed methods and results of heterosis evaluation on this plant material were described by Wolko et al. [38]. In the current study, we focused on mid-parent heterosis, which was estimated by comparing of the particular hybrid with the trait mean over two direct parents.

2.3. Molecular Analyses

Genomic DNA from fresh young leaves of all studied genotypes was isolated and purified using the modified CTAB method [40] already standardized in our laboratory. A detailed description can be found in Dobrzycka et al. [41].

Sequences of a total 89 pairs of SSR primers were obtained from Li et al. [15] (Table S1). Selected markers are distributed on every chromosome of *B. napus*. For genotyping microsatellites, the multiplex PCR method using M13-tailing was applied [42]. This approach is based on universal fluorescent-labeled primer (M13), which has the same sequence as added at the 5′-end of each forward primer. During the PCR reaction, this fluorescent primer hybridizes with the complementary region added in the early PCR cycles. In this way, there is no need for fluorescent labeling of each SSR primer. The PCR reaction mixture contained three primers: SSR forward primer modified by adding at its 5′-end nucleotide sequence (GTTTTCCCAGTCACGAC) identical to the M13 sequence, M13 forward primer

labeled with one of the following fluorescent dyes (VIC, FAM, NED, PET—Applied Biosystems, Waltham, MA, USA), and SSR reverse primer (synthesized by Sigma Aldrich, Saint Louis, MO, USA).

The PCR was performed in 96-well plates with a final volume of 6 µL. The composition of the mixture was as follows: 10 ng of DNA template, 0.25 µM of forward-M13 primer, 0.5 µM of fluorescent-labeled M13 forward primer, 0.75 µM of reverse primer, 2.5 µL of Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany), and an amount of deionized water to refill 6 µL of the reaction mixture. The amplification was performed using a thermal cycler with the following program: initial denaturation at 95 °C for 5 min; 30 cycles with 95°C denaturation for 30 s, 55–60 °C annealing for 1.5 min, 72 °C elongation for 30 s; 60 °C final elongation for 30 min. The amplification products were automatically separated by a capillary electrophoresis system on an ABI PRISM 3130 XL genetic analyzer (Applied Biosystems) with GeneScan-600 LIZ size standard. Due to the use of different dyes, four primers were separated simultaneously. This method allows increasing efficiency and reduces costs. SSR analyses were conducted in Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland.

In the first step, polymorphism of 89 SSR markers was tested on parental lines (324/2, 622/3, CMS *ogura*, *Rfo*) since they constitute the gene pool for this study. The DH population and two groups of hybrids contain a set of parental alleles in different configurations. The 43 microsatellite markers that demonstrated polymorphisms in the first step were chosen to further analysis of all genotypes. Results of SSR genotyping across all genotypes were scored as presence (1) or absence (0) of given allele. Data were analyzed with Peak Scanner Software v1.0 (Applied Biosystems).

2.4. Statistical Analysis

The normality of the distribution of the observed traits was tested using Shapiro–Wilk's normality test to check whether the analysis of variance met the assumption that the analysis of variance model residuals followed a normal distribution. The homogeneity of variance was tested using Bartlett's test. Box's M test was used to check multivariate normality and homogeneity of variance-covariance matrices.

The polymorphic information content (PIC) for each marker was calculated using the:

$$\mathrm{PIC}_i = 1 - \sum_{j=1}^{k} {p_{ij}}^2, \quad (1)$$

where p_{ij} is the frequency of the j-th allele for i-th marker and summation extends over k alleles. *PIC* values ranged from 0 (in the case of fixation of one allele) to 1 (when the frequencies of both alleles were equal) [43].

The association between molecular markers and observed traits was estimated using regression analysis [44].

$$y_i = \mu + a \cdot m_i + e_i, \quad (2)$$

where y is the observed quantitative trait, μ is the general mean, a is the regression coefficient for the main effect of the marker m_i, m_i is the indicator variable of the marker genotypes, e_i is an error of observations. The marker observations were tested as independent variables and considered in individual models. We used the critical significance level equal to 0.05, resulting from a Bonferroni correction, for each regression model [45]. The percentage of phenotypic variation (coefficient of determination, R^2) was estimated. The coefficient of determination was used to measure how the model fits data and, in this study, the amount of the phenotypic variance explained by the marker effects. Association analysis between identified SSR markers and eight observed phenotypic traits were made for each year separately. Additionally, associations between heterosis effects for plant height, the number of branches per plant, the number of siliques per plant, silique length,

the number of seeds per silique, thousand seed weight, and SSR markers were estimated based on regression analysis for each year of study independent by model:

$$h_i = \mu + a \cdot m_i + e_i, \quad (3)$$

where h is the heterosis effect of the observed quantitative trait.

All analyses were conducted in Genstat 18.2 (VSN International Ltd., Hemel Hempstead, England, UK).

