Turkish Journal of FIELD CROPS

Turk J
Field Crops
2022, 27(2), 235-241
DOI: 10.17557/tjfc.1126296

SELECTION OF ALFALFA (*Medicago sativa* L.) HYBRID PARENTS AND HETEROSIS ANALYSIS OF F1 HYBRIDS

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Received: 05.06.2022

ABSTRACT

Heterosis is an effective way to increase yield and improve quality in alfalfa breeding. The key issue for efficiently use of heterosis is to identify parents with potential to produce hybrid combinations with higher performance. This study aimed to analyze the genetic distance between the 45 alfalfa accessions, and combine different hybrid combinations. The results showed that the mating ability of hybrid combinations in which both parents are tetraploid is greater than that in which both parents are diploid or in hybrid combination with different parental ploidy. In terms of compatibility, combinations were ranked from largest to smallest as follows: diploids x tetraploids, tetraploids x tetraploids, diploids x diploids, tetraploids x diploids. For parents of the same ploidy level analysis, the mating ability of the hybrid combinations in which both parents were tetraploid was greater than those in which both parents were diploid. For parents of the different ploidy level analysis, the mating ability of alfalfa hybrid combinations with a diploid as female parent was better than those of alfalfa hybrid combinations with a tetraploid as female parent. Moreover, CYK2 x HH10 with high heterosis and CYK4xHH10 with high relative seed setting rate should be used as candidate materials for further investigation.

Keywords: Alfalfa, Genetic distance, Heterosis, Seed setting

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most widely used perennial legume species and is widely distributed all over the world. It has numerous important agronomic qualities such as good palatability, high nutritional value, high yield and high forage value, and has received strong interest and popularity in breeding (Li-Jun et al., 2013). Breeders exploit the heterosis that occurs between crosses of alfalfa from different genetic groups to improve production and quality (Groose et al., 1989). Although traditional breeding has gained attention in breeding new varieties, this method has the limitations of highly uncertainty, a long breeding cycle and low efficiency. To increase breeding efficiency and shorten breeding cycles, it is important to predict the potential heterosis of parents. Certain hybrids show desirable yield, biomass, resistance, properties or other agronomic traits compared with their parents, a phenomenon known as heterosis. Heterosis has been widely used for the improvement of agronomic traits in a variety of crops and forages (Labroo et al., 2021). Since heterosis is a complex genetic phenomenon, it is difficult to describe quantitively. The vast majority of research examines the traits of the hybrid F1 generation and selects high-quality parental combinations. However, using this method to select high-quality hybrid combinations requires many field experiments. With the development of molecular marker technology, molecular markers have become extremely popular among breeders and scientists involved in cross-breeding (Herrmann et al., 2017). Achieving a combination of phenotypic and genotypic selection using both traditional breeding and modern biotechnological approaches is the key to cultivating new varieties. Therefore, molecular markers are used to explore the relationship between the genetic distance between parents and heterosis, ultimately speeding up the breeding cycle (Jordan et al., 2003; Geng et al., 2021). The genetic difference between parents presumably contributes to the genetic basis of heterosis. The genetic distance is an index used to measure the comprehensive genetic difference of each trait between different parents between parents. It can be used to quantify the genetic differences between two parents in order to guide parental selection. Han et al. (2020) used SSR (Simple Sequence Repeat) molecular markers to construct a Populus tomentosa (*Populus tomentosa Carrière*) breeding parent population, generating hybrid combinations with potentially high combining ability. Wang et al. (1994) also verified the degree of dissimilarity between parental lines using a molecular tool to search for a superior parental rice

combination. Mukri et al. (2022) demonstrated genetic distance as a basis for heterosis breeding in field maize, and identified of the suitable parents for heterosis breeding, thereby augmenting the productivity and quality of maize. In recent years, heterosis in conjunction with molecular marker technology has been used to understand genetic diversity of alfalfa (Annicchiarico et al., 2017). SSR markers are polymerase chain reaction (PCR) based, highly polymorphic, repeatable and simple to use, and are unaffected by environment and the reproductive period (Rui et al., 2004; He et al., 2009).

In this study, we aimed to use SRAP (Sequence related amplified polymorphism) markers to analyze genetic distance between 45 alfalfa germplasm materials and assess combinations of varieties with differences in ploidy and fertility. We further defined combinations with greater crossability by comparing seed-setting among different cross combinations and determine the authenticity of hybrids. Hybrid combinations with potentially strong heterosis were screened out according to the crossability of hybrid offspring for yield traits and their heterosis performance, which can provide a solid foundation for creating new alfalfa cultivars with high yield and good quality traits.

