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Full Length Research Paper

Genetic relationships among alfalfa gemplasms resistant to common leaf spot and selected Chinese cultivars assessed by sequence-related amplified polymorphism (SARP) markers

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Genetic relationships among 26 alfalfa cultivars, of which, 12 were of high resistance to common leaf spot (CLS), were assessed using sequence-related amplified polymorphism (SRAP) markers. 34 SRAP primer combinations were selected for fingerprinting of these cultivars and a total of 281 bands were observed, among which 115 were polymorphic (40.93%). Based on molecular data, 26 cultivars were classified into 5 groups. Group I included 12 Chinese cultivars, most of which had a low CLS resistance and were planted in cold and/or drought region in China, while 10 of 11 cultivars with a high CLS resistance were put in group II or group IV respectively. Furthermore, the clustering pattern was, on the whole, consistent with their CLS resistance or geographic origins. In addition, there was a low genetic diversity among alfalfa cultivars from China. In conclusion, SRAP markers may serve as a quick tool to analyze the genetic relationships and genetic diversity among alfalfa cultivars in conjunction with DNA-bulking method. The information produced by this study on the genetic relationships and genetic diversity among 26 cultivars could be useful to select parents in a CLS resistance breeding program of alfalfa.

Key words: Lucerne, SRAP, *Medicago sativa*, common leaf spot, genetic relationships

NTRODUCTION

Alfalfa (*Medicago sativa*) is the most important forage species globally in temperate climates (Barnes et al., 1988) and the most important forage legume in China. The mainly cultivated alfalfa belongs to two sub-species of this species, *M. sativa subssp. sativa* and *M. sativa subssp.× varia*, and is the autotetraploid that is naturally outcrossing, and thus is susceptible to inbreeding depression. So, the great majority of alfalfa cultivars are synthetic populations that have been developed from successive generations of random mating of selected clones and their progeny. The complex genetic nature of alfalfa makes breeding for improved yield very difficult.

A foliar disease called common leaf spot (CLS), is one of the most serious diseases occurring on alfalfa throughout the world. It is caused by the fungus *Pseudopeziza medicaginis* (Lib.) Sacc. CLS usually not only causes substantial yield losses but also affects forage quality by reducing carbohydrate and protein content (Mainer and Leath,1978; Morgan and Parbery, 1980; Hwang et al., 2006), with reduced protein levels generally having the greatest negative impact on feed value. Yuan and Zhang (2000) evaluated the CLS resistance in 250 alfalfa populations representing an extensive geographic origin and found that few of cultivars from China were of high resistance to CLS. It is an effective method which

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improves the resistance to CLS of Chinese cultivars using cultivars with high resistance to CLS identified by Yuan and Zhang (2000) as breeding parents. However, efficient use of these gemplasm requires accurate characterization of genetic variation within and among populations. Molecular-marker-based genetic diversity assessments of alfalfa cultivars offers a promising approach to desigh more effective strategies to use these gemplasms.

An interesting modified marker technology termed as sequence-related amplified polymorphism (SRAP) (Li and Quiros, 2001) was similar to random amplified polymorphic DNA (RAPD), but it was a preferential random amplification of coding regions in genome. SRAP had been applied extensively in genetic linkage map construction (Li and Quiros, 2001; Yeboah et al., 2007), genetic diversity analysis (Ferriol et al., 2003; Zhao et al., 2009; Yang et al., 2010), and comparative genetics (Li et al., 2003) of different species. In the genetic diversity analysis, the information derived from SRAP marker was more concordant to the agronomic and morphological variability and to the evolutionary history of the morphotypes than that from other molecular markers (Ferriol et al., 2003). Recently, SRAP had been also applied in estimating genetic relationships among alfalfa germplasm and selected cultivars (Vandemark et al., 2006).

The objective of this experiment was to use SRAP markers to estimate genetic relationships among 14 selected alfalfa cultivars (landraces or developed varieties) from China and 12 cultivars with high resistance to CLS originated from other countries in the world. In addition, some of DNA fragments found polymorphic were sequenced and analyzed in order to determine the nature of the amplified fragments using SRAP markers and provide sequence information for developing of gene markers of alfalfa.