2.5. Physical Mapping

The fifteen SSR markers that associated with the same trait or with heterosis for the same trait in both years, and had an effect with the same sign, were mapped on the *Brassica napus* reference genome—cultivar ZS11, Bra_napus_v2.0, GCF_000686985.2 [26]. The genome localization of markers were established by alignment of primer sequences using Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ accessed on 31 January 2022), with the following settings: no target sequences, both primer sequences were passed in the Primer Parameters section, in the Primer Pair Specificity Checking Parameters section were selected *Brassica napus* L. (taxid:3708) as source organism and Refseq representative genomes as database. In the next step, the localization of markers was verified using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 31 January 2022) search of the sequence fragments identified in the first step. This BLAST search was performed with the following settings: database—Whole-Genome-Shotgun contigs, source organism—*Brassica napus* L. (taxid:3708), program—Highly similar sequences (megablast) with default settings. The alignment to WGS database allowed localized markers in Da-Ae cultivar (GCA_020379485.1, unpublished; https://www.ncbi.nlm.nih.gov/nuccore/2106577125 accessed on 31 January 2022). The genomic localization of the markers enabled the designation of chromosome regions of interest and specify candidate genes based on reference genome annotation.

3. Results

For initial testing of the parental lines, 89 pairs of SSR primers were used (Table S1). Forty-three of them demonstrated polymorphism and were used to test all the genotypes. Table S2 presents characteristics of polymorphic SSR markers. A total of 115 alleles were identified, from 2 to 6 per marker, with an average of 2.67. Range of the obtained product sizes was from 122 bp to 511 bp. The polymorphic information content (PIC) calculated for these markers ranged from 0.602 for BrGMS1804 to 1.000 for BrEMS0015 and BnGMS0662.

3.1. Association Analysis for Phenotypic Traits

Association analysis between 43 polymorphic SSR markers and eight observed phenotypic traits demonstrated that every marker was linked with one or more of the studied traits, and some of these associations were repeated in both years. In the first year of study, 42 SSR markers were associated with 1 to 5 traits, and in the next year, 41 markers were associated with 1 to 6 traits (Table S3). We identified in total 354 significant associations, of which 168 in year 2015 and 186 in 2016 (Table 1). They are described in detail in Table S4, which contains an estimation of regression, p-values, and phenotypic variance explained by the markers. These values are presented for every association of the discussed markers. The percentage of phenotypic variance explained by the markers in year 2015 ranged from 1.6% to 15.9% with an average of 4.64%, and in 2016 from 1.6% to 11.7% with an average of 4.25% (Table S4). Most of these associations (especially those related to flowering traits) had an opposite sign of estimation in both years of study. The positive or negative sign of association values means that the marker correlates with respectively increasing or decreasing effect of phenotypic trait.

Table 1. The number of total significant associations of detected alleles with studied quantitative traits. Both years—the same allele of a particular marker that repeats association in both years.

Trait	2015	2016	Both Years
Beginning of flowering	30	28	4
Length of flowering	60	28	21
Plant height	9	21	5
No. of branches per plant	2	23	1
No. of siliques per plant	1	54	0
Silique length	11	1	0
No. of seeds per silique	16	15	2
Thousand seed weight	39	16	3
Total	168	186	36

For further detailed analysis, we selected only those markers whose same allele was associated with the same trait in both years of research and had an effect with the same sign (Table 2). There are eight such markers linked with four traits. With plant height associated five SSR markers (BrGMS3837, BnEMS1119, BrGMS2901, BoGMS0454, BnGMS0386), with the number of branches per plant one marker (BrGMS0086), with the number of seeds per silique two markers (BrGMS0086, BoGMS0454), and with thousand seed weight two markers (BoGMS1740, BnGMS0509). Two of these markers (BoGMS0454 and BrGMS0086) were associated with two traits simultaneously. The percentage of total phenotypic variance explained by markers for plant height ranged in year 2015 from 1.8% to 8.3%, and in 2016 from 1.6% to 5.9%. For the number of branches per plant, these values were 2.0% and 1.9% (in 2015 and 2016, respectively). This parameter for the number of seeds per silique ranged from 2.3% to 5.0% and from 2.2% to 4.9% (respectively), and for thousand seed weight it was from 2.0% to 3.7% and 1.8% (respectively). The PIC values for these markers ranged from 0.836 to 0.986.

Table 2. Characteristics of SSR markers associated with studied traits, with the same allele recurring and having the same sign of estimation in both years of field trials.

Trait	Marker	Allele	2015			2016			PIC
			Effect	*p*-Value	%	Effect	*p*-Value	%	
PH	BrGMS3837	313	5.03	0.033	1.9	6.92	0.003	4.3	0.982
	BnEMS1119	224	4.09	0.040	1.8	4.42	0.023	2.3	0.836
	BrGMS2901	277	3.67	0.012	2.9	2.82	0.050	1.6	0.964
	BoGMS0454	232	−6.46	<0.001	8.3	−5.41	<0.001	5.9	0.920
	BnGMS0386	229	4.72	0.014	2.8	5.69	0.003	4.4	0.986
BPP	BrGMS0086	313	−0.46	0.033	2.0	−0.41	0.033	1.9	0.882
SPS	BrGMS0086	317	−1.65	0.023	2.3	−2.13	0.002	4.9	0.882
	BoGMS0454	232	−1.26	0.001	5.0	−0.83	0.024	2.2	0.920
TSW	BoGMS1740	241	0.29	0.005	3.7	0.26	0.038	1.8	0.842
	BnGMS0509	227	0.10	0.033	2.0	0.12	0.038	1.8	0.934

Effect—estimates of regression coefficients, %—The percentage of total phenotypic variance explained by markers, PIC—polymorphic information content, PH—plant height, BPP—no. of branches per plant, SPS—no. of seeds per silique, TSW—thousand seed weight.