MATERIALS AND METHODS

Plant material

The alfalfa materials used in the study consisted of 5 varieties collected from Inner Mongolia Agricultural University, 19 wild alfalfa collected from Xinjiang and 21 provided by the National Medium-term Gene Bank of Forage Germplasm of the Institute of Grassland Research of the Chinese Academy of Agricultural Science (CAAS), Inner Mongolia, Hohhot, China (Additional file 1: Table S1). 45 alfalfa materials were grown in an experimental field in Hohhot on April, 2020, including *M. varia* (3), *M. sativa* (12) and *M. falcata* (30). Hohhot (Latitude 40°83' and Longitude 111°73') is located in the central part of Inner Mongolia and has a temperate continental monsoon climate. The elevation ranges from 900 to 1050 m, annual rainfall is 335.2 to 534.6 mm, and the monthly average temperature is 17 to 22.9°C. Twenty-seven hybrid combinations were constructed based on the genetic distance between materials and the ploidy of the materials themselves. Pollinating time was between 9:00 and 10:00 a.m. during the blossoming period. Pollination was performed using conventional methods. After removing the unhealthy florets of the female parent, three florets were retained for pollination without emasculation. The pollinated flowers were covered with thin cotton for protect. The cotton was removed the next day. For each hybrid combination, three sets of replications were included in the analysis, approximately 150 flowers were hand cross-pollinated in each repeat to test for cross compatibility. Mature pods were harvested at 40 days after pollination, and the numbers of seeds per pod were counted. Pod setting represents the ratio of the total number of pods setting to the total number of fertilized florets. Relative seed setting represents the ratio of the number of seeds per pod for hybrid seeds to seeds per pod for open-pollinated females. Furthermore, hybrid progeny populations were used for hybridization verification and parents were grown under the same greenhouse conditions. The leaves of parents and F1 populations were used for molecular identification. In a glasshouse the true hybrids of the population and parents were singly transplanted into the soil. The soil surface pH was slightly alkaline, ant the spacing between plants was 45-50 cm with 65-70cm between rows. Early in the flowering stage, aboveground biomass was harvested, and biomass yield was measured.

DNA extraction and SRAP analysis

Genomic DNA was extracted from 10 young leaves of each accession using the cetyl trimethyl ammonium bromide (CTAB) method (Stewart et al., 1993). DNA quality and concentration were evaluated using 1% agarose gel electrophoresis and NanoDrop 2000. Genomic DNA was diluted in TE buffer to a concentration of 20 -30ng/μL and samples were preserved at -20°C for further use. Thirty-six pairs of primers in the study were synthesized by Shanghai Sangon Biotech Co., Ltd (Table 1). The 20μL total volume of the reaction system contained 10μL of 2×Taq PCR Master Mix (Tian Gen Biotech, Beijing), 7μL of double-distilled H2O, 1μL each of primer (10μM), and 1μL of diluted DNA. The following amplification program was used: denaturation at 95°C for10 min, followed by 5 cycles of denaturation at 94 °C for 1min, annealing at 35 °C for 1min, extension at 72 °C for1 min, and 35 cycles of denaturation at 94 °C for 1min, annealing at 50 °C for 1min, extension at 72 °C for1 min (Yun-Tana et al., 2015) and a final extension at 72 °C for 7min. After that, the products of PCR amplification were analyzed by 8 % polyacrylamide gel electrophoresis which was carried out at a voltage of 100 V for 1.2 h in 1× TBE running buffer and products were visualized by staining with AgNO3 (Qian et al., 2021).