MATERIALS AND METHODS

Genetic materials

14 selected alfalfa cultivars from China, 11 cultivars with high resistance to CLS originated from other countries in the world (Table 1) and 1 famous cultivar, “Rambler”, with medium resistance to CLS originated from Canada were used in this study (Yuan and Zhang, 2000). In cultivars from China, 4 belonged to synthetic varieties including “Gannong No.3”, “Zhongmu No. 1”, “Gongnong No. 1” and “Xinmu No. 2” while all other were landraces. The resistant cultivars mostly come from America and Canada, and only 2 originated from England and New Zealand respectively.

DNA isolation

DNA was extracted from the bulked trifoliate leaf tissues of 30 seedlings from each cultivar using the CTAB (cetyltrimethyl-ammonium bromide) procedure of Doyle and Doyle (1990). Finally, the quality and quantity of DNA were analyzed by running 1% agarose gel electrophoresis containing λ-DNA standards.

SRAP assay

All SRAP reactions were performed in 20 µl volume containing 20 ng DNA, 200 µM each dNTP, 1.5 mM $MgCl_2$, 1 unit Taq DNA Polymerase (Takara, Japan) and 0.25 µM of both forward and reverse primers. Amplification was carried out in an Eppendorf Mastercycler Gradient Thermocycler with the PCR program of Li and Quiros (2001). PCR products were resolved by electrophoresis on 2% agarose gel with ethidium bromide and photographed in SYNGENE Automated Gel Documentation System.

Sequencing of SRAP fragments and sequence analyzing

To order to get good sequencing results, part cultivar/primer pairs were separated on 4% denaturing polyacrylamide gels and silver stained. Some of the amplified fragments using SRAP markers were recovered from the dried acrylamide gel and re-amplified. The fragments were then ligated into the pBS-T vector and the recombinant plasmids were transformed into *Escherichia coli,* DH5α. 3 Transformants with insert each recovered fragments were sequenced at Shanghai Sangon Co. Ltd. Sequence similarity searches were performed at GeneBank database (http://www.ncbi.nlm.nih.gov) and *Medicago truncatula* Database (http://www.jcvi.org/), with the program BLASTN or BLASTX.

Data analysis

SRAP bands behave as dominant markers, and the band profiles of each primer pair were manually scored for the presence (1), absence (0) or missing (-1) of co-migrating fragments for all cultivars. Only fragments which had a molecular weight ranged from 100 bp to 2000 bp and were of medium or high intensity were considered for data analysis. Polymorphism information content (PIC) provide an estimate of the discriminatory power of a marker and the PIC for each SRAP primer pairs was determined as described by Smith et al. (1997). Pair-wise genetic distances among the studied cultivars were calculated using Jaccard’s genetic dissimilarity coefficient, estimated as $d_{ij} = c / (a+b+c)$, where, d_{ij} is the measure of distance between sample i and j, a is the number of fragments present in i and absent in j, and b is the number of fragments present in j and absent in i, c is the number of shared present fragments by i and j. The resulting pairwise dissimilarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA) using SAS9.0. The 0/1 matrix is available to readers upon request.

RESULTS

SRAP analysis

The selected primers were based on previous reports of Li and Quiros (2001), Budak et al. (2004) and Vandemark et al. (2006). There were 224 sets of primer combinations that were combined by 16 forward primers and 14 reverse primers (Table 2). Based on preliminary test, 34 sets of primer combinations, which steadily produced well-defined and scorable amplification products, showed polymorphisms in all 26 cultivars (Table 3). Figure 1 was the amplification profile of primer combination F14/R9 and it produced the most bands and the most polymorphic bands in 34 primer combinations. A total of 281 bands were observed, among which 115 were

Table 1. the alfalfa materials in this study and their CLS resistance.