The eight SSR markers described in Table 2 were further mapped on the *Brassica napus* reference genomes (ZS11 and Da-Ae).

3.2. Association Analysis for Heterosis Effect

Association analysis between 43 polymorphic SSR markers and heterosis effect for six phenotypic traits in two generations of hybrids (CMS × DH and CMS/DH × *Rfo*) demonstrated that most of the markers were linked with heterosis effect for one or more traits, and some of these associations were repeated in both years. Number of associated markers for single cross hybrids in year 2015 was 26 and in year 2016 was 27 (Table S5). For three-way cross hybrids it was 15 and 16, respectively. The number of total associations for heterosis is summarized in Table 3. We identified 32 significant associations in year 2015 and 55 in 2016 for CMS × DH hybrids, and for CMS/DH × *Rfo* hybrids there were 29 and 24 associations, respectively. These associations are described in detail in Tables S6 and S7, which contain an estimation of regression, *p*-values, and phenotypic variance explained by the markers. These values are presented for every association of the discussed markers. The percentage of phenotypic variance explained by the markers for CMS × DH hybrids in year 2015 ranged from 5.2% to 28.1% with an average of 10.23%, and in 2016 from 4.9% to 25.4% with an average of 10.22% (Table S6). The percentage of phenotypic variance explained by the markers for CMS/DH × *Rfo* hybrids in year 2015 ranged from 4.9% to 17.6% with an average of 7.3%, and in 2016 from 5% to 10.9% with an average of 7.57% (Table S7).

Table 3. The number of total significant associations of detected alleles with heterosis effect for CMS × DH and CMS/DH × *Rfo* hybrids. Both years—the same allele of a particular marker that repeats association in both years.

Heterosis for Trait	2015	2016	Both Years
CMS × DH			
Plant height	3	6	1
No. of branches per plant	1	3	0
No. of siliques per plant	1	5	0
Silique length	13	14	1
No. of seeds per silique	13	21	9
Thousand seed weight	1	6	0
Total	32	55	11
CMS/DH × *Rfo*			
Plant height	6	7	0
No. of branches per plant	4	1	0
No. of siliques per plant	7	0	0
Silique length	5	6	0
No. of seeds per silique	6	4	0
Thousand seed weight	1	6	0
Total	29	24	0

For further investigation, we selected only markers whose same allele was associated with heterosis effect for a given trait in both years of research and had an effect with the same sign (Table 4). There are nine such markers for CMS × DH hybrids, linked with three traits. With plant height associated one SSR marker (BoGMS1897), with silique length one marker (BrGMS2252), and with the number of seeds per silique nine markers (BrGMS1490, BrGMS4252, BrGMS3688, BrGMS0086, BrGMS4057, BrGMS2252, BoGMS1897, BrGMS2901, BnGMS0749). Two of these markers (BoGMS1897 and BrGMS2252) associated with two traits simultaneously. For CMS/DH × *Rfo* hybrids, there were no markers whose same allele repeated the association in both years. The percentage of total phenotypic variance

explained by markers for heterosis for plant height was 5.7% in year 2015 and 5.3% in 2016. For silique length it was 8.7% and 22.4%, respectively. These values for heterosis for the number of seeds per silique ranged from 5.3% to 9.7% in 2015 and from 10.0% to 25.4% in 2016. The range of PIC values for these markers was from 0.630 to 0.998.

Table 4. Characteristics of SSR markers associated with heterosis effect for CMS × DH hybrids, with the same allele recurring and having the same sign of estimation in both years of field trials.

Heterosisfor Trait	Marker	Allele	2015			2016			PIC
			Effect	*p*-Value	%	Effect	*p*-Value	%	
PH	BoGMS1897	219	−5.28	0.036	5.7	−5.91	0.043	5.3	0.912
SL	BrGMS2252	206	−3.70	0.012	8.7	−4.15	<0.001	22.4	0.630
SPS	BrGMS1490	358	−2.45	0.009	9.7	−3.80	<0.001	25.4	0.998
	BrGMS4057	209	2.37	0.012	9.0	3.39	<0.001	19.8	0.994
	BrGMS2252	206	−1.89	0.009	9.6	−1.96	0.007	10.2	0.630
	BrGMS4252	290	4.04	0.028	6.5	4.86	0.008	10.0	0.996
	BrGMS3688	261	4.04	0.028	6.5	4.86	0.008	10.0	0.898
	BrGMS0086	317	4.04	0.028	6.5	4.86	0.008	10.0	0.882
	BoGMS1897	219	−1.92	0.043	5.3	−2.71	0.004	12.1	0.912
	BrGMS2901	237	4.04	0.028	6.5	4.86	0.008	10.0	0.964
	BnGMS0749	303	−4.04	0.028	6.5	−4.86	0.008	10.0	0.898

Effect—estimates of regression coefficients, %—The percentage of total phenotypic variance explained by markers, PIC—polymorphic information content, PH—plant height, SL—silique length, SPS—no. of seeds per silique. The nine SSR markers described in Table 4 were mapped on the *Brassica napus* reference genomes (ZS11 and Da-Ae).