Table 1. Primer information used for SRAP analysis in the study

Forward primer	Sequence(5'to3')	Reverse primer	Sequence(5'to3')
F1	5’-TGAGTCCAAACCGGAGC-3’	R1	5’-GACTGCGTACGAATTTGC-3’
F2	5’-CGAATCTTAGCCGGCAC-3’	R2	5’-GACTGCGTACGAATTAAC-3’
F3	5’-CGAATCTTAGCCGGAAT-3’	R3	5’-GACACCGTACGAATTGAC-3’
F4	5’-GTAGCACAAGCCGGAGC-3’	R4	5’-GACACCGTACGAATTTGA-3’
F5	5’-CGAATCTTAGCCGGATA-3’	R5	5’-CGCACGTCCGTAATTCCA-3’
F6	5’-TGAGTCCAAACCGGATA-3’	R6	5’-GACTGCGTACGAATTAAT-3’

Data analysis

Amplified SRAP fragments were evaluated as present (1) or absent (0) bands and the resulting matrix of binary values were used for further analyses. The observed Polymorphism bands, Polymorphic loci rate, the coefficient of Shannon's information index (I), and the polymorphic information index were calculated using PopGene 32 and Excel. The value of PIC showed highly polymorphic loci (PIC > 0.5), moderately polymorphic loci (0.25 PIC 0.5), and lowly polymorphic loci (PIC <0.25) (Rongxi et al., 2016). A dendrogram was generated using arithmetic mean (UPGMA) cluster analysis. Analysis of variance (ANOVA) was performed with SAS software (SAS Institute Inc). The calculation formulae were as follows: Pod setting (%) = (number of pod setting / number of fertilized florets) × 100%. Relative seed setting= (seeds per pod for hybrid combinations/seeds per pod for open-pollinated female) × 100%. Heterosis was estimated as follows: Mid-parent heterosis (%)=(F1－MP)/MP×100%; high-parent heterosis (%)=(F1－HP)/HP×100%; and low-parent heterosis (%) =(F1－LP)/LP×100%; where F1 is the hybrid value, and MP = (P1 + P2) 2, in which P1 and P2 are the performance of parental lines, HP /LP is the high/low (better) parent performance.

RESULTS AND DISCUSSION

Genetic distance analysis based on SRAP markers

The number of amplified bands in 45 alfalfa materials from thirty-six primer combinations ranged from 4 to 9 with an average of 5.33 bands per primer pair. Table 2 lists the polymorphism of thirty-six SRAP primer pairs. Among them, primer F1R1 and primer F5R4 regulated the largest number of amplified bands. The number of polymorphic bands varied from 1 to 7, and the highest polymorphic bands were generated from primers F1R1. The overall mean for polymorphism bands was 2.89. The highest percentage of polymorphic loci was 100% in primers F1R2, F3R2 and F6R6, followed by F6R2 (83%), with a mean of 51.81% polymorphic loci per primer. The polymorphism information content (PIC) values ranged from the lowest 0.69 (F3R2) to the highest 0.98 (F2R4), with an average of 0.88. For all thirty-six primer pairs, PIC was greater than 0.5, indicating that all amplified polymorphic sites have high polymorphism. Shannon's information index (I)ranged from 0.47 (F3R2) to 0.03 (F2R4), and the genetic diversity of alfalfa material amplified by primer F3R2 was relatively large. Similar results were also reported by Singh (2016), who assessed the genetic diversity of rice using Shannon's information index and the percentage of polymorphic bands (PPB), where a greater Shannon's information index (I) indicates a greater difference in genetic diversity.

The sample 199 bands amplified from the SRAP data were used to assess the genetic distance among the 45 alfalfa accessions (Additional file 1: Table S2). Pairwise genetic distances between the 45 accessions ranged from 0.03 to 0.25. The cluster analysis grouped the 45 accessions into four groups, as shown in Figure 1. Group-I was comprised of 6 accessions, followed by 32, 5, and 2 accessions respectively in group-II, III, and group-IV. Group-I included XJ4k, CY4k, CY2k and CY4b. Germplasm material from the same variety with different fertility and ploidy clustered together, showing allows that clustering results were not affected by the fertility and ploidy (Rao et al. 2014). *Medicago falcata* (No.10) and *Medicago sativa* (No.11) from Xinjiang City formed the second group (II). The third group (III) consisted of 5 wild diploid *Medicago falcata* (Nos. 7, 8, 9, 12, and 13) from Xinjiang City. The rest of the materials from Hohhot City, Hulunbeier, Xilingol League, and other places were clustered in the group-IV. Those that clustered into the same group also displayed closer genetic proximity in the cluster map. Geographical, ecological, climate and reproductive isolation have a material effect on the level of genetic diversity (Kumar et al., 2009).