Assay number	Cultivar	Species[1]	Gerplasm No. [2]	Origin	Seed source [3]	CLS Reistance [4]	SRAP group
1	Baoding	Ms	0130	Hebei, China	B	MR	I
2	Gannong No.3	Ms	--	Gansu, China	C	--	I
3	Longdong	Ms	--	Gansu, China	C	--	I
4	Xinjiangdaye	Ms	2712	Xinjiang, China	C	MR	III
5	Yongji	Ms	0456	Shanxi, China	B	HS	I
6	Zhongmu No. 1	Ms	2758	Beijin, China	B	--	I
7	Changwu	Ms	0208	Shănxi, China	B	MS	I
8	Gongnong No. 1	Ms	128	Jilin, China	B	MS	I
9	Qianxian	Ms	0060	Shănxi, China	B	MR	I
10	Yanggao	Ms	0133	Shanxi, China	B	MS	I
11	Zihua	Ms	1149	Heilongjiang China	B	MS	I
12	Aohan	Ms	2761	Neimenggu, China	B	MR	I
13	Xinmu No. 2	Ms	2715	Xinjiang, China	B	--	I
14	Weinan	Ms	0932	Shănxi, China	B	MS	II
15	Rambler	Mv	72-10	Canada	B	MR	III
16	Victoria	Ms	--	Canada	A	HR	IV
17	PG sutter	Ms	88-44	America	B	HR	IV
18	Oranga	Ms	84-759	New Zealand	B	HR	IV
19	Glacier	Mv	83-228	Canada	B	HR	V
20	America(1)	Ms	2325	America	B	HR	II
21	America	Ms	0064	America	B	HR	II
22	Spredor No. 2	Ms	85-77	America	B	HR	IV
23	Superstan	Ms	83-130	America	B	HR	II
24	Valor	Ms	83-241	Canada	B	HR	II
25	England(3)	Ms	1872	England	B	HR	II
26	WL202 G3057	Mv	83-128	Canada	B	HR	II

[1]Ms, *Medicago sativa subssp. sativa*; Mv, *Medicago sativa subssp. x varia*. [2] collection code of Institute of Animal Science, Chinese Academy of Agricultural Science. [3] A, Beijing Clover Group; B, Institute of Animal Science, Chinese Academy of Agricultural Science; C, Pratacultural College of Gansu Agricultural University. [4] Yuan and Zhang (2000); HR, high resistance; MR, Medium resistance; MS, Medium susceptibility; HS, High susceptibility.

polymorphic (40.93%), ranging between 1 and 8 per primer combination, with an average of 3.4 bands per primer combination. The size of scored bands ranged from 100 to 2000 bp. The mean of the PIC value over the 34 combinations averaged 1.12, ranging from 0.35 for F8/R10 to 3.36 for F14/R9. In order to assess the reproducibility of the band profiles, two PCR amplifications each primer combination were carried out for the cultivar "Weinan". The results show that 98.93% of the scorable bands were reproducible across two PCR replicates, indicating the SRAP assay was of high reproducibility between PCR replicates.

Genetic relationships

Pairwise comparison was made between all the

Table 2. The primer sequences of SRAP used in this experiment.

Primer	Type	Sequence (5´-3´)
F1	Forward	TGAGTCCAAACCGGATA
F2	Forward	TGAGTCCAAACCGGAGC
F3	Forward	TGAGTCCAAACCGGAAT
F4	Forward	TGAGTCCAAACCGGACC
F5	Forward	TGAGTCCAAACCGGAAG
F6	Forward	TGAGTCCAAACCGGACA
F7	Forward	TGAGTCCAAACCGGACG
F8	Forward	TGAGTCCAAACCGGACT
F9	Forward	TGAGTCCAAACCGGAGG
F10	Forward	TGAGTCCAAACCGGAAA
F11	Forward	GTAGCACAAGCCGGAGC
F12	Forward	GTAGCACAAGCCGGACC
F13	Forward	CGAATCTTAGCCGGATA
F14	Forward	CGAATCTTAGCCGGAGC
F15	Forward	CGAATCTTAGCCGGCAC
F16	Forward	CGAATCTTAGCCGGAAT
R1	Reverse	GACTGCGTACGAATTAAT
R2	Reverse	GACTGCGTACGAATTTGC
R3	Reverse	GACTGCGTACGAATTGAC
R4	Reverse	GACTGCGTACGAATTAAC
R5	Reverse	GACTGCGTACGAATTGCA
R6	Reverse	GACTGCGTACGAATTCAA
R7	Reverse	GACTGCGTACGAATTCAC
R8	Reverse	GACTGCGTACGAATTCAT
R9	Reverse	GACTGCGTACGAATTCTA
R10	Reverse	GACTGCGTACGAATTGTC
R11	Reverse	CGCACGTCCGTAATTAAC
R12	Reverse	CGCACGTCCGTAATTCCA
R13	Reverse	CGTAGCGCGTCAATTATG
R14	Reverse	CGTAGCGCGTCAATTAAC