Comparing association analysis for traits and heterosis, it can be noticed that two markers were common (Table 5). Marker BrGMS2901 was associated simultaneously with plant height and heterosis effect for the number of seeds per silique. Marker BrGMS0086 was associated with the number of branches per plant and the number of seeds per silique, as well as with heterosis effect for the number of seeds per silique. Interestingly, in the case of the number of seeds per silique, the same allele (317 bp) of this marker was associated with the trait and with the heterosis effect for this trait.

Table 5. SSR markers chosen for physical mapping and their associations with observed traits and heterosis effect.

Marker	Number of Alleles	Product Size (bp)	Associations
BrGMS1490	4	300, 358, 400, 404	Heterosis for the no. of seeds per silique
BrGMS4057	3	201, 207, 209	Heterosis for the no. of seeds per silique
BrGMS2252	2 (2 loci)	206, 219	Heterosis for silique length, Heterosis for the no. of seeds per silique
BrGMS3837	2	313, 332	Plant height
BrGMS4252	3	288, 290, 292	Heterosis for the no. of seeds per silique
BrGMS3688	2	261, 265	Heterosis for the no. of seeds per silique
BrGMS0086	2	313, 317	No. of branches per plant, No. of seeds per silique, Heterosis for the no. of seeds per silique
BoGMS1897	3	215, 217, 219	Heterosis for plant height, Heterosis for no. of seeds per silique

Table 5. *Cont.*

Marker	Number of Alleles	Product Size (bp)	Associations
BnEMS1119	3	224, 227, 233	Plant height
BoGMS1740	4 (2 loci)	226, 237, 241, 511	Thousand seed weight
BrGMS2901	3	234, 237, 277	Plant height, Heterosis for the no. of seeds per silique
BoGMS0454	3	232, 234, 236	Plant height, No. of seeds per silique
BnGMS0749	2	283, 303	Heterosis for the no. of seeds per silique
BnGMS0386	2	229, 235	Plant height
BnGMS0509	2 (2 loci)	215, 227	Thousand seed weight

3.3. Physical Mapping

The 15 microsatellite markers that strongly associated with the traits or heterosis for traits were mapped on the *B. napus* reference genome (cultivar ZS11, Bra_napus_v2.0) by sequence local alignment method. BLAST primer sequences alignment for these markers is presented in Table 6. The location of six markers was different from that reported previously in the literature [15,26]. However, a more detailed analysis showed that the differences in the location of the markers on the chromosomes were also observed between two oilseed rape cultivars genomes (ZS11 and Da-Ae) which are accessed in the GenBank database.

The genomic localization of the markers enabled the designation of chromosome regions of interest and, based on genome annotation, specified candidate genes.

Table 6. BLAST primer sequences alignment for 15 markers associated with studied traits or heterosis for traits and the potentially most important candidate genes in the marker vicinity.

Marker	*B. Napus*	ZS11	Da-Ae	Localization	Candidate Gene
BrGMS1490	A3	A3	A3	30279113-30279456	**at 3′ side:** 1024 bp—auxin-responsive protein iaa7, LOC106439612 10,158 bp—probable E3 ubiquitin protein ligase DRIPH, LOC106444148
BrGMS4057	A3	A3	A3	11248112-11248298	**at 5′ side:** 745 bp—protein bonzai 2-like, LOC106437781 **at 3′ side:** 4468 bp—centromere/kinetochore protein zw10 homolog, LOC106437783
BrGMS2252	A5	A5 and A6	contig ScsI-HWf_2680;HRSCAF = 3438	25862970-25863163	**inside gene:** surfeit locus protein 1-like isoform x2, LOC111215684 **at 5′ side:** 2888 bp—uncharacterized protein, LOC106390143
BrGMS3837	A7	A7	A7	8357225-8357524	**at 5′ side:** 863 bp—s-protein homolog 5-like, LOC106355185 **at 3′ side:** 1058 bp—e3 ubiquitin-protein ligase sirp1, LOC106358094 1946 bp beta-1,3-galactosyltransferase GALT1, LOC106358093
BrGMS4252	A9	absent	A9	63006113-63006113	**at 5′ side:** 2420 bp—protein of unknown function HID58_034852 **at 3′ side:** 1555 bp—similar to Uncharacterized protein HID58_034853
BrGMS3688	A10	A10	A10	13277024-13277267	**inside gene:** probable mediator of RNA polymerase II transcription subunit 19b, LOC106371805 **at 5′ side:** 1182 bp—translation initiation factor eIF-2B subunit gamma-like LOC106371804 **at 3′ side:** 4664 bp—elongation factor 1-beta 2-like LOC106371803