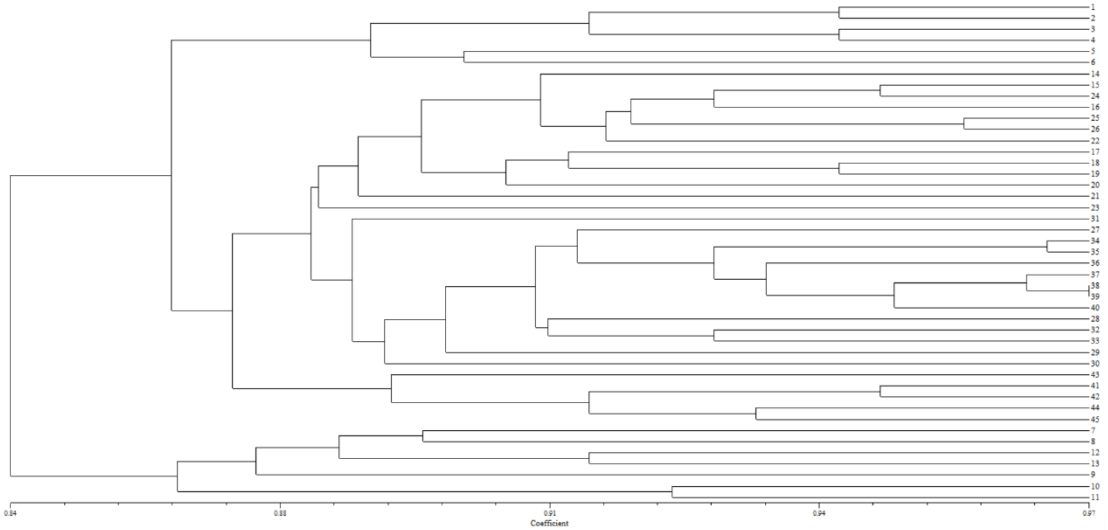

Figure 1. SRAP clustering of 45 *Medicago* materials

Table 2. Polymorphism of 36 SRAP primer pairs

Primer	Amplified bands	Polymorphism bands	Polymorphic loci rate	PIC	Shannon's information index
F1R1	9	7	77.78%	0.8213	0.2856
F1R2	5	5	100.00%	0.8847	0.2278
F1R3	4	1	25.00%	0.8756	0.1727
F1R4	4	2	50.00%	0.9091	0.1518
F1R5	4	3	75.00%	0.8608	0.2191
F1R6	6	4	66.67%	0.8104	0.2927
F2R1	5	3	60.00%	0.9056	0.1718
F2R2	6	2	33.33%	0.8601	0.2032
F2R3	3	1	33.33%	0.9461	0.1000
F2R4	4	1	25.00%	0.9891	0.0267
F2R5	4	2	50.00%	0.7773	0.3186
F2R6	8	4	50.00%	0.8654	0.2167
F3R1	5	3	60.00%	0.9269	0.1401
F3R2	6	6	100.00%	0.6912	0.4686
F3R3	6	3	50.00%	0.9065	0.1573
F3R4	5	2	40.00%	0.8301	0.2461
F3R5	6	4	66.67%	0.8279	0.2678
F3R6	5	1	20.00%	0.9415	0.0936
F4R1	6	1	16.67%	0.9259	0.1061
F4R2	5	2	40.00%	0.8997	0.1515
F4R3	5	3	60.00%	0.8961	0.1737
F4R4	5	2	40.00%	0.9079	0.1562
F4R5	5	1	20.00%	0.9605	0.0698
F4R6	7	4	57.14%	0.7994	0.2915
F5R1	7	2	28.57%	0.9404	0.1018
F5R2	7	3	42.86%	0.8914	0.1730
F5R3	7	3	42.86%	0.9094	0.1467
F5R4	9	5	55.56%	0.8617	0.2265
F5R5	7	2	28.57%	0.9263	0.1178
F5R6	5	1	20.00%	0.9474	0.0864
F6R1	4	2	50.00%	0.8178	0.2709
F6R2	6	5	83.33%	0.9598	0.0759
F6R3	5	4	80.00%	0.7242	0.4133
F6R4	6	4	66.67%	0.8100	0.3060
F6R5	4	2	50.00%	0.9027	0.1591
F6R6	4	4	100.00%	0.8469	0.2421
mean	5.53	2.89	51.81%	0.8766	0.1952

