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IDENTIFICATION BY SSR AND SRAP MARKERS AND HETEROSIS ANALYSIS OF F1 HYBRIDS (*Medicago ruthenica* L.)

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ABSTRACT

Medicago ruthenica L. could be used as the crossbreeding material in forage crops to provide rich resistance gene resources based on its strong resistance to adversity stress. This study aimed to identify the authenticity and analyze heterosis of the intraspecific hybrids acquired from two Medicago ruthenica L. materials. The results showed 85 true hybrids in 118 F1 progenies identified by one SSR marker and five SRAP markers. Besides, the SRAP markers (13.89%) indicated higher identification efficiency than SSR markers (7.69%), and the rate of true hybrids in HZ population (100%) was higher than that in ZH population (36.54%). The six agronomic traits varied to different degrees, and their variation coefficients ranged from 18.53% to 45.72% in 13 hybrids of ZH population in 2019 and 2020. Moreover, ZH7 and ZH8 presented excellent agronomic performance, which could be used as candidate materials for further research. The mid-parent heterosis (Hm) of all agronomic traits was between -20.55% and 36.46%, and heterobeltiosis (Hh) showed negative values of 13 hybrids.

Key words: Heterosis, intraspecific hybridization, Medicago ruthenica L., molecular marker

INTRODUCTION

Medicago ruthenica L. is a perennial forage in the Medicago family. It has a wide distribution in the grassland of Siberia, Mongolia, and northern China, due to its remarkable ability to resist cold and drought under unfavorable environmental conditions (Campbell et al., 1997). It is of great significance to maintain grassland health and realize the sustainable development of grassland. However, it is urgent to breed high yield and good quality cultivars of Medicago forage from Medicago ruthenica L., which could provide abundant and diverse genetic resources.

Crossbreeding is an effective method to screen the offspring with excellent performance. The morphological characteristics, such as plant height, seed size, and leaf length, have rich genetic diversity in different Medicago ruthenica L. resources (Shi et al., 2009; Li et al., 2012), which has the potential advantages for breeding and domesticating cultivated varieties. Due to its strong resistance to abiotic stress, Medicago ruthenica L. and Medicago sativa L. 'zhaodong' were used as parents to obtain Medicago sativa L. 'Longmu 801' with strong cold resistance (Liu et al., 2015), disease resistance (Ma et al., 2017), and drought resistance(Liu et al., 2009). However, the intraspecific hybridization research was limited in Medicago ruthenica L., and studies on agronomic traits performance needed to be strengthened in their hybrids.

Identifying hybrids is a primary and necessary concern in developing conservation and management strategies after crossbreeding. Besides, it is essential to verify the accuracy of hybrids at the molecular level, especially for the hybrids obtained from intraspecific hybridization. It has been developed different kinds of molecular markers to identify hybrids, such as RAPD (Random Amplification Polymorphic DNA) (Hashemi et al., 2009), SSR (Simple Sequence Repeat) (Saxena et al., 2010), and other DNA markers based on sequencing (Du et al., 2010; Havelka et al., 2017). Sequence Related Amplified Polymorphism (SRAP) has the advantages of good repeatability, high polymorphism, and uniform distribution in the genome, which has been widely used in various research, including genetic diversity (Guenni et al., 2016; Zheng et al., 2017), construction of a genetic map (Liang et al., 2015), DNA fingerprinting analysis (Qi et al., 2010), as well as hybrids identification.

The phenomenon of heterosis has proved to be an important genetic tool in evaluating agronomic traits of hybrids (Patel et al., 2012). Heterosis analysis has been widely reported in alfalfa breeding studies to improve their production and quality. (Xue et al., 2015; Cai et al., 2013). Medicago ruthenica L. Sojak 'zhilixing' is a cultivar with

strong resistance, good feeding value and high yield (Shu et al., 2018; Campbell and Xia, 2002; Li, 2007), which has been introduced to the alpine region. Compared with the wild Medicago ruthenica L., Medicago ruthenica L. Sojak 'zhilixing' has a relatively high forage yield. And there were differences in their plant natural height, leaf length, and flower color. The current study developed the non-castration hybridization and acquired F1 hybrid populations from crossing Medicago ruthenica L. Sojak 'zhilixing' with wild Medicago ruthenica L.. We determined the authenticity of hybrids and analyzed their agronomic traits growth in the first and second years. The results of their agronomic traits could guide agricultural production, and some hybrids could be selected as candidate materials for future molecular breeding, genetic map construction, and QTL mapping.