Table 6. *Cont.*

Marker	*B. Napus*	ZS11	Da-Ae	Localization	Candidate Gene
BrGMS0086	A10	C9	C9 (A10 imperfect copy)	46523704-46523994	**inside gene:** uncharacterized protein LOC106372251 **at 5′ side:** 876 bp—exocyst complex component EXO70B1-like, LOC106372250 **at 3′ side:** 1450 bp—uncharacterized protein, LOC106372252
BoGMS1897	C1	C1	C1	43605468-43605672	**inside gene:** mRNA-U1 small nuclear ribonucleoprotein A, LOC106376916 **at 5′ side:** 3466 bp—mitogen-activated protein kinase kinase 5-like, LOC106374862 **at 3′ side:** 2105 bp—ATP synthase subunit epsilon, mitochondrial-like, LOC106376917 6662 bp glutamyl-tRNA reductase-binding protein, chloroplastic-like, LOC106376915
BnEMS1119	C2	C2	C2	8371767-8371978	**inside gene:** mRNA-B3—domain-containing protein At5 g60130-like, LOC106347474 **at 3′ side** 737 bp—AP2-like ethylene-responsive transcription factor TOE2, LOC106347473
BoGMS1740	C3	C3	C3	14545140-14545412 14540291-14540517	**at 5′ side:** 3630 bp—uncharacterized protein bnac03 g15280 d 5140 bp putative aminoacrylate hydrolase RutD, LOC106437504 **at 3′ side:** 10,336 bp—side: uncharacterized protein, LOC106437503 11 824 bp—calcium-transporting ATPase 8, plasma membrane-type-like
BrGMS2901	C6	A7	C6	8094885-8095092	**at 5′ side:** 1470—bp at: leaf rust 10 disease-resistance, LOC106353895 **at 3′ side:** 8907—bp at: uncharacterized protein, LOC106357121
BoGMS0454	C7	C6	C7	8094885-8095092	**at 5′ side:** 6722 bp—at rho guanine nucleotide exchange factor 8, LOC106430314 **at 3′ side:** 7789 bp—dynamin-related protein 4c-like isoform x1, LOC106430290
BnGMS0749	C7	C7	C7	28505712-28505955	**at 5′ side:** 24,650 bp—at 5′ side: transcription factor FAMA, LOC106409757 **at 3′ side:** 16,979 bp—at 3′ side: uncharacterized protein loc111208025, LOC111208025
BnGMS0386	C7	C7	C7	50580906-50581119	**at 5′ side:** 7286 bp—uncharacterized protein, LOC106421058 **at 3′ side:** 16,154 bp—plasma membrane-associated cation-binding protein 1, LOC106421190
BnGMS0509	C8	C5 and C8	C8	5155507-5155709	**inside gene:** B3 domain-containing protein At3g17010-like, LOC111208599 **at 3′ side:** 949 bp polygalacturonase At1g48100-like, LOC11120859

B. napus—linkage group according to Li et al. [15], ZS11—chromosomes according to BLAST analysis with ZS11 reference genome (GCF_000686985.2), Da-Ae—chromosomes according to BLAST analysis with Da-Ae WGS assembles (GCA_020379485.1).

The results of alignments indicated that the BrGMS4252 was located on A9 chromosome in genome Da-Ae; however, in the ZS11 genome, this microsatellite locus could not be identified. Marker BrGMS0086 could be mapped on chromosome C9 in both genomes, though an imperfect copy of this sequence (89% sequence covering and 95% of identity) was situated on chromosome A10 of cultivar Da-Ae, too. The microsatellite locus of marker BrGMS2901 in the cultivar ZS11 was found on chromosome A7, but in Da-Ae the same sequence was located on C6. Marker BoGMS0454 in the genome ZS11 was located on chromosome C6, but the same locus in genome Da-Ae was situated on C7. The region containing marker BnGMS0509 in the genome of ZS11 showed two loci (on chromosomes C5 and C8), whereas cultivar Da-Ae should give one PCR product, just from the C8 locus, due to substitution within the primer binding site on chromosome C5. There were also

some minor inaccuracies in the location of two other markers. There were two copies of BrGMS2252 in ZS11 genome (A5 and A6), but mapping in Da-Ae showed just one localization, on the contig, without chromosome assignation. The fragment containing BoGMS1740 was duplicated in both genomes and the copies were located in one close region of the C3 chromosome.

4. Discussion

Association studies for agronomic traits with the use of microsatellite markers have been conducted on many crops but have rarely been used in research on rapeseed. This project focused on identifying SSR markers significantly associated with yield-related traits or heterosis effect for these traits in winter oilseed rape and searching for candidate genes involved in forming these features. To test parental lines of studied populations, 89 SSR primer pairs were used. Some of these primers were also tested in rapeseed genetic diversity studies [17,19,46,47]. After initial analysis, 43 markers proved to be polymorphic and were used to analyze all genotypes. We obtained a total of 115 alleles, from 2 to 6 per marker, with an average of 2.67. A similar level of polymorphism was described by Zhu et al. [4], who obtained a score of 43 polymorphic markers out of 100 tested, with comparable values of allele number per marker (2–7, mean value of 3.59). A similar mean number of alleles per marker (2.1) was obtained by Raza et al. [18], who studied six *Brassica* species (including *B. napus*) using 10 SSR markers, which jointly produced 21 alleles. However, a higher number of alleles was achieved by Qu et al. [48] (from 2 to 11 per marker, with an average of 5.29), who tested 217 genotypes of *B. napus* with 37 SSR markers, receiving a total of 196 alleles. The above-mentioned values show that the number of primers used by us and the number of polymorphisms obtained are comparable to other studies.