Seed setting rate of hybrid combinations

Successful crosses between diploids × tetraploids and tetraploids × tetraploids were conducted in addition to diploid × diploid crosses. Results are presented in Table 3 for pollination efficiency (%), seeds per pod and relative setting rate (%). Among the cross-pollinated 27 parental combinations, the combination of CY4k×HH10k had the highest outcrossing seed setting rate (87.18%) and mean number of seeds per pod. The average relative seed setting rate of tetraploid alfalfa interspecific and intraspecific hybrids was 63.86%. The average relative seed setting rate of diploid alfalfa interspecific and intraspecific hybrids was 52.16%. Cluster analysis showed that 7 hybrid combinations with high mating ability are clustered into category 1, including the parents of 5 hybrid combinations that are tetraploid. The tetraploid as a female parent with diploid as a male parent were assembled in 10 hybrid combinations, while 6 hybrid combinations did not produce pods (Figure 2). The relative seeding rate of all other combinations was 22.95%-78.21%, with an average value of 38.3%. Four hybrid combinations with diploid as a female parent and tetraploid as a male parent produced pods, and the relative seed setting rate was 53%-88.44%, with an average value of 69.86%. These results show that for parents of the same ploidy level analysis, the mating ability of the hybrid combination in which both parents were tetraploid was greater than that in which both parents were diploid. For parents of the different ploidy level analysis, the mating ability of alfalfa hybrid combinations with a diploid as female parent was better than that of alfalfa hybrid combinations with a tetraploid as female parent.

Table 3. The seed setting rate of 27 hybrid combinations

Ploidy groups	Parental combination		Seed	Seeds per pod	Relative seed
	Female	Male	%	N	%
4x x 4x	XJk^4	HH10k^4	61.54±8.88bc	1.9±0.12a	43.79±4.13g
		ZH11k^4	53.85±7.69c	1.13±0.13bc	25.98±3.02h
	HH6k^4	HH10k^4	15.38±4.44d	1.13±0.03bc	83.15±4.58ab
		ZH11k^4	20.51±2.57d	1.23±0.03bc	90.29±2.30a
	CYk^4	HH10k^4	87.18±5.13a	2.17±0.19a	82.00±5.90ab
		ZH11k^4	53.85±7.69c	1.47±0.20b	57.96±13.70defg
	CYb^4	HH10k^4	74.36±2.56ab	1.17±0.03bc	87.99±4.00ab
		ZH11k^4	87.18±5.13a	1.13±0.09bc	84.87±0.97ab
2x x 2x	XJk^2	HH7k^2	48.72±2.57c	1.13±0.09bc	51.24±4.91efg
		HH9k^2	48.72±9.25c	1.34±0.20bc	60.83±9.58def
		HH28k^2	28.21±5.13d	1.1±0.06bc	49.49±1.98efg
		HH29k^2	15.38±4.44d	1.03±0.03c	47.08±5.41fg
		HH23k^2	0	-	-
4x x 2x	XJk^4	HH7k^2	25.64±2.56d	1±0c	22.95±0.77h
		HH9k^2	0	-	-
		HH28k^2	0	-	-
		HH29k^2	23.08±4.44d	1.23±0.23bc	28.26±5.25h
		HH23k^2	23.08±0d	1.03±0.03c	23.77±1.56h
	CYb^4	HH7k^2	0	-	-
		HH9k^2	0	-	-
		HH28k^2	0	-	-
		HH29k^2	0	-	-
		HH23k^2	17.95±6.79d	1.03±0.03c	78.21±5.78abc
2x x 4x	XJk^2	HH10k^4	25.64±6.78d	1.47±0.09b	65.86±1.76cde
		ZH11k^4	17.95±2.57d	1.17±0.09bc	53.00±6.23efg
	CYk^2	HH10k^4	23.08±4.44d	1.33±0.17bc	88.44±3.01ab
		ZH11k^4	17.95±5.13d	1.07±0.07bc	72.14±6.06bcd

Note: XJ: *M. sativa* L. 'Xinjiang Daye'; CY: *M. varia* Martin. 'Caoyuan No.1'; HH: *M. falcata* L.; ZH: *M. sativa* L.*;* k^4: Fertile tetraploid; b^4: Male sterile tetraploid; k^2: Fertile diploid; Different letter in the same line represent significant differences (P<0.05).