MATERIALS AND METHODS

Plant material

The F1 hybrid populations were named as ZH population (Medicago ruthenica L. Sojak 'zhilixing'♀ × wild Medicago ruthenica L.♂) and HZ population (wild Medicago ruthenica L. ♀× Medicago ruthenica L. Sojak 'zhilixing' ♂), which included 52 individuals and 66 individuals, respectively. The parents grew in an experimental field in Hohhot, China (Figure 1). Hohhot (Latitude 40°83' and Longitude 111°73') is located in the central part of Inner Mongolia and dominated by a temperate continental monsoon climate. The pollination was performed at 9:00~11:00 a.m. during the blossoming period in 2017 and 2018. After removing the unhealthy florets of female parent, three florets were retained for pollination without emasculation. The pollinated flowers were covered with thin cottons for defensive and protective functions. The cottons were removed on the next day, and the seeds were harvested when they were ripe.

Figure 1. The photos of hybrid parents
Note: The left is wild *Medicago ruthenica* L., and the right is *Medicago ruthenica* L. Sojak 'zhilixing'.

The HZ population was planted in pots in April 2018 and transplanted into an experimental field in July 2018. The parents and ZH population were planted in pots in April 2019 and transplanted into a different experimental field in July 2019. Each individual was spaced 50cm apart from others. The leaves of parents and F1 populations were used for molecular identification, and the true hybrids of ZH population and parents were used to determine of agronomic traits.

DNA extraction and detection

The total genomic DNA was extracted by using plant genomic DNA kit DP305 (TianGen Biotech, Beijing) following the manufacturer's instructions. DNA concentration was determined by NanoDrop 2000. DNA integrity was evaluated by agarose gel electrophoresis. The DNA samples were diluted to 40 ng μL-1 and stored at -20℃.

SSR analysis

Thirteen pairs of primers were used for SSR analysis (Table 1). The PCR reaction was 20 μL containing 1 μL DNA, 1 μL forward primer (10μM), 1 μL reverse primer (10μM), 10 μL 2×Taq PCR MasterMix (TianGen Biotech, Beijing), and 7 μL ddH2O. PCR was performed by 2720 thermal cycler (Applied Biosystems, USA) and programmed for initial denaturation of 2 min at 94℃; 30 cycles of 45 s of denaturing at 94℃, 40 s of annealing at the specific annealing temperature, and 45 s of elongation at 72℃; final extension at 72℃for 7 min (Wu et al., 2015; Wang et al., 2016). The PCR products were fractionated by electrophoresis on 8% polyacrylamide gel in 1×TBE buffer at 12.5 V cm-1 for 1.5 h and stained with AgNO3 (Bassam et al., 1991; Talebi et al., 2011).

Table 1. SSR primer information

Primer name	Primer sequence (5'to3')		Annealing temperature
	Forward sequence	Reverse sequence	
W6018	AGC AGG ATT TGG GAC AGT TGT	ACC GTA GCT CCC TTT TCC A	55℃
Alf1	CTT GGA ACT ATT GTT GAG T	ACC GTT TCC CAA AAC ATA CTT	50℃
Alf4	GGG GAT TCT TGA ATA GAT G	TGG TTC GCT GTT CTC ATG	50℃
A1f2	TTT TCC CAC CTC ATT AG	TTG AGA TTC AAA GGG TTA C	46℃
A1f3	CCC ATC AAC ATT TTC A	TTG ATT GGA ACG AGT	43.2℃
W6002	CAT ATT GTT AGA TTT GTG G	GTG AGC GTT AAG TTG GTA GAG	45℃
W6007	GAT TTG GGC CTC ATT CCT TCT TGT	CCT GAA GGG GGA AAA TTG CCC AC	58℃
W6019	TGG AAT TTG GGA TAT AGG AAG	GCC ATA AGA ACT TCC ACT T	49.2℃
AFca1	CGT ATC AAT ATC GGG CAG	TGT TAT CAG AGA GAG AAA GCG	51℃
AFca11	CTT GAG GGA ACT ATT GTT GAG T	AAC GTT TCC CAA AAC ATA CTT	52℃
MTR58	GAA GTG GAA ATG GGA AAC C	GAG TGA GTG AGT GTA AGA GTG C	52℃
AFctt1	CCC ATC ATC AAC ATT TTC A	TTG TGG ATT GGA ACG AGT	49.5℃
MTLEC2A	CGG AAA GAT TCT TGA ATA GAT G	TGG TTC GCT GTT CTC ATG	50℃