Polymorphic Information Content (PIC) describes the marker quality in genetic studies, characterizing its ability to detect the polymorphism among genotypes. PIC values for codominant markers range from 0 (monomorphic) to 1 (very highly informative, with several alleles of equal frequency). Markers with PIC values greater than 0.5 are considered to be very informative [49]. PIC values calculated for markers in our study ranged from 0.602 for BrGMS1804 to 1.000 for BrEMS0015 and BnGMS0662. This means that all these markers are highly informative and are useful for assessing the variation between the studied individuals. Chen et al. [19] obtained, in their study, lower PIC values (from 0.16 to 0.59) using 30 microsatellite markers to test 537 individuals of feral *B. napus*. In Raza et al. [18]'s study, polymorphic information content was also lower and varied from 0.37 to 0.71, with an average of 0.66 per primer, while in Tsuge et al. [17]'s study it ranged from 0.04 to 0.68 with an average of 0.36 for 24 markers tested on 22 *Brassica* genotypes.

For studied phenotypic traits, we identified in total 354 significant associations, of which 168 in year 2015 and 186 in 2016, and only 36 of them were common in both years. Differences in the associations probably resulted from the influence of the environment on the observed features, which is also reflected in their variability [39]. There are few published studies about the association of SSR markers with phenotypic traits. Only one of them concerns oilseed rape, describing research similar to ours, and was conducted by Cai et al. [50]. They tested 192 genotypes of *B. napus*, and despite the use of a more significant number of SSR markers (451), they obtained a smaller number of associations for the six quantitative traits (a total of 43 and 71 associations across three years and in particular years, respectively). Similar analyses for maize were conducted by Vathana et al. [51]. They studied associations for eight agronomic traits with 50 SSR markers and found nine related with seven traits. Kim et al. [52] also studied associations in maize using 200 SSR markers and found 32 markers associated with all eight traits (some of them with few traits simultaneously). Association analyses for genotyping 420 *Paeonia rockii* accessions using 58 pairs of polymorphic EST-SSR markers and 24 yield quantitative traits were performed by Liu and Cheng [53]. The authors identified 141 significant associations involving 17 traits and 41 EST-SSRs. Comparing the above data, we can conclude that

taking into account the number of analyzed genotypes and the primers used, the number of associations obtained in our study is significant.

Phenotypes may be the result of gene expression, impact of environmental factors, or a combination of both. Phenotypic variation, then, is the variability of phenotypes that exists in a population. The percentage of phenotypic variance explained by the markers (R^2) in our research in 2015 ranged from 1.6% to 15.9% with an average of 4.64%, and in 2016 from 1.6% to 11.7% with an average of 4.25%. The highest values were observed in both years for length of flowering. R^2 for plant height was higher in 2015 (8.3%) than in 2016 (5.9%), while Cai et al. [50] obtained a similar average value for this trait (7.33%) across three years. Almost the same level of phenotypic variance was observed for the number of seeds per silique (5% in 2015, 4.9% in 2016 in our study, and 5.22% in Cai et al. [50]'s study). On the other hand, these authors achieved a higher level of R^2 for seed weight (8.03%), while in our research it was 6.3% and 3.7% in the following years. Taken together, we conclude that the estimates of values of phenotypic variance explained by markers noted in our study are not overestimated.

Some associations of a given marker were repeated for a specific trait in both years of research. A large number of such associations appeared for the traits connected with flowering, but most of them had opposite signs of estimation in following years. A positive or negative sign means the marker's correlation with the increasing or decreasing effects of phenotypic traits. Differences in estimation signs were probably an expression of an environmental impact—weather conditions caused significant variation in flowering time of plants. For some features such as the number of branches per plant, siliques per plant, and silique length, a large variation was observed in the number of associations occurring in individual years—many associated markers in the first year and none or just a few in the second. This resulted in a small number of recurring associations, which may also be related to the high variability of these features depending on environmental conditions. We consider that the most promising are those markers, for which a specific allele was associated with the same trait in both years of research with the same sign value (+/−). We believe these markers are the most stable and not significantly influenced by the environment. There are eight such markers linked with four studied phenotypic traits: with plant height associated five SSR markers (four with positive effect and one with negative), with the number of branches per plant one marker (with negative effect), with the number of seeds per silique two markers (both with negative effect), and with thousand seed weight also two markers (both with positive effect). A greater number of associations for oilseed rape genotypes was observed in Cai et al. [50]'s research, who received fourteen markers linked with plant height, six with silique length, seven with the number of seeds per silique, and nine with the seed weight.

Apart from the association analysis for traits, it was also performed for the mid-parent heterosis effect in two generations of hybrids for six studied morphological features (excluding flowering traits). For CMS × DH hybrids 87 significant associations were identified, of which 32 in year 2015 and 55 in 2016, and 53 significant associations were identified for CMS/DH × *Rfo* hybrids (29 and 24, respectively). Several alleles associated with the heterosis effect for single cross hybrids for a particular trait were repeated in both years of research, while the associations for the same alleles for three-way cross hybrids did not repeat. This may be due to the fact that the heterosis effect is difficult to capture, as it results from the interaction of parental genomes and the influence of the environment on a particular feature. Contrary to the trait association analysis, not all markers were linked with the heterosis effect for a given trait and there were more of them for single cross hybrids (33) than for three-way cross hybrids (23). The values of phenotypic variance explained by the markers for CMS × DH hybrids were almost the same in both years (on average 10.23% in 2015 and 10.22% in 2016). These values for CMS/DH × *Rfo* hybrids were slightly lower but also very similar—on average 7.3% and 7.57% (respectively). It follows that, despite the smaller number of associations for the heterosis effect, these markers accounted for a higher percentage of the explained variance than markers for the traits.