Chromosome ploidy has a direct impact on the plant compatibility. In the present study, we compared compatibility between alfalfa of the same ploidy level as well as between alfalfa of different ploidy levels. In terms of compatibility, combinations were ranked from largest to smallest as follows: diploids × tetraploids, tetraploids × tetraploids, diploids × diploids, tetraploids × diploids. Cross affinity of different ploidy has been reported in different plant species, but there were great differences in results of cross-compatibility due to different cultivars. Our research shows that the mating ability of hybrid combinations in which both parents were tetraploid was greater than those in which both parents were diploid. These results parallel those of a study conducted by He et al. (2019), which might be an effect of the abnormal behavior of chromosomes during meiosis of diploid megaspore mother cell (MMC), but this will require further study. The mating ability of alfalfa hybrid combination with diploid as female parent is better than that of alfalfa hybrid combination with tetraploid as female parent. These results are in agreement with those obtained by Jing et al. (2021), which determined that diploids are more suitable as female parents by analyzing differences between different ploidy levels in red clover (*Trifolium pratense*. L). However, interspecific hybrids with different ploidy levels often manifest hybrid incompatibilities that cause reproductive isolation between species. Wang et al. (2016) studied the affinity of different ploidy hybrids of myrtle (*Myrtus communis* L.), and showed that in the hybridization of diploids and tetraploids, the tetraploid had higher affinity as the female parent, which is inconsistent with the results of this study.

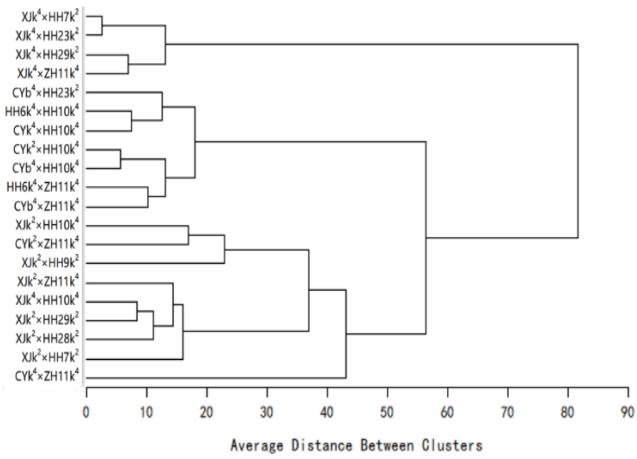

Figure 2. Cluster diagram of relative seed setting rate of 20 hybrid combinations

Note: XJ: *M.sativa* L. 'Xinjiang Daye'; CY: *M. varia Martin*.'Caoyuan No.1'; HH: *M. falcata* L.; ZH: *M. sativa* L.; k4: Fertile tetraploid; b4: Male sterile tetraploid; k2: Fertile diploid.

Hybrid identification and heterosis analysis

True hybrids were obtained from these hybrid offspring using SRAP molecular markers. When hybrids had the

specific locus of the male parent, they could be identified as true hybrids (Huang et al., 2014). 24,16 and 8 F1 seeds were generated from crosses XJk4 x HH10k4, XJk4 × ZH11k4, and XJk2 × HH7k2 respectively, of which 13 (XJk4 x HH10k4) ,14 (XJk4 × ZH11k4) and 7 (XJk2 × HH7k2) true hybrids were identified using the F6R6 primer combination (Figure 3). All hybrid progeny obtained from the remaining 11 hybrid combinations were true hybrids. When male-sterile female parents were used, the offspring were all true hybrids. False hybrids occurred in F1 progeny when male fertile female parents were used. There may be self-pollination during hybridization.