SRAP analysis

Six forward primers and six reverse primers were paired randomly for a total of 36 combinations for SRAP analysis (Table 2, Table 3) (Devran and Baysal, 2012). The PCR reaction was the same as that in the SSR analysis. The PCR was carried out with the following conditions: initial denaturation at 94℃ for 10 min; 5 cycles of 1 min of denaturing at 94℃, 1 min of annealing at 35℃ and 2 min of elongation at 72℃; 30 cycles with annealing temperature at 50℃; elongation at 72℃ for 7 min. The PCR products were followed the same procedure as above SSR analysis.

Table 2. Information of SRAP primer

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

Table 3. Information of SRAP combinations

Primer number	Primer combination	Primer number	Primer combination	Primer number	Primer combination
FR1	F1R1	FR13	F1R5	FR25	F5R5
FR2	F1R2	FR14	F2R5	FR26	F6R1
FR3	F1R3	FR15	F3R5	FR27	F6R2
FR4	F1R4	FR16	F4R1	FR28	F6R3
FR5	F2R1	FR17	F4R2	FR29	F6R4
FR6	F2R2	FR18	F4R3	FR30	F6R5
FR7	F2R3	FR19	F4R4	FR31	F1R6
FR8	F2R4	FR20	F4R5	FR32	F2R6
FR9	F3R1	FR21	F5R1	FR33	F3R6
FR10	F3R2	FR22	F5R2	FR34	F4R6
FR11	F3R3	FR23	F5R3	FR35	F5R6
FR12	F3R4	FR24	F5R4	FR36	F6R6

Agronomic traits analysis

The plant absolute height, natural height, number of primary branches, leaf length, leaf width, and plant above-ground biomass of parents and 13 F1 hybrids in ZH population were measured in September 2019 and June 2020, respectively. Approximate leaf area was the product of leaf length and width. Plant type index was the ratio of plant natural height to absolute height. Variation coefficient (%) was the ratio of standard deviation to mean value. Five

biological replicates were randomly selected in parents. The temperature information was shown in Table 4 at growing periods in 2019 and 2020. All the materials were consistent in field management.

Data analysis

EXCEL 2007 was used for heterosis analysis. Mid-parent heterosis (Hm) was determined based on percent increase or decrease of mean value in hybrids against their mid-parent value. Hm = (F1-MP)/MP×100%, where MP was the mid-parent value. Heterobeltiosis (Hh) was calculated based on percent increase or decrease of mean value in hybrids over their better parent value (Abro et al., 2009). Hh = (F1-BP)/BP×100%, where BP was the better parent value.

RESULTS AND DISCUSSION

Screening of molecular markers and identification of F1 populations

Six molecular markers were selected with different loci in parents (Figure 2), including Alf3, which was screened from 13 SSR markers, and FR4, FR9, FR25, FR28, as well as FR36 which were screened from 36 SRAP combinations. The identification efficiency of SRAP combinations (13.89%) was higher than SSR markers (7.69%). When hybrids had the specific locus of male parent, they could be identified as true hybrids (Xue et al., 2009). There were 19 true hybrids in ZH population identified by Alf3, FR4, and FR28. The HZ population was all true hybrids identified by FR9, FR25, and FR36. There were a total of 85 true hybrids in 118 individuals of two F1 populations.

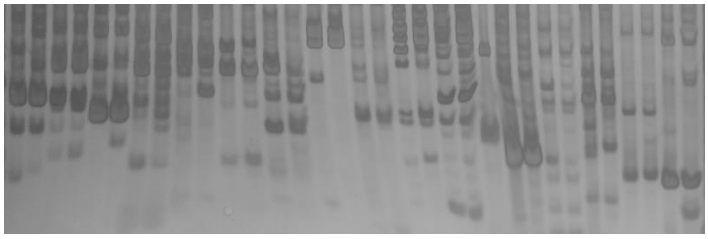

Figure 2. The part results of selection of SRAP and SSR markers in parents
Note: Two swim lanes represent a molecular marker; the former swim lane is *Medicago ruthenica* L. Sojak 'zhilixing' and the latter is wild *Medicago ruthenica* L..

The rate of true hybrids in ZH population (36.54%) was much lower than that in HZ population (100%). The result was similar to Hong et al. (Hong et al., 2012), who found different rates of true hybrids in reciprocal Arachis Hypogaea L. F1 populations. The corolla color of Medicago ruthenica L. Sojak 'zhilixing' is yellow at front and purplish yellow abaxially, while wild Medicago ruthenica L. has yellow corolla at front and back (Li et al., 2015). Therefore, the internal factors and regulatory mechanisms of the two parent flower may be different, which may contribute to the difference of the true hybrid rates. We will perform further study to verify it. New loci were detected in hybrids, which were different from the parents, indicating that the crossbreeding caused gene recombination (Figure 3). Similar results were observed in Zoysia (Xue et al., 2009) and rice hybrids (Hashemi et al., 2009), which also had abundant variation in hybrids.

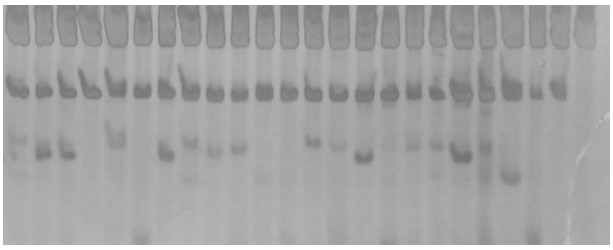

Figure 3. The part results of identification of HZ population by FR9
Note: the last swim lane is wild *Medicago ruthenica* L.; the penultimate is *Medicago ruthenica* L. Sojak 'zhilixing'; the rest are F_1 hybrids.

Performance of different agronomic traits in hybrids of ZH population

The 13 individuals of ZH population had large variation in many agronomic traits (Table 5). The variation coefficients varied to different degrees ranging from 18.93% to 45.72% in 2019 and from 18.53% to 22.48% in 2020. There were relatively high variation coefficients of plant above-ground biomass in 2020 and leaf-stem ratio in 2019, which were 45.72% and 36.68%, respectively. The number of primary branches also showed a relatively high variation coefficient, which was similar to the results in hybrid progenies of alfalfa cultivars (Wei et al., 2009). The variation of these agronomic traits is significant for improving forage yield.

Table 5. Performance of different agronomic traits in F_1 hybrids of ZH population

F_1 hybrid	Number of primary branches			Approximate leaf area (cm^2)			Plant type index			Leaf-stem ratio			Plant absolute height (cm)			Plant above-ground biomass (g)		
	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean
ZH1	8	17	12.5	0.98	1.31	1.145	0.19	0.22	0.205	0.96	0.53	0.745	103.5	90	96.75	41.52	118	79.76
ZH2	8	18	13	1.49	1.12	1.305	0.4	0.5	0.45	0.94	0.69	0.815	79.4	62	70.7	27.35	95.81	61.58
ZH3	8	15	11.5	0.91	0.88	0.895	0.32	0.37	0.345	1.17	0.54	0.855	69.3	60	64.65	13.13	86	49.565
ZH4	8	18	13	0.99	0.93	0.96	0.29	0.33	0.31	0.5	0.42	0.46	91.3	80	85.65	37.06	103	70.03
ZH5	7	14	10.5	1.39	1.51	1.45	0.34	0.34	0.34	0.73	0.53	0.63	87.9	62	74.95	38.82	90	64.41
ZH6	8	16	12	1.21	1.81	1.51	0.48	0.4	0.44	0.51	0.66	0.585	89.2	65	77.1	40.83	48.97	44.9
ZH7	13	20	16.5	1.12	1.22	1.17	0.38	0.43	0.405	0.35	0.44	0.395	111.3	94	102.65	68.84	109	88.92
ZH8	12	19	15.5	1.62	1.52	1.57	0.42	0.44	0.43	0.5	0.73	0.615	78.2	64.5	71.35	41.46	90.48	65.97
ZH9	6	13	9.5	1.52	1.38	1.45	0.35	0.4	0.375	0.81	0.64	0.725	82.9	58	70.45	28.04	90	59.02
ZH10	9	19	14	1.42	1.45	1.435	0.32	0.36	0.34	1.21	0.61	0.91	72.8	55	63.9	26.69	84	55.345
ZH11	6	14	10	1.08	1.14	1.11	0.29	0.34	0.315	1.13	0.56	0.845	66.3	50	58.15	21.98	77	49.49
ZH12	9	18	13.5	1.39	1.24	1.315	0.23	0.31	0.27	0.54	0.39	0.465	104.3	90	97.15	31.78	116	73.89
ZH13	4	7	5.5	1.1	1.03	1.065	0.47	0.46	0.465	0.96	0.56	0.76	33.8	52	42.9	9.51	72	40.755
Mean	8.15	16	12.075	1.25	1.27	1.26	0.35	0.38	0.365	0.79	0.56	0.675	82.32	67.88	75.1	32.85	90.79	61.82
Variation coefficient (%)	29.13	21.8	25.465	18.93	20.76	19.845	24.85	19.4	22.125	36.68	18.53	27.605	24.36	22.48	23.42	45.72	20.72	33.22

Table 6. Heterosis and Heterobeltiosis for different agronomic traits in ZH population

Population	Number of primary branches			Approximate leaf area (cm^2)			Plant type index			Leaf stem ratio			Plant absolute height (cm)			Plant above-ground biomass (g)		
	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean	2019	2020	Mean
P_1	10.31	16.25	13.28	1.38	1.22	1.3	0.38	0.58	0.48	0.57	0.47	0.52	90.45	82.02	86.235	105.61	129.47	117.54
P_2	9.13	19	14.065	0.59	0.52	0.555	0.25	0.29	0.27	0.73	0.93	0.83	65.5	44.08	54.79	37.85	31.06	34.455
F_1(Mean)	8.15	16	12.075	1.25	1.27	1.26	0.35	0.38	0.365	0.79	0.56	0.675	82.32	67.88	75.1	32.85	90.79	61.82
Hm(%)	-16.12	-9.22	-12.67	26.74	46.18	36.46	10.9	-14.15	-1.625	21.95	-19.72	1.115	5.58	7.67	6.625	-54.21	13.11	-20.55
Hh(%)	-20.9	-15.79	-18.345	-9.73	4.24	-2.745	-7.96	-35.65	-21.805	8.5	-39.84	-15.67	-8.98	-17.23	-13.105	-68.9	-29.88	-49.39

Note: P_1 is *Medicago ruthenica* L. Sojak 'zhilixing'; P_2 is wild *Medicago ruthenica* L..

The performance of agronomic traits in the second growth year was greater than that in the first year, which was consistent with the reports of Onat et al. (2017) and Ilker et al. (2018). Plant above-ground biomass and absolute height showed more excellent performance than plant type index and leaf-stem ratio in ZH1 and ZH12. However, the plant type index affects forage yield and agricultural management conditions. Improving plant type index could realize high yield at the unit area (Salek and Fakhrvaezi, 2011). Therefore, ZH7 and ZH8 can be used as candidate materials for further research based on their comprehensive performance in 2019 and 2020.

Heterosis analysis of ZH population

The Hm and Hh were characterized by negative values of 13 individuals in ZH population. The Hm was from -54.21% to 26.74% in 2019 and from -19.72% to 46.18% in 2020, while the Hh was from -68.90% to 8.50% in 2019 and from -39.84% to 4.24% in 2020 (Table 6). There was no apparent heterobeltiotic effect, which was an important factor restricting their yield improvement (Zhang et al., 2010). Rajeev et al. (2018) also found the negative heterosis in interspecific hybrids of cotton, which indicated that non-additive genes dominated in these agronomic traits under genetic control. Many different loci in parents contributed to the positive heterosis in F1 hybrids (Peng et al., 2013; Zhang et al., 2016; Chen et al., 2009). It has been reported that farther relationship the crossing parents had, the better heterosis effect observed in hybrids (Jiang et al., 2017; Zhao et al., 2011). However, the crossing parents both belonged to Medicago ruthenica L. in this study, and they were intraspecific hybridization. Therefore, the close relationship of parents could result in no apparent heterobeltiotic effect in F1 hybrids. Only six specific loci were identified from 13 SSR markers and 36 SRAP markers in parents, and the low ratio of specific loci may be related to their negative heterosis.

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