There are no published studies on the association of microsatellite markers with the heterosis effect for agronomic traits in oilseed rape. Cho et al. [54] conducted a similar study of heterosis for grain yield in rice hybrids. In their research, 25 SSR markers were associated with mid-parent heterosis (MPH), of which 22 markers increased MPH, while three markers decreased MPH. Authors regarded them as HV (hybrid vigor) and HW (hybrid weakness) markers, respectively. Similarly, in our previous research on this plant material, we observed the heterosis effect, conventionally referred to as positive or negative [38]. Among the markers identified in the current study, for which the same allele was associated in both years with the heterosis effect for the same trait, there were those with a positive or a negative sign of estimation. As mentioned before, markers with a positive sign of estimation cause an increase in the value of the given feature. In our study, five markers with a positive effect (combined with the number of seeds per silique) and four with a negative effect (combined with the number of seeds per silique, silique length, and plant height) were observed. Two of these markers were associated with heterosis for two features simultaneously and both traits were negatively affected. Hence, we believe that the heterosis in hybrids could be improved by selective breeding to introduce alleles associated with the positive effect and eliminate those associated with the negative.

After performing association analyses for quantitative traits and the heterosis effect, we focused our attention on those markers whose specific alleles were associated with the same feature/heterosis in both years of research and had an effect of the same sign. There were eight such markers: five for plant height (BrGMS3837, BnEMS1119, BrGMS2901, BoGMS0454, BnGMS0386), one for the number of branches per plant (BrGMS0086), two for the number of seeds per silique (BrGMS0086, BoGMS0454), and two for thousand seed weight (BoGMS1740, BnGMS0509). Two of these markers (BoGMS0454 and BrGMS0086) were associated with two traits simultaneously. For heterosis effect, there were nine such markers for CMS × DH hybrids, linked with three traits: one for plant height (BoGMS1897), one for silique length (BrGMS2252), and nine for the number of seeds per silique (BrGMS1490, BrGMS4252, BrGMS3688, BrGMS0086, BrGMS4057, BrGMS2252, BoGMS1897, BrGMS2901, BnGMS0749). Two of these markers (BoGMS1897 and BrGMS2252) were associated with the heterosis effect for two traits simultaneously. Additionally, two markers were linked with phenotypic trait and heterosis effect at the same time. Marker BrGMS2901 was associated with plant height and heterosis for the number of seeds per silique, and marker BrGMS0086 was associated with the number of branches per plant, the number of seeds per silique, and, interestingly, heterosis for the same trait, that is, the number of seeds per silique.

All primers used in our study were selected from Li et al. [15], who collected 3890 SSR markers from previous studies and developed 5968 SSR markers from genomic sequences of *B. rapa*, *B. oleracea*, and *B. napus*. The authors created a set of 230 single-locus markers; however, in our research some of them showed multiple loci. Single-locus markers have advantages over multi-locus in genetic studies since they facilitate analyses. Only a small number of SSR markers in *B. napus* genetic maps are considered single-locus, and some of these were accompanied by redundant monomorphic amplicons. Nevertheless, these markers might be polymorphic in other genotypes; hence, these alleged single-locus SSR markers can only be called population-specific single-locus. In Li et al. [15]'s study, 78 of the 81 BrGMS markers were assigned to the A genome (*B. rapa*) and 69 of the 70 BoGMS markers to the C genome (*B. oleracea*) of *B. napus*, which suggests that these single-locus markers conservatively amplify loci from their source genome. The BnGMS and BnEMS markers were evenly distributed in both genomes. However, one BoGMS marker was mapped in the A genome and three BrGMS markers (inter alia BrGMS2901, associated and mapped in our study) were localized in the C genome. Authors indicated that these four markers could be successfully amplified in both A and C genomes, suggesting that the 'misassignment' of these markers might be caused by a mutation that occurred at the target primer binding sites in the corresponding cognate genome in *B. napus* [15]. We observed a similar phenomenon in the results of BLAST alignments. For example,

BrGMS2901 marker was found in the cultivar ZS11 on chromosome A7, but in Da-Ae the same sequence was located on C6 chromosome. Likewise, marker BrGMS0086 could be mapped on chromosome C9 in both genomes, though an imperfect copy of this sequence was situated on chromosome A10 of cultivar Da-Ae, too.

The described above occurrence of differences in localization of microsatellite loci in references genomes (ZS11 and Da-Ae) seems puzzling, and could further complicate the study on marker association with traits and future use of these markers in breeding. The instability and high variability of the *Brassica napus* genome have already been described [27–29]. They can result from rearrangement between alloploid genomes by homoeologous exchange (HE) which occurs during meiosis. Recently, Lee et al. [27] demonstrated that a high degree of rearrangements was found between published genomic assembly sequences from different rapeseed cultivars. The observed dislocations of markers between chromosomes in our study could be affected by HE. However, the homologous recombinations concerning large genome fragments should not disrupt the regional collinearity of the marker surroundings with the closest genes in the genome region. Therefore, the association between marker and trait could not be disturbed regardless of chromosomal rearrangements.