cultivars included in this study. Genetic dissimilarity coefficient (d_{ij}) calculated from SRAP data varied from 0.300 between “Aohan” and “Yanggao”, to 0.678 between “Rambler” and “Weinan”, with a mean of 0.525. The mean of *dij* of 14 Chinese cultivars (susceptibility group), the mean of *dij* of 12 forage cultivars (resistance group) and the mean of *dij* among the cultivars of these two groups were 0.477, 0.532 and 0.548 respectively.

A dendrogram based on the dissimilarity coefficients of the 26 cultivars was constructed (Figure 2). According to the data of “Cluster History” of SAS (data not shown), when 5 groups became 4 groups in course of joining, both SPRSQ (semipartial R-square) value (from 0.045 to 0.107) and PST2 (pseudo t2) value (from 1.4 to 3.4) had a relatively great increase while the reverse was the fact for RSQ (R-square) value (from 0.327 to 0.220). Moreover, PSF (pseudo F) value for 5 groups was a local maximum (2.6). Therefore, it was appropriate that 26 cultivars were separated into 5 groups (Figure 2). Among them, group I included 12 of 14 Chinese cultivars most of which had a low CLS resistance and were planted in cold and/or drought region in China. 10 of 11 cultivars with a high CLS resistance were put in group II and group IV respectively. Group II included the cultivars adapted to humid environment, for example, “WL202 G3057” which was planted in Canada irrigation soil and “Weinan” which came from humid Guanzhong regions of Shănxi in China. Furthermore, the cultivars in Group IV were more cold-resistance and/or drought-enduring than Group II, for example, “Victory” which adapted to the environmental conditions of northern America.

Sequence analysis of SRAP fragments

In order to determine the nature of the amplified fragments using SRAP markers, 9 randomly selected polymorphic fragments, obtained with different primer combinations, were sequenced and the GC content of 8 (89%) of them was over 35% (Table 4). The results of

Table 3. SRAP primer combinations used in this study and their polymorphism information.

Primer pair	NF[1]	NPF[2]	PFP[3]	PIC[4]
F1/R5	10	5	0.500	1.06
F1/R14	6	2	0.333	0.70
F2/R1	11	1	0.091	0.48
F2/R11	12	3	0.250	1.33
F2/R13	8	6	0.750	1.68
F3/R6	9	5	0.556	1.30
F3/R8	7	3	0.429	1.23
F3/R9	10	5	0.500	1.57
F3/R12	9	4	0.444	1.07
F3/R14	10	2	0.200	0.75
F4/R12	8	3	0.375	0.85
F5/R11	6	4	0.667	1.17
F6/R3	6	2	0.333	0.67
F7/R2	6	2	0.333	0.84
F7/R9	7	4	0.571	1.58
F8/R4	9	5	0.556	1.56
F8/R10	7	2	0.286	0.35
F9/R4	9	2	0.222	0.64
F9/R9	10	3	0.300	0.68
F11/R3	7	3	0.429	0.99
F11/R7	9	3	0.333	1.37
F11/R11	6	3	0.500	1.07
F13/R9	10	2	0.200	0.86
F14/R3	7	3	0.429	1.32
F14/R5	8	3	0.375	1.38
F14/R9	14	8	0.571	3.36
F14/R11	6	4	0.667	1.24
F14/R13	8	5	0.625	1.04
F15/R1	7	2	0.286	0.63
F16/R3	7	2	0.286	0.54
F16/R7	9	4	0.444	1.24
F16/R8	8	3	0.375	1.11
F16/R9	9	5	0.556	1.87
F16/R13	6	2	0.333	0.63