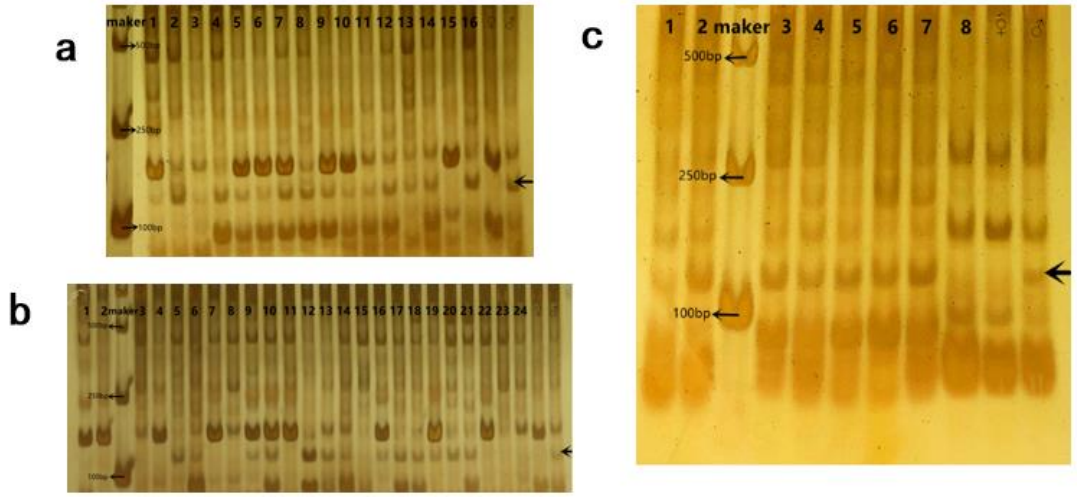

Figure 3. Partial results of identification of hybrids by F6R6. (a) Amplification map of XJk4×HH10k4 hybrid. (b) Amplification map of XJk4×ZH11k4 hybrid. (c) Amplification map of XJk2×HH7k4 hybrid.
Note: →: The location of the male parent special band; ♀: Female parent; ♂: Male parent;
Number: Represents the number of the hybrid offspring.

Mid-parent heterosis was from 0.96 % to-0.95, high-parent heterosis was from 0.88% % to－0.95%, low-parent heterosis was from 1.04% % to－0.93% (Table 4). F1 plants from six different hybrid combinations (1, 2, 4, 8, 10) showed a middle parent advantage and super-parent advantage compared with parental lines, indicating high heterosis levels for their biomass per plant. The biomass per plant of three hybrid combinations (3, 9, 11) was significantly lower than that of both parents. There is no simple linear relationship between heterosis and genetic distance. It has been reported that the exploitation of heterosis can only be maximized by increasing the genetic distance between parental populations (Sang et al., 2020). Stupar et al. (2007) found that the modes of heterosis between different maize plants was similar regardless of the genetic distance between parents. On the contrary, this appears to contribute towards the carrot hybrid yield prediction by estimation of the genetic distances between parents in a study by Jagosz et al. (2011). The effect of genetic distance in predicting heterosis may be related to the selected material and the number of markers used. In this study, genetic distance was used to divide the groups, and partial hybrid combinations with the farther distances had higher yield and heterosis, but this was only estimated in terms of yield. Genetic distance could not be used as a good predictor of alfalfa heterosis, but helped with the initial selection of combinations.

Table 4. Heterosis of biomass per plant in 12 cross combinations

Number	Female parent	Male parent	Hay yield (g)	Mid-parent heterosis (%)	Transgressive higher heterosis (%)	Transgressive lower heterosis (%)
1	XJk^4	HH10k^4	0.132±0.018abc	0.222	0.128	0.333
2		ZH11k^4	0.186±0.040a	0.958	0.879	1.044
3	XJk^2	HH10k^4	0.090±0.028bcde	-0.341	-0.423	-0.231
4		HH7k^2	0.176±0.020a	0.323	0.128	0.6
5		HH9k^2	0.142±0.016ab	0.214	-0.09	0.821
6		HH28k^2	0.063±0.012cdef	-0.36	-0.596	0.537
7		HH29k^2	0.021±0.004ef	-0.759	-0.865	0.167
8	CYk^4	HH10k^4	0.157±0.020ab	0.271	0.208	0.342
9		ZH11k^4	0.024±0.011ef	-0.783	-0.815	-0.736
10	CYk^2	HH10k^4	0.181±0.049a	0.356	0.207	0.547
11	HH6k^4	HH10k^4	0.008±0.001f	-0.944	-0.953	-0.932
12		ZH11k^4	0.113±0.015abcd	-0.134	-0.335	0.242

Note: XJ: *M. sativa* L. 'Xinjiang Daye'; CY: *M. varia* Martin. 'Caoyuan No.1'; HH: *M. falcata* L.; k^4: Fertile tetraploid; k^2: Fertile diploid. Different letters in the same line represent significant differences (P<0.05).

ACKNOWLEDGMENT

This research on molecular identification was developed at the Key Laboratory of Grassland Resources of the Ministry of Education of China. This research was funded by the National Natural Science Foundation of China (32160323), Key Projects in Science and Technology of Inner Mongolia (2021ZD0031) and Inner Mongolia Autonomous Region Science and Technology Program Project (2020GG0063).

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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