Based on gene annotations of *Brassica napus* genome, the regions shrouding the markers were analyzed to find candidate genes potentially linked to the studied quantitative traits or heterosis effect. Beta-1,3-galactosyltransferase (GALT1, LOC106358093) was one of the genes identified within a short distance of the marker BrGMS3837 on chromosome A7 (1946 bp at 3′ side). This gene has been identified to have an influence on fiber development and demonstrated an impact on the growth and development of plants [55,56]. Markers BnEMS1119 and BnGMS0509 were located in close proximity to gene-coding proteins with DNA-binding domains characteristic for transcription factors B3 and AP2 (LOC106347474, LOC106347473 and LOC111208599). All these genes may play a role in expression regulation and could have a great impact on the key traits of a plant yield [57–59]. Two genes situated close to BoGMS0454, rho guanine nucleotide exchanged factor 8 (LOC106430314) and dynamin-related protein 4c-like (LOC106430290), could also have an influence on plant development [60,61]. In the immediate vicinity of the marker BrGMS2901, there were genes that may be associated with resistance to leaf rust (LOC106357121) and receptor-like kinases (RLKs). RLKs proteins have been described as involved in a diverse array of plant responses including development, growth, hormone perception, and the response to pathogens [62]. Marker BrGMS0086 is situated near the gene-coding exocyst complex component EXO70B1-like (LOC106372250). This protein is a component of an exocyst subcomplex specifically involved in autophagy-related, Golgi-independent membrane traffic to the vacuole [63]. Marker BoGMS1897 was situated inside the gene involved in the splicing process—mRNA-U1 small nuclear ribonucleoprotein A (LOC106376916). Marker BrGMS1490 is adjacent to the gene of auxin-responsive protein iaa7 (LOC106439612), which protein product can play a crucial role in auxin response [64]. Marker BrGMS3688 is located inside the gene which codes the probable mediator of RNA polymerase II transcription subunit 19b (LOC106371805). These proteins are coactivators involved in the regulated transcription of nearly all RNA polymerase II-dependent genes and have been indicated as key regulators of plant growth [65,66]. Additionally, in the proximity of this marker on chromosome A10, two genes involved in the translation process are situated: translation initiation factor eIF-2B subunit gamma-like (LOC106371804) and elongation factor 1-beta 2-like (LOC106371803). The gene of protein bonzai 2-like (LOC106437781), which is situated near marker BrGMS4057, codes a calcium-dependent phospholipid binding protein, which has an effect on promoting growth and development in *Arabidopsis* [67,68]. The BrGMS2252 is localized within the gene of surfeit locus protein 1-like isoform x2 (LOC111215684). This gene encodes a protein localized in the inner mitochondrial membrane and is involved in the biogenesis of the cytochrome c oxidase complex. In *Arabidopsis*, this gene showed a link to early plant development and hormonal growth responses [69]. Marker BnGMS0749 is

situated in the proximity of the transcription factor FAMA (LOC106409757), which show essential role in differentiation of *Arabidopsis* guard cells [70,71].

5. Conclusions

From the pool of SSR markers we studied in detail, almost all that were linked to genes that could be responsible for the development and growth of plants showed a positive estimation effect in our association analysis and thus increased the value of a given trait or heterosis effect. These are the following markers: BrGMS3837, BnEMS1119, BrGMS2901, BnGMS0509, BrGMS3688, and BrGMS4057. According to PIC values, all these markers are highly informative, which is useful for detecting the polymorphism among studied genotypes. In addition, each of these markers had exactly the same allele repeated in both years of observation, which indicates their relative stability and insensitivity to environmental conditions. In conclusion, we can state that the relationship between these markers and studied phenotypic traits can be a powerful diagnostic tool in rapeseed selection breeding. As further steps, the microsatellite markers selected in our research require additional testing on other genotypes to confirm their usefulness. Quantitative agronomic traits can be improved by combining alleles associated with the positive effect and eliminating those associated with the negative. Enhancing the positive effect is possible through the accumulation of the favorable alleles from a few significant loci, even though the associated markers individually explain a small amount of phenotypic variance [15,72]. Accumulation of valuable alleles with minor effects is an efficient way to improve the productivity of rapeseed varieties for yield-related traits, which results in applications in molecular breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12071544/s1, Table S1: Total sets of 89 SSR primers used for the initial polymorphism analysis (Li et al., 2013); Table S2: Characteristics of 43 polymorphic SSR markers; Table S3: Markers associated with observed traits in 2015 and 2016; Table S4: Characteristics of SSR markers significantly associated with studied quantitative traits; Table S5: Markers associated with heterosis effect in 2015 and 2016; Table S6: Characteristics of SSR markers associated with heterosis effect for phenotypic traits in CMS×DH hybrids; Table S7: Characteristics of SSR markers associated with heterosis effect for phenotypic traits in CMS/DH × *Rfo* hybrids.

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