[1] NF, Number of fragments. [2] NPF, Number of Polymorphic fragments. [3] PFP, Polymorphic fragments percent. [4] PIC, polymorphism information content.

BLAST search showed that all of the sequenced fragments shared significant similarity to CDS (coding sequences) or gene sequences stored in the Genbank database and *Medicago truncatula* Database. Furthermore, two fragments, 13-090 and 16-092, showed a high similarity with Polynucleotide transferase of *M. truncatula* and Receptor protein kinase CLAVATA1 precursor of *Ricinus communis* respectively. The DNA sequence information of sequenced SRAP fragments is available to readers upon request.

DISCUSSION

Alfalfa cultivars are genetically heterogeneous and commercial cultivars of alfalfa seed are composed of thousands of plants of different genotypes. Popularly, genetic distance estimates among alfalfa cultivars were carried out by evaluation of individual genotypes within cultivars (Pupilli et al., 2000; Zaccardelli et al., 2003; Flajoulot et al., 2005). However, the studies of Yu and Pauls (1993), Segovia-Lerma et al. (2003) and Vandemark et al. (2006) indicated that the hierarchical patterns of diversity among alfalfa cultivars by using bulk DNA templates were associated with their geographic, subspecific, and intersubspecific hybrid origins. Although, some allelic information was likely lost as a result of DNA bulking from a population perspective, the DNA-bulking method permits sampling of a greater number of

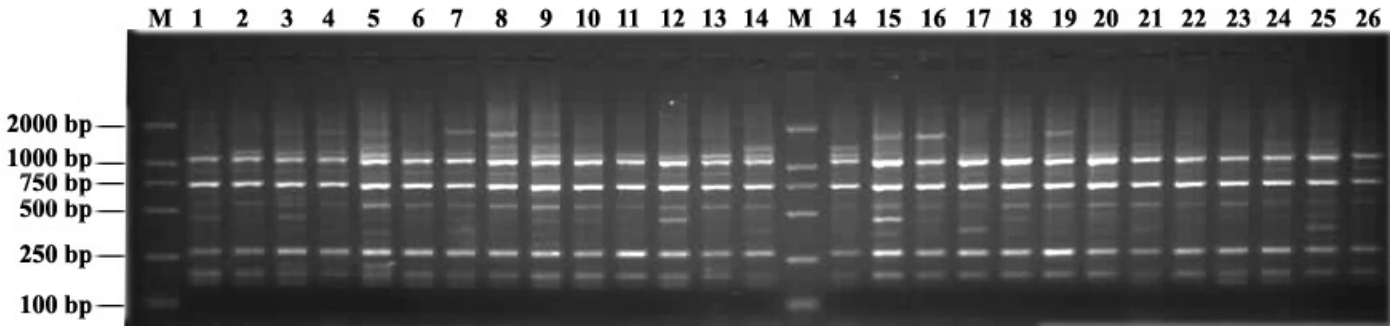

Figure 1. Fingerprint patterns generated using SRAP primer pair F14R9 from the genomic DNA of the 26 alfalfa cultivars. M, DNA molecular weight standard; Codes of lanes corresponding to that of the 26 alfalfa cultivars in Table 1.

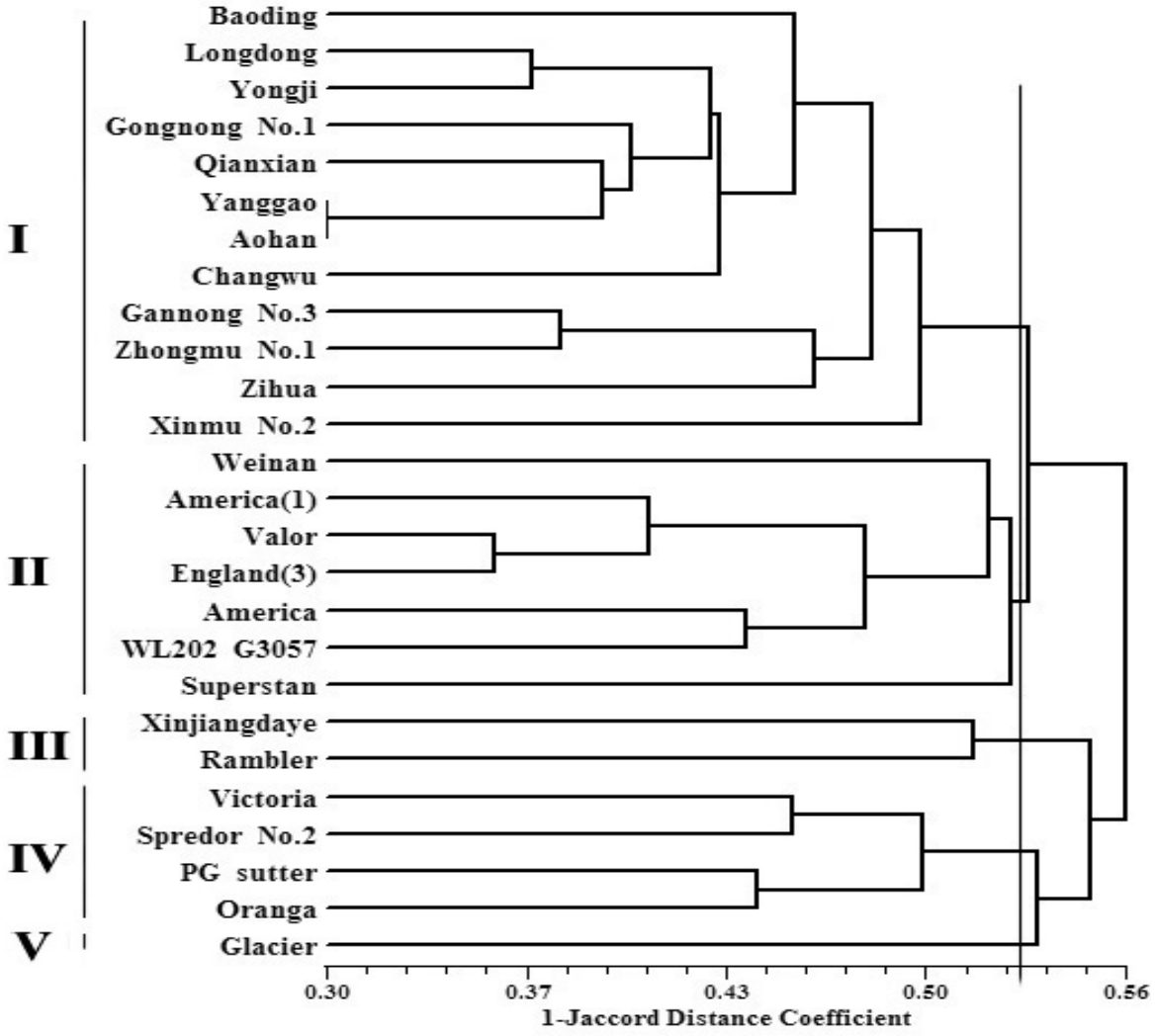

Figure 2. UPGMA dendrogram of 26 alfalfa cultivars based on SRAP markers.

individuals in heterogeneous populations. This approach may more accurately reflect a population's general genetic composition compared with evaluation of fewer individual genotypes. It also permits greater population number to be evaluated with comparable resources. This study used DNA-bulking method in conjunction with

Table 4. Sequence analysis of 9 of SRAP polymorphic fragments isolated from acrylamide gels.

Marker name	Primer pair	Size (bp)	GC (%)	BLAST N/X (database)	Score (bit)	Accession number
03-080	F3R8	485	41.7	BLASTN (refseq_ma)	46.4	gi|NM_001159170
07-020	F7R2	910	45.9	BLASTN (Mt3.0 CDS)	67.3	IMGA|Medtr2g005010
07-092	F7R9	613	41.8	BLASTN (Mt3.0 CDS)	103.0	IMGA|Medtr2g005010
11-030	F11R3	455	36.9	BLASTN (refseq_ma)	69.8	gi|NM_001061890
13-090	F13R9	901	41.1	BLASTX (nr)	228.0	gi|ABN08587
14-092	F14R9	133	48.9	BLASTN (refseq_ma)	37.4	gi|XM_002284796
16-091	F16R9	242	48.4	BLASTN (Mt3.0 CDS)	97.9	IMGA|Medtr5g047120
16-092	F16R9	491	35.2	BLASTX (nr) BLASTN (Mt3.0 CDS)	237.0 356.8	gi|XP_002297907 IMGA|Medtr5g097160
16-093	F16R9	245	33.1	BLASTN (refseq_ma)	37.4	gi|XM_002262976

SRAP to estimate genetic relationships among the studied alfalfa cultivars, and showed a similar result to that of the studies of Yu and Pauls (1993), Segovia-Lerma et al. (2003) and Vandemark et al. (2006). In general, the distances among these cultivars and their clustering pattern were consistent with their CLS resistance or geographic origins. For example, both cultivar pairs “Longdong”/ “Yongji” and “Yanggao” and “Aohan” showed low genetic distance, and this was accordant with resembling environment between their origin regions. Moreover, the UPGMA dendrogram, basically, separated between cultivars with a high CLS resistance and cultivars with a low CLS resistance, and distinguished between Chinese cultivars and cultivars from other countries in the world.

In this study, 12 Chinese cultivars, were classified as a group (Figure 1, group I) and they had a mean distance of 0.455, which was obviously lower than that of other 14 cultivars (0.542), indicating that there was possibly a low genetic diversity among alfalfa cultivars. The main reason for it was that all of these 12 Chinese cultivars came from cold and/or drought regions in north China. Therefore, introducing CLS resistance genes into new varieties and improving genetic diversity should be two important goals in breeding in these regions. Remarkably, 4 cultivars included in group IV not only had a high CLS resistance, but were cold-resistance and/or drought-enduring. Using these cultivars as one of breeding parents could both make easy selecting of ideal plants, which adapt to cold and/or drought environment and have a high CLS resistance, and increase genetic diversity.

The sequenced fragments not only had a high GC content, but also shared significant similarity to reported CDS or gene sequences. This finding confirms that a large proportion of the bands generated by SRAPs include exons in ORFs, which are expected to be evenly distributed along all chromosomes, and agreed with the results reported in previous studies with other species (Li and Quiros, 2001; Ferriol et al., 2003). ORFs may be involved in the agronomic and morphological traits, and thus the information derived from SRAP markers was more concordant to the agronomic and morphological variability. Ulteriorly, this also was made sure by the fact that the information on genetic relationships among 26 alfalfa germplasms produced by this study was well consistent with their CLS resistance or geographic origins. Since the mainly cultivated alfalfa was an autotetraploid, co-dominant markers would be more useful than dominant ones. The nucleotide sequences of SRAP markers sequenced in this study could be use to develop co-dominant markers targeting a gene.

In conclusion, the genetic relationships and genetic diversity information derived from SRAP markers were more concordant to the agronomic and morphological variability than other molecular markers such as amplified fragment length polymorphism (AFLP) and RAPD. Furthermore, SRAP markers may serve as a quick tool to analyze the genetic relationships and genetic diversity among alfalfa cultivars in conjunction with DNA-bulking method. The information of the genetic relationships and genetic diversity among CLS resistance alfalfa germplasms and Chinese cultivars would be useful to select parents in a CLS resistance breeding program of alfalfa. In addition, the molecular tools developed in this study can be applied for characterizing other germplasm collections of alfalfa.

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