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Development and Characterization of SSR Markers and Their Use to Assess Genetic Relationships among Alfalfa Germplasms

C. He, Z. L. Xia, T. A. Campbell, and G. R. Bauchan*

ABSTRACT

Simple sequence repeat (SSR), or microsatellite markers, are codominant, abundant and hypervariable molecular markers from eukaryotic genomes that are being widely used in genetic mapping, phylogenetic studies, and marker-assisted selection. Currently, the number of SSR markers available from alfalfa (*Medicago sativa* L.) genomic libraries is limited. This study was conducted to identify additional SSR markers in the alfalfa genome and to evaluate their ability to separate the nine historically recognized progenitors of North American cultivated alfalfa (African, Chilean, Falcata, Flemish, Indian, Ladak, Peruvian, Turkistan, and Varia), as well as seven additional accessions of *M. sativa* ssp. *sativa*, *falcata,* and *coerulea* and the model legume *M. truncatula*. Genomic DNA from the autotetraploid alfalfa germplasm W10 was used to develop 81 primer pairs, which amplified SSRs containing AC, AT, CT, CTT, GAT, and GGT motifs. The majority (96%) of the primer pairs were functional and 61 (78.2%) detected 2 to 11 polymorphic fragments among the accessions. A dendrogram was constructed using cluster analysis from these data, representing three main clusters: (i) diploid *ssp. falcata*; (ii) *M. truncatula*; and (iii) all remaining entries. Additional separation of some accessions [*M. truncatula* ('Jemalong'), Ladak ('Ladak'), Fall dormancy 11 (UC-1465), Indian (Sirsa Type 9), Flemish ('Dupuit'), Peruvian ('Hairy Peruvian'), and African 2 ('Moapa')] was obtained through multiple correspondence analysis. These genomic alfalfa SSRs have excellent utility for polymorphic assessment with potential application for phylogenetic and genetic mapping studies of alfalfa.

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Abbreviations: AFLP, amplified fragment length polymorphism; CCC, cubic clustering criterion; EST, express sequence tag; GC, Guanine Cytosine; GD, Genetic distances; MCA, multiple correspondence analysis; PCR, polymerase chain reaction; PI, plant introduction; PIC, Polymorphism information content; QTL, quantitative trait loci; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat.

Cultivated alfalfa (*Medicago sativa* L.) is the most important forage crop in North America (Barnes et al., 1988) and is the fourth most widely grown crop in the United States behind corn, wheat, and soybeans (USDA Agricultural Statistics: http://www.nass.usda.gov; verified 9 Sept. 2009). Primary alfalfa breeding objectives include enhancing nutritional value, increasing biomass production, and improving pest resistance. Improving forage quality and yield is difficult because of the complex obligate outcrossing and autotetraploid ($2n = 4x = 32$) nature of cultivated alfalfa. In addition, quality and yield components are conditioned by quantitative trait loci (QTL) that are subject to environmental variation (Robins et al., 2007b), which can make selection for these traits difficult or ineffective without the use of molecular markers (Hospital and Charcosset, 1997). Alfalfa improvement may be facilitated through the use of marker-assisted selection

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since markers are associated with plant genotype instead of plant phenotype, which often is highly influenced by environmental factors (Luo et al., 1997). Molecular markers have been utilized as effective tools for plant breeding (Obert et al., 2000), variety identification (Mengoni et al., 2000), and genome mapping (Echt et al., 1993; Barcaccia et al., 1999; Diwan et al., 2000; Kaló et al., 2000). In addition, these markers can be used for positional cloning of targeted regions in plant genomes (Yan et al., 2003).

Simple sequence repeats (SSRs), or microsatellites, are short tandem repeats of two to five nucleotides of DNA sequences. The number of repeats is highly variable, whereas the regions flanking SSRs are highly conserved (Davierwala et al., 2000; Gur-Arie et al., 2000). SSR markers are polymerase chain reaction (PCR) -based, abundant, codominant, highly reproducible, and are distributed evenly across plant genomes (Powell et al., 1996). Other molecular markers have been developed for alfalfa including restriction fragment length polymorphisms (RFLPs) (Brummer et al., 1993; Tavoletti et al., 1996; Brouwer and Osborn, 1999; Pupilli et al., 2000), random amplified polymorphic DNA (RAPDs) (Kiss et al., 1993; Yu and Pauls, 1993; Brummer et al., 1995), and amplified fragment length polymorphisms (AFLPs) (Obert et al., 2000; Segovia-Lerma et al., 2003). SSR from genomic libraries of alfalfa (Diwan et al., 1997), express sequence tag (EST) (Julier et al., 2003; Eujayl et al., 2004; Sledge et al., 2005; Robins et al., 2007a, 2007b), and bacterial artificial chromosome (BAC) (Choi et al., 2004a, 2004b; Zhu et al., 2005) from *M. truncatula* Gaertn. have been used in genetic mapping of alfalfa and comparative mapping studies of leguminous species.

Akkaya et al. (1992) were the first to determine that SSR existed in plant species and thus have been used extensively in several crop species including beans (*Phaseolus* spp.; Gaitán-Solís et al., 2002), cotton (*Gossypium hersutum* L.; Saha et al., 2003), eggplant (*Solanum aethiopicum* L.; Nunome et al., 2003a, 2003b), soybean [*Glycine max* (L.) Merr.; Cregan et al., 1999], tomato (*Lycopersicum esculentum* Mill.; Smulders et al., 1997; He et al., 2003), and wheat (*Triticum aestivum* L.; Eujayl et al., 2001; Zhang et al., 2002). Diwan et al. (1997) were the first to demonstrate that SSR markers could effectively distinguish among *Medicago* germplasms. Eujayl et al. (2004) developed 455 useful EST-SSR markers from *M. truncatula* EST sequences. SSR markers have been used in phylogenetic analyses to assess genetic diversity among germplasm sources and to distinguish among plant genotypes or species (Mengoni et al., 2000; He et al., 2003; Zhu et al., 2005).

Melton et al. (1990) developed separate populations of the nine historically recognized alfalfa germplasm types: African, Chilean, Falcata, Flemish, Indian, Ladak, Peruvian, Turkistan, and Varia. These populations were developed by intercrossing a number of plants selected from various cultivars and/or plant introductions (PIs). An example of this is the Ladak population, which was developed by intercrossing 22 "Ladak type" cultivars and/or PIs. These nine populations were to be used "for basic studies related to genetic diversity, heterozygosity, and heterosis in alfalfa" (Melton et al., 1990). However, genetic diversity studies of these historical germplasm collections by Kidwell et al. (1994) using RFLPs, and Bauchan et al. (2003) using chromosome banding detected few differences among populations, and the authors concluded that the historical populations had been inadvertently intermated during the seed increase process. To overcome the possibility of using mixed germplasm sources, the nine historical alfalfa accessions evaluated in this study were chosen from an individual cultivar which was used by Melton et al. (1990) to develop the original historical populations.

This study was conducted to identify additional genomic SSR markers for the genetic improvement of alfalfa. The objectives were to (i) identify new SSR markers in the alfalfa genome; and (ii) assess their utility for separating *Medicago* species and subspecies, especially *M. sativa* ssp. *sativa* genotypes.

MATERIALS AND METHODS

DNA Isolation and Plant Materials

A tissue bulk of five leaves from young actively growing seedlings in the greenhouse was collected from 20 individual plants from an autotetraploid alfalfa germplasm, W10. W10 is a multiple pest resistant germplasm developed by USDA-ARS, Beltsville, MD (Elgin, 1982). Twenty plants were used to represent the population, as was determined in previous studies by Kidwell et al. (1994) and Segovia-Lerma et al. (2003) to be sufficient to represent diversity levels within alfalfa populations. Total genomic DNA was extracted from the bulk tissue samples for DNA library construction using the method described by Doyle and Doyle (1989).

Construction of Genomic Libraries, Cloning, and Screening for SSRs

Purified W10 genomic DNA was digested with restriction enzymes *Sma* I, *Alu* I, *Rsa* I, *Nac* I, *Hin*c II, and *Xmn* I (Promega, Madison, WI), and fragments were electrophoresed in 1.5% agarose gels. Fragments of 400 to 900 bp length were excised from the gel for ligation into the *Sma* I site of vector pUC19. Ligated DNA was used for transformation into the ultracompetent cells, XL2-Blue cells (Stratagene, La Jolla, CA), through heat shock at 42°C for 60 s. To every 100 μL of transformed cells, 900 μL of LB (Luria-Bertani) media was added followed by incubation at 37°C with light shaking for 1 h. Colonies were grown and transferred to NYTRAN nylon transfer membranes (Midwest Scientific, Valley Park, MO) according to the protocol of Sambrook et al. (1989).

Probes were made by ^{32}P-labeling of oligo-nucleotides with 15 repeats of AC, AT, or CT and with 10 repeats of CTT, GAT, or GGT (Song et al., 2002). After hybridization,

membranes were placed on filter paper in a cassette for 24 h before exposure to 35 × 43 cm BioMax film (Eastman Kodak Comp., Rochester, NY).

Primer Design and Fragment Sizing

Inserts were sequenced in an ABI PRISM 3100 Genetic Analyzer using the BigDye DNA sequencing kit (Applied Biosystems, Foster City, CA). The DNA sequences were manually checked for the presence of SSRs and those sequences containing SSRs, not only with sufficient flanking regions but also with a minimum of seven repeats for dinucleotide or six repeats for trinucleotide SSR, were used for PCR primer design. Primers were designed using the software Primer 3 with the following core parameters: (i) primer length of 18 bp to 25 bp; (ii) 40 to 60% Guanine Cytosine (GC) content; (iii) Tm of ≥50°C; and (iv) predicted PCR product length of 100 bp to 300 bp. In addition, primer internal structures, such as hairpin loops, possible primer dimmers and length of single base pair run at the 3′ end, also were taken into consideration during primer design. Primers were commercially synthesized by Invitrogen Life Technologies (Carlsbad, CA).

PCR amplifications were conducted in 96-well or 384-well plates using PTC-225 Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). Each reaction mixture contained 20 ng of DNA, 0.2 μM of forward and reverse primers, 250 μM of each dNTP, 1 μL of 10 × PCR buffer containing 100mM Tris-HCl, pH 8.3, 500mM KCl, and 1 unit of Taq polymerase (Invitrogen). PCR began with denaturation at 94°C for 2 min, followed by 30 cycles of PCR amplification, each consisting of 40 s at 94°C for denaturation, 40 s at the Tm°C for each primer pair for annealing and 40 s at 68°C for extension. The PCR products were finally extended at 72°C for 5 min. DNA fragments were sized using high-resolution 3% TREVIGEL agarose gels (Trevigen, Inc., Gaithersburg, MD) or the ABI PRISM 3100 Genetic Analyzer.

Nomenclature for SSR Markers

SSR names were prefixed with an "A" (for "alfalfa"), followed by the repeat motif in lowercase and ascending numbers for each distinct repeat motif (He et al., 2003). For example, Act001 and Agat001 designated SSR markers at two different loci with the repeat motif "ct" and "gat", respectively. For the imperfect or multiple repeats, the most prevalent motif was used. For example, SSRs markers with motifs of (ac)16 + (at)6 and (ctt)5 + (ct)6 were named Aac and Act, respectively. When two or more different motifs were repeated the same number of times, the motif at the 5′ end was used in the designation. For example, a locus with the SSRs (CT)8 + (CA)8 was designated as Act.

Germplasm

A total of 15 *M. sativa* subspecies accessions, as well as a *M. truncatula* accession, were used for assessing SSR loci for genetic diversity (Table 1). The nine historical alfalfa accessions (African, Chilean, Falcata, Flemish, Indian, Ladak, Peruvian, Turkistan, and Varia) evaluated here were selected from the individual original cultivars used by Melton et al. (1990) in the development of the historically recognized germplasm sources in the USDA-ARS National Germplasm System. The USDA-ARS system was searched using the Germplasm Resources Information Network (GRIN) for the oldest nine historically recognized alfalfa germplasm sources. Accessions with NSL (National Seed Storage Laboratory) numbers are samples from original seed collections. A *M. sativa* ssp. *falcata* germplasm release Wisfal (Bingham, 1993), two very non-dormant alfalfa germplasms (fall dormancy class 10 and 11) (Taggard et al., 2000), an accession of diploid *M. sativa* ssp. *falcata*, and an accession of diploid *M. sativa* ssp. *coerulea* and *M. truncatula,* cv. Jemalong, also were included in this study (Table 1). Diploid species of *M. sativa* (ssp. *coerula* and ssp. *falcata*) are considered to be the progenitors of tetraploid alfalfa, whereas *M. truncatula* was included due to its importance as a model plant, which is closely related to alfalfa. Since the time of the original study of

Table 1. *Medicago* germplasm utilized for assessing the utility of SSR loci derived from alfalfa for conducting genetic diversity analyses in this genus.

Number	Germplasm source	Cultivar name	Accession number	Seed source†	Abbreviation
1	African 1	African	NSL 4142	Arizona, 1961	Af1
2	African 2	'Moapa'	NSL 4123	Nevada, 1961	Af2
3	Chilean	Arizona Common	NSL 4144	Arizona, 1961	Ch
4	Falcata	'Wisfal'	PI 560333	Wisconsin, 1990	Fa
5	Wild 4x Falcata	Wild Tetraploid ssp. *falcata*	PI 214218	Denmark, 1954	W4fa
6	Flemish	'DuPuit'	PI 206103	France, 1953	Fl
7	Indian	Sirsa Type 9	PI 235736	India, 1956	In
8	Ladak	'Ladak'	NSL 4164	Maryland, 1961	La
9	Peruvian	Hairy Peruvian	NSL 4143	Arizona, 1961	Pe
10	Turkistan	Kayseri	PI 171721	Turkey, 1948	Tu
11	Fall Dormancy 10	UCDOR-CUF$(H)_4$	UC-1887	California, 2000	Ar1
12	Fall Dormancy 11	UCDOR-WADI QURAYAT $(H)_2$	UC-1465	Saudi Arabia California, 2000	Ar2
13	Varia	'Grimm'	NSL 4162	Maryland, 1972	Va
14	Wild 2x *coerulea*	Diploid ssp. *coerulea*	PI577548	Russia, 1962	W2co
15	Wild 2x *falcata*	Diploid ssp. *falcata*	PI258754	Russia, 1959	W2fa
16	*M. truncatula*	'Jemalong'	PI442895	Australia, 1980	Mt

†Seed source: State or country where the accession was obtained and the year the accession was donated to the U.S. germplasm collection.

the historical germplasm sources of cultivated alfalfa by Barnes et al. (1977), new germplasm sources were collected in the Arabian Peninsula (Smith et al., 1991) that have been used in the development of very non-dormant alfalfa cultivars (represented by the germplasm UC-1465 in this study). Germplasm was selected to represent the range of genetic diversity in alfalfa to identify polymorphism levels for genomic SSRs within the *M. sativa* subspecies.

Twenty seeds per accession were planted, one seed per pot in the greenhouse. Greenhouse temperatures ranged between 28 and 32°C. Once the seedling had five to six fully expanded leaves, three leaves per plant were harvested. Three leaves from each of the 20 plants were bulked together for DNA extraction, which was performed as described for W10.

Data Analysis

The potential for using SSR markers to separate genotypes was assessed by using each genotype/primer combination to amplify PCR fragments in each accession, which were scored for presence (1) or absence (0) and combined into a presence/absence data matrix. Polymorphism information content (PIC) for each locus was calculated using the following equation (Saal and Wricke, 1999; Shete et al., 2000):

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} p_i^2$$

where p_i is the frequency of the ith genotype out of the total number of genotypes at a locus, and k is the total number of different genotypes for the specific locus. The PIC of a marker is the probability that the marker genotype of the progeny of a heterozygous parent with a dominant trait allows for the deduction of which marker allele the progeny will inherit from the parent. Thus, it is a measure of a marker's usefulness for linkage analysis.

Genetic distances (GD), based on all pairwise comparisons, were computed for the combined marker data, using the formula of Nei and Li (1979): $GD = 1 - [2N/(N_i + N_j)]$, where N is the number of common bands, and N_i and N_j are the total number of bands for genotypes i and j, respectively. This formula disregards 0/0 matches, which could be caused by factors other than genetic similarity (Campbell, 2000), and weighs 1/1 matches by a factor of 2 to better separate genotypes. A multiple correspondence analysis (MCA) was performed on the GD data using PROC CORRESP (SAS Institute, 2000). This analysis produces weighted principal components (dimensions) from a contingency table assuming Euclidean distances. Genotypes were clustered using Ward's Minimum Variance (PROC CLUSTER; SAS Institute, 2000) where the distance between two clusters is the analysis of variance sum of squares between the clusters added over all variables. For interpretation, the sums of squares are converted to R^2 values (PROC CLUSTER; SAS Institute, 2000). Dendrograms were produced using PROC TREE and enhanced using PROC GPLOT (SAS Institute, 2000). Ward's procedure was bootstrapped (100 cycles) using a program written in the Interactive Matrix Language (SAS Institute, 2000). The higher the boot strap value the more likely the accessions are related compared to the other accessions in this study. MCA three-dimensional results were plotted using Sigma Plot (Sigma Plot, V8.0, Chicago, IL).

RESULTS

Isolation of SSRs from the Alfalfa Genomic DNA Library

A total of 158 colonies with intense hybridization signals were isolated from the alfalfa genomic library. On the basis of sequence motifs, 81 DNA sequences were determined to be suitable for SSR primer design. Fifty-nine primer pairs were designed for dinucleotide repeats, whereas 22 were designed for trinucleotide repeats. Primer-evaluations using 3 of the 16 alfalfa DNA samples indicated that 78 (96.3%) generated PCR fragments, whereas three of the SSR loci could not be amplified (Table 2).

Allelic Variation and Characterization of SSR Loci

Seventeen (22%) of the SSR loci were monomorphic, whereas 61 (78%) generated polymorphism among different DNA samples of the 16 accessions (Table 2). The percentage of SSR markers that were polymorphic was similar for both dinucleotide and trinucleotide loci (Table 2).

Table 3 lists the SSR motifs, primer sequences, number of alleles, expected size of PCR products, and PIC of all SSR loci. Of the 78 SSR loci evaluated, the majority was polymorphic, whereas 17 loci generated monomorphic fragments (Table 3). Polymorphic loci showed 2 to 11 visible fragments while most of them had 2 to 4 fragments visible. Primers for di- and trinucleotide SSRs amplified a total of 228 and 82 fragments, respectively, with an average of 5.2 and 4.8 fragments per primer, respectively. PIC ranged from 0.12 to 0.92 and PIC values were highly correlated with number of alleles detected ($r = 0.77$, $p < 0.001$) (Table 3).

Although these 78 SSR primers all amplified fragments in cultivated alfalfa, eight *M. sativa* ssp. *coerulea* and two *M. sativa* ssp. *falcata* primer pairs did not produce fragments for *M. truncatula* or for diploid *M. sativa* ssp. (Table 4). A total of 70 (89.7%) SSR primer pairs annealed to *M. truncatula* sequences and 76 (97.4%) amplified the *M. sativa* ssp. *coerulea* and ssp. *falcata*, indicating a close syntenic relationship among *Medicago* spp. Coincidentally, Eujayl et al. (2004) found that 89% of the EST-SSRs were transferable from *M. truncatula* to alfalfa and annual medics. In addition, the total number of PCR fragments amplified by the polymorphic SSR primers for the individual accessions ranged from 69 for *M. truncatula* (Jemalong) to 103 for African 2 (Moapa) (Fig. 1).

For germplasm differentiation, 26 (37.1%) loci had unique polymorphisms that distinguished *M. truncatula* from the other germplasm, whereas only 17 (22.4%) and 6 (7.9%) loci had unique polymorphisms that separated diploid *M. sativa* ssp. *falcata* and ssp. *coerulea,* respectively, from the remaining genotypes (Table 2).

Germplasm Differentiation and Analysis of Genetic Diversity

Local maxima for the cubic clustering criterion (CCC) (SAS Institute, 2000) are often associated with a reasonable estimate of the true number of population clusters. The CCC reached a local maximum at three clusters for these data, indicating that the genotypes were clustered into three groups comprised of *M. truncatula*, diploid *M. sativa* ssp. *falcata*, and the remaining genotypes (Fig. 1). Ladak (La), *M. truncatula* (Mt) and Fall Dormancy Class 11 (Ar2) are clearly distinguished from each other as well as the rest of the accessions. Turkistan (Tr) is very similar but distinguishable from the cluster of eight which includes African 1 (Af1), Chilean (Ch), Falcata (Fa), Wild 4x Falcata (W4fa), Fall Dormancy Class 10 (Ar1), Varia (Va), and wild 2x *coerulea* (W2co) and 2x *falcate* (W2fa). Peruvian (Pu) and African 2 (Af2) are similar, but distinguishable from each other and are distinct from the cluster of eight, and the cluster containing Indian (In) and Flemish (Fl). SSRs successfully discriminated among 8 of the 16 accessions evaluated. Diploid *M. sativa* ssp. *falcata* was clustered with the remaining ssp. *falcata* in contrast to its placement in a separate cluster by the Ward's minimum variance analysis; however, the support for placing it in a separate cluster was weak due to the relatively low bootstrap values (Fig. 1). The bootstrap value listed for the 2 *falcata* branch was 100 as listed in Fig. 1.

Table 2. Number of SSRs developed from an alfalfa genomic library of di- and trinucleotide motifs, and their utility for polymorphism detection based on screening 16 *Medicago* germplasms.

Type	SSR group† (5′ ~ 3′)	Number of SSRs	Polymorphism			
			Polymorphic	%	Total fragments	PIC‡
Dinucleotide	$(ct/ga)_n$	43	44	78.6	228	0.53
	$(ac/tg)_n$	10				
	$(at/ta)_n$	3				
Trinucleotide	$(gat/cta)_n$	14	17	77.3	82	0.43
	$(ggt/cca)_n$	5				
	$(ctt/gaa)_n$	3				
Total or average		78	61	78.2	310	0.50

†SSR motifs used for making probes for colony hybridization.

‡PIC = polymorphism information content.

DISCUSSION

Of the 78 SSR loci identified, 78% generated polymorphisms, which included 44 dinucleotide and 17 trinucleotide SSR loci. This result indicated that SSR markers are highly variable among *Medicago* germplasm. The polymorphism rates were similar for dinucleotide (78.6%) and trinucleotide (77.3%) SSR loci; however, we did not search for many trinucleotide SSRs in this study. Trinucleotide SSRs are preferred because the differences among trinucleotide-based microsatellite fragments can be more easily detected because of the clarity of the bands during sequencing (Song et al., 2002). Although these 78 SSR primers all amplified fragments in cultivated alfalfa, eight and two primer pairs did not produce fragments for *M. truncatula* or for diploid *M. sativa* ssp. *coerulea* and ssp. *falcata*, respectively (Table 4).

The 61 polymorphic SSR loci generated a total of 310 fragments, including 228 and 82 for di- and trinucleotide SSR markers, respectively (Table 2), which should be sufficient for differentiating this set of alfalfa germplasm. Zhang et al. (2002) reported that a minimum of 167 alleles is sufficient for differentiating among 43 wheat varieties, although they suggested that more polymorphic alleles could help construct a stable dendrogram. In addition, the number of fragments generated among the SSR loci ranged from 1 to 11 with an average of 5.1 fragments per SSR locus and PIC values ranged from 0.12 to 0.92 with an average of 0.50 (Table 2 and 3), indicating a higher polymorphism rate than that of other species, including tomato (He et al., 2003) and wheat (Eujayl et al., 2001). In addition, PIC values were not significantly correlated with the number of repeats among 61 polymorphic *Medicago* SSR loci ($r = 0.07$, $p = 0.59$), suggesting that the minimum numbers of repeats of seven and six for di- and trinucleotide SSRs, respectively, should be sufficient for conducting genetic diversity analysis in alfalfa. Again, the heterozygous nature of alfalfa, which is an outcrossing species that displays inbreeding depression, results in a large amount of variability within and among germplasm, which could require additional molecular markers to distinguish cultivars and subspecies from each other.

Some unsuccessful amplification in the non-*sativa* subspecies could be related to reduced sequence homology (Table 4), which could be further related with genetic distance (Fig. 1 and 2). Gaitán-Solís et al. (2002) reported that a range of 9 to 26% of the SSR primer pairs that were developed from *Phaseolus vulgaris* L. failed to amplify DNA from other species including *P. coccineus* L., *P. acutifolius* A. Gray, *P. polyanthus* Greenm., and *P. lunatus* L. Also, the total number of PCR fragments amplified by polymorphic primers for individual accessions did not appear to be associated with genetic diversity, except for *M. truncatula*, which had the lowest total number of fragments (69), compared with the rest of the *Medicago* accessions (Fig. 1 and 2).

In this study, 26 (37%) SSR primers generated one or more unique PCR fragments for *M. truncatula*, and 6 (8%) and 17 (22%) primers produced unique bands for the two diploid ssp. *coerulea* and ssp. *falcata*, respectively. Cluster analysis on the basis of the SSR polymorphism produced three main clusters: (i) wild diploid ssp. *falcata* (PI 258754), (ii) *M. truncatula* (Jemalong), and (iii) the remaining

Table 3. SSR primer name, core motifs and the flanking primer sequences, melting temperatures, number of fragments detected, expected fragment sizes of the PCR products and polymorphism information content (PIC) of microsatellite markers developed from an alfalfa genomic library.

Primer name	Motif	Primer sequence† (5′– 3′)	Tm	No. of fragments	Expected size‡	PIC¶
			°C		bp	
Aac001	$(ac)_{12}$	f: act acg act cta gac act tta tta g r: ctt ctc tct ttc tca tac tct tc	47.7 46.6	10	138	0.92
Aac002	$(ac)_{16}(at)_6$	f: ttg aaa ttg gca aac cca c r: aaa ggt gca gaa acc ctc	50.7 46.2	11	112	0.91
Aac003	$(ac)_{12}$	f: agc aac aaa ctt taa ggc ag r: ggg acc cat tct tat tta ag	46.9 45.1	4	121	0.67
Aac004	$(ac)_{10}$	f: aag gca aat aag cta gac cca r: agc tcg gga ccc att ctt at	58.0 59.9	9	246	0.83
Aac005	$(tcc)_3(ca)_2+(ac)_9$	f: aca caa tag caa cca ggg tg r: tcg gtt gcg tgt gta act c	49.8 49.1	4	234	0.48
Aac006	$(ac)_{7+5}$	f: gat tca ctt gcc att cga gct tgc r: tcc tgg ccg ttg atg tgt gtt g	60.9 60.2	5	170	0.33
Aac007	$(ac)_{6+5+4}$	f: cat acc att aca tgc tcc ac r: ggt acc cac gtg taa tat aat tg	45.1 48.0	3	157	0.50
Aac008	$(ac)_{15}(ta)_4$	f: act ctt agg agc agg atc ac r: gca gaa gct cta gtg gta tg	46.7 46.7	11	231	0.91
Aac009	$(ac)_{11}(ca)_2$	f: tgt tga ggt cat ggt ggc ta r: agc tcg gga ccc cta aaa t	60.1 59.9	9	220	0.80
Aac010	$(ac)_{4+4+5+7}$	f: atg ttg tga ggt gtc taa ttt atg g r: ata taa att agg gac tcc act ttg g	51.2 51.4	8	201	0.45
Aat001	$(tc)_2+(at)_5$	f: gca tac tcc cag cta atc cg r: cga gct cgg tac cga ttc	64.3 62.3	5	236	0.65
Aat002	$(at)_8+(ca)_4$	f: aat taa tgg att gac acc c r: tgt gtg tgt gtg tgt ggg	43.5 45.3	9	143	0.90
Aat003	$(at)_2+(ca)_2(at)_4$	f: cac cct aga taa gat tgt aaa g r: gga tcg ccc tgt tag tag	44.1 45.2	3	111	0.50
Act001	$(ct)_{25}$	f: tac aca agc aat tca agg aag g r: cac acg act att gcg ctt atg	51.4 51.2	4	172	0.22
Act002	$(ct)_8+(ca)_8$	f: tcc tat ctt acc tac aat gcg aac r: ccc aca tct cac tcc atc c	51.6 49.4	3	167	0.12
Act003	$(ta)_4+(ct)_9$	f: taa ctt cca ttc ttc caa cct g r: ttc tac atc tgc tct ctg ttg aat c	50.4 51.5	5	163	0.32
Act004	$(ct)_{10+2+20}$	f: aca tga ttt aag gtt gag aca gag r: ttt agg gtg tta cag gag gaa c	49.0 49.5	4	146	0.56
Act005	$(ct)_{25}$	f: caa tcc gtg agt ggt gag aa r: ttg gac cga act ggg taa ac	59.7 59.8	3	111	0.23
Act006	$(ctt)_5+(ct)_6$	f: ctg gaa cat gga acc tgg at r: gtt ttg cgt tga cct tgg ta	59.8 58.7	2	255	0.42
Act007	$(ct)_{4+3}+(ct)_3(ggt)_2$	f: ctt ccc ctt cgt ttt tct cc r: gat gca aac atg tgc cag ac	60.0 60.1	3	221	0.56
Act008	$(ct)_{13+4}$	f: cac ttc acc aac cca aaa ca r: ccc tct ata cac ctc cca cg	59.4 59.4	4	239	0.43
Act009	$(ct)_{12+5}$	f: aag caa ccg aac aac gat tt r: agt gac agt tat ggg ggt gg	59.6 59.7	10	203	0.62
Act010	$(tccc)_3(ct)_{20}$	f: atc ttg aaa gcc aga cac cg r: cca ttg tat tcc cta agt ttg cc	60.3 60.9	6	268	0.48
Act011	$(ct)_{12}$	f: gca tga tca agc tag aaa ccc t r: tct cag act cga att atc caa gc	59.8 59.9	6	288	0.89
Act012	$(ct)_{10}$	f: gtt tgt gca gcc ctt tga tt r: atg caa acc aag att aag gc	60.1 56.9	3	130	0.70
Act013	$(ct)_{20}$	f: aca tgc cat tta gcc cat tc r: aag ttc act cgc gaa aga gg	59.8 59.6	4	224	0.56
Act014	$(ct)_{19}$	f: aat tta tta tgg ccc ctc cg r: cca cct tca atg taa gcc gt	60.0 60.0	2	295	0.12
Act015	$(ct)_{11}$	f: act ccc tcc ttg ctc cat tt r: cat gga tgg att cgt gaa ga	60.1 59.5	1	101	0.0
Act016	$(ct)_{4+7+4}$	f: atg tgt aga cct tga ctc ttg atg r: gtt tca cct ttg tca cct gc	49.5 49.4	2	160	0.12

Table 3. Continued.

Primer name	Motif	Primer sequence† (5′– 3′)	Tm	No. of fragments	Expected size‡	PIC¶
			°C		bp	
Act017	$(ct)_{10}$	f: cgg gcc tct ttt cac tta ca r: ctt ctc tca cca ctt tcc cg	60.2 59.8	4	293	0.61
Act018	$(ct)_{19}(ta)_6+(ct)_7$	f: agt gtg tga taa ata agt ttg tgg r: cgt aat tgt tac tgg ttt gaa ag	61.1 62.0	5	242	0.48
Act019	$(ct)_{14}+(ta)_4$	f: ccg gat ttc ttt cat tgt gg r: gaa aca atg gat ttt tgc cg	60.3 60.3	6	218	0.71
Act020	$(ct)_{19}$	f: tgt tca gag tta agt gca gcg r: tca agt gct cac cca aac aa	59.3 60.3	7	276	0.92
Act021	$(ct)_9$	f: gca tgt gca gtg gaa gaa ga r: aat ggt acg cgt gta tgc aa	60.0 60.0	2	235	0.23
Act022	$(ct)_{6+8}$	f: gct caa att tca cgt cac ga r: tcc att cac ttc aac atc gc	59.9 59.7	1	288	0.0
Act023	$(ct)_{16}$	f: tgc att gta tac tta cct ttc att g r: ccc aca cac tca aat ctc ctt ac	50.4 52.0	1	171	0.0
Act024	$(ct)_7(ctcc)_3$	f: tgt cga aag cac ttg gaa tg r: tgc aat cta gca tcg gag aa	59.8 59.5	5	200	0.71
Act025	$(ct)_{6+5}$	f: atc ggt tct gag atc gcc ta r: caa acc ctt caa aac cct ca	59.8 59.9	4	207	0.35
Act026	$(ct)_9$	f: aaa cca ccc ttc cat ctt cc r: agg gtg gag aag aag cat ga	60.2 59.8	7	222	0.58
Act027	$(ct)_{13}$	f: gtg gat gac ctt ggt gaa g r: ttc aat taa tca caa gca ata tc	47.0 46.4	1	168	0.0
Act028	$(ct)_{4+5}$	f: ttc att tct tct tcg tct tc r: gct att aaa tca aac gaa cg	43.6 45.0	2	123	0.38
Act029	$(ct)_{10}$	f: cct ctc gtt tac ctc cct tg r: tcc aat cat aat tgt ccg ca	58.8 59.9	6	282	0.66
Act030	$(ct)_{15}$	f: agg agc atc tgc cca tag tg r: agg gag atc gag gga caa gt	60.2 60.1	1	254	0.0
Act031	$(ct)_{4+5+4}$	f: gga aaa agg gga agg caa ta r: tgt ttt cct ttt tgc tta att c	60.3 55.5	1	225	0.0
Act032	$(ga)_8+(ct)_{20+9}$	f: gac acc tgt ttt tga acc cg r: ggc gag aaa cac ttg tgt gg	60.4 61.7	1	281	0.0
Act033	$(ct)_{10+3}$	f: ggg ctc aga aac aca gat cc r: aac ccg tta aca att ccc aa	59.7 59.2	1	175	0.0
Act034	$(ct)_4t(ct)_3t(ct)_4t(ct)_2$	f: gaa aga ctt gtg tgg acc agg r: tgt aga cca ggg acc aaa cc	59.6 59.8	1	163	0.0
Act035	$(ct)_{11}$	f: cgc aat gaa tta ggt cga ttt r: tgg att acc ttg gtg aag gc	59.1 59.9	4	226	0.23
Act036	$(ct)_{10}$	f: gga ttg tgg atg gaa cct g r: atc aga gtt cag agt ctt atg cag	49.6 49.2	1	100	0.0
Act037	$(ct)_8at(ct)_{10}at(ct)_9$	f: gcc aca gat caa caa ttt gc r: act tct tac cca ccg gaa cc	59.1 60.2	3	242	0.41
Act038	$(ct)_{15}$	f: att ccc atg agt gcc aag ac r: tgt gtt tga gca ttc cat cc	59.9 59.5	2	296	0.12
Act039	$(ct)_{25}$	f: ttc agg atc cct aaa tga gtg aa r: gtt ttg gat gca caa cat gg	56.0 59.8	3	189	0.48
Act040	$(ct)_5(cca)_4+(at)_3$	f: cgg tgg gtg aga gat gga tat g r: ccc gca tga atg cat ata ttg	55.6 53.1	8	233	0.50
Act041	$(ct)_{4+4}+(cct)_3$	f: atg agt cca tgg gtg ata act g r: tac ttt gtg tct tgt gaa tat cct c	50.5 49.2	1	213	0.0
Act042	$(tct)_3+(ct)_4+(cca)_3$	f: gtt aat ctc tgt tct ctt ctt ctg r: ttg ttc gtc gaa gaa gat g	46.0 45.6	8	298	0.75
Act043	$(ct)_{4+4}+(tg)_3$	f: tgg aat tga aac act taa acc tct c r: gat aca tag tgc gaa aga tta aag g	52.8 50.8	1	222	0.0
Actt001	$(ctt)_{24}+(tct)_5$	f: aat tgt gag ctt tcc tct tcc r: tac gac tct aga cac ttt att agg c	49.5 48.6	11	235	0.86
Actt002	$(ct)_6+(ctt)_{3+6}$	f: aga tgc tct gga gga tga ttc ac r: cac aca ctt agg gta agc att g	53.8 49.4	9	223	0.67

Table 3. Continued.

Primer name	Motif	Primer sequence† (5′– 3′)	Tm	No. of fragments	Expected size‡	PIC¶
			°C		bp	
Actt003	$(ctt)_4+(tc)_3$	f: aca cac aca cac aca cac tta g r: gac aag gtg ttg caa tct c	45.2 44.5	11	189	0.88
Agat001	$(gat)_5(ag)_2$	f: tat ctg att cca act tgg gta tg r: tac aaa gca tgc aac gaa ac	50.3 49.0	4	231	0.48
Agat002	$(gat)_6$	f: tca agt tcc gta acc taa ttt cc r: aga aca tcc tac aaa caa aca aca c	51.4 51.2	4	228	0.73
Agat003	$(gat)_8$	f: acc tag taa caa taa tgt tgg tgg r: tac gcc att tct tct ctt tga c	49.2 50.7	2	236	0.12
Agat004	$(gat)_7$	f: caa tag atg cag tat cag gta tc r: gtt aca cct ttg aag aat cca g	45.5 47.6	2	232	0.22
Agat005	$(gat)_8$	f: agg gta aca aac atg gtc tat g r: cca cac cat aca ata atg aac c	47.8 48.8	1	108	0.0
Agat006	$(gat)_7$	f: aac agt ctc att caa atc cag tc r: ctt gtt gat gtt att gac ctg g	49.3 49.0	4	100	0.63
Agat007	$(gat)_{10}$	f: ggc tgc aaa gga ttc ttc tga ag r: ggt cca tga tgt tga tcc ttc tta tg	56.2 56.8	1	102	0.0
Agat008	$(gat)_2gac(gat)_4$	f: aac aga agc cac cgt aag cga c r: aag tgt tca tgc ctg acc aat ctc	57.2 56.1	3	104	0.12
Agat009	$(gat)_8$	f: cgt tcg agt tat tgt tgt tgg tg r: cac tga aac tga atc gga aag aag	53.9 54.0	2	207	0.12
Agat010	$(gga)_5+(ggt)_3(gat)_5$	f: atc tag cag tac cac cac cat atg r: ttg cac aga cac aga ttg tac ata g	51.8 51.5	1	172	0.0
Agat011	$(gat)_8+(gtt)_2(att)_7$	f: cga gga ggt gag ata ata gag g r: ccc ttc tac aat atc tca tcc tc	49.3 48.4	1	184	0.0
Agat012	$(gat)_{3+5}$	f: gat agt ccg tac ctt ggc tct g r: tgt tca gct ctt cat cta cat ctt c	52.3 51.5	6	106	0.23
Agat013	$(gat)_6+(ga)_4$	f: cat tac agt atc tct aaa caa gtt cct g r: tta tag cca cta tcc tcg ttc c	51.6 49.9	1	150	0.0
Agat014	$(gat)_3(tc)_2+(ct)_3$	f: ttt gct tgt ttg aac tca ttg c r: gtt aca tgc agg gtc gac tc	52.3 49.3	2	141	0.12
Aggt001	$(ggt)_{6+4}$	f: cct gtt ggt tgt tgt aga tat gg r: agt tcg cca cca aat gta atc	50.7 50.9	4	177	0.37
Aggt002	$(ggt)_{3+6}$	f: gga gct cag atg gtg gtg gtt gta g r: ctc cag tgt tcc ttc tct cac ctc	59.8 55.1	3	238	0.23
Aggt003	$(gct)_3+(ggt)_6$	f: ggt gct gca act tct tca aat tc r: tga tac gct gaa ccc aat tgt aaa c	54.7 56.2	3	221	0.48
Aggt004	$(ggt)_2+(caa)_2(tcc)_3$	f: aaa tga tat tgt agg aag atc gtg c r: agt tat gag gga gca ccg ag	52.8 50.9	10	164	0.86
Aggt005	$(ggt)_4+(ga)_3(ttg)_4$	f: aac taa cac att aca ttt gag ag r: cta tgt tcc aat gtt cca tc	43.0 44.1	2	114	0.12

†f = forward primer; r = reverse primer.

‡Expected size is the expected PCR fragment length of the alfalfa germplasm W10.

¶PIC = polymorphism information content.

entries (Fig. 1). The third cluster separated into two subgroups, one of which had higher bootstrap values (100%) than the other (Fig. 1). The higher the bootstrap value the more confidence there is in there being a close relationship between accessions in that group or between accessions at each end of the branch points (Li, 1997). Also, some of the accessions did not cluster as expected (Fig. 1). For instance, all of the ssp. *falcata* accessions, PI 560333, PI

Table 4. PCR amplification profiles of 78 SSR primers for *Medicago truncatula* and three *M. sativa* subspecies.

PCR amplification	*M. truncatula*	*M. sativa*		
		ssp. *coerulea*	ssp. *falcata*	ssp. *sativa*
No fragment	8	2	2	0
≥1 fragment(s)	70	76	76	78
% primers amplified fragments	89.7	97.4	97.4	100
Showing unique fragments†	26	6	17	40
%	37.1	7.9	22.4	51.3

†The number of SSR primers generating species- and subspecies-specific fragments for distinguishing that specific species/germplasm from the rest germplasm.

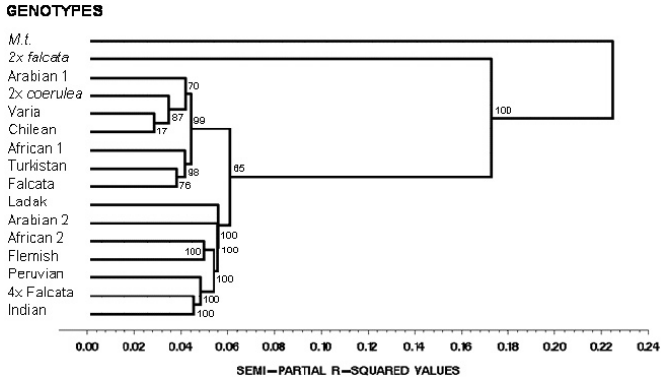

Figure 1. Dendrogram constructed through cluster analysis based on PCR products from 61 polymorphic SSR primers to establish phylogenetic relationships among the nine historically recognized alfalfa germplasm sources, as well as germplasm collected in the wild. Numbers at nodes are bootstrap values (%) that measure the reliability of the derived tree. Af1 = African 1, Af2 = African2 'Moapa', Ch = Chilean, Fa = Falcata, W4fa = Wild 4x Falcata, Fl = Flemish, In = Indian, La = Ladak, Pe = Peruvian, Tu = Turkistan, Ar1 = Fall Dormancy 10, Ar2 = Fall Dormancy 11, Va = Varia, W2co = Wild 2x *coerulea*, W2fa = Wild 2x *falcata*, Mt = *M. truncatula*.

214218, PI 258754 were not closely clustered, which may reflect their different countries of origin or ploidy levels. Because alfalfa is an autotetraploid there can be multiple alleles at each locus thus increasing their polymorphisms. In addition, the very non-dormant accessions with similar fall dormancy levels, Fall dormancy 10 (Ar1) and Fall dormancy 11 (Ar2) were not closely clustered (Fig. 1 and 2), although fall dormancy class 10 (UC-1887) (a selection from cv. CUF 101, which is the standard check cultivar

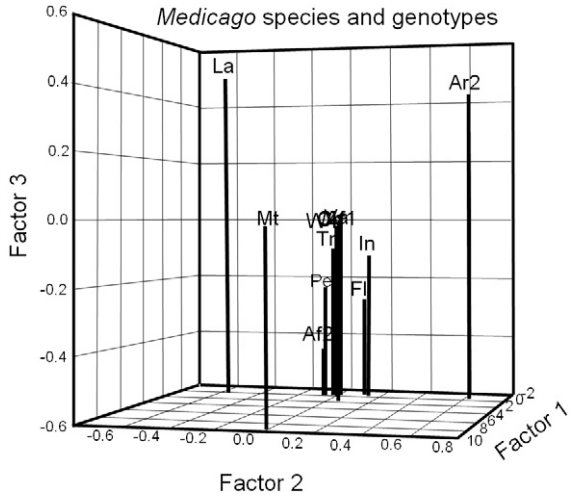

Figure 2. 3D scatter plot after multiple correspondence analyses of genetic distance among 16 germplasms based on diversity assessment using 78 SSR markers. Genotypes were clustered using Ward's minimum variance analyses. Af1 = African 1, Af2 = African2 'Moapa', Ch = Chilean, Fa = Falcata, W4fa = Wild 4x Falcata, Fl = Flemish, In = Indian, La = Ladak, Pe = Peruvian, Tu = Turkistan, Ar1 = Fall Dormancy 10, Ar2 = Fall Dormancy 11, Va = Varia, W2co = Wild 2x *coerulea*, W2fa = Wild 2x *falcata*, Mt = *M. truncatula*.

for fall dormancy class 9) was closely clustered to African 1, which is a fall dormancy class 9.

Segovia-Lerma et al. (2003) used AFLP markers from 30 genotypes within the nine historically recognized alfalfa germplasms, which generated two main clusters: *M. sativa* ssp. *falcata* vs. *M. sativa* ssp. *sativa* and ssp. *varia*. In our data where the hierarchical structure was somewhat less well-defined based on cluster analysis, principal correspondence analysis was used to differentiate among accessions. On the basis of this approach, a few additional entries were separated, including *M. truncatula* (Jemalong) (Mt), Ladak (Ladak) (La), Fall dormancy 11 (UC-1465) (Ar2), Indian (Sirsa Type 9) (In), Flemish (Dupuit) (Fl), Peruvian (Hairy Peruvian) (Pe), African 2 (Moapa) (Af2), and Turkistan (Kayseri) (Tr) (Fig. 2). However, the diploid ssp. *falcata* was no longer separated, and clustered with the remaining germplasms. Other authors also reported different degrees of inconsistency between cluster analysis and multiple correspondence analyses (Campbell, 2000) or principal components analysis (Kidwell et al., 1994). Similarly, Kidwell et al. (1994) reported in a study of the historically recognized alfalfa germplasm sources that only two germplasms could be separated, *M. sativa* ssp. *falcata* and "Peruvian type" *M. sativa* ssp. *sativa*. However, the remaining genotypes representing seven different *M. sativa* ssp. *sativa* germplasm could not clearly be distinguished on the basis of the principal component analysis with RFLP markers, this was probably due to the intermating of the germplasm sources evaluated. Bauchan et al. (2003) could only separate the Indian germplasm source from the other three (African, Chilean, and Peruvian) nondormant alfalfa germplasm sources using chromosome banding patterns of all eight alfalfa chromosomes.

Our inability to differentiate among 8 of the 16 alfalfa germplasms evaluated using SSR markers could be because of the limited number of polymorphic markers used or because the original germplasm sources may have not been very distinct when introduced into the U.S. Although alfalfa was first cultivated nearly 9000 years ago (Ivanov, 1977), most of the distribution throughout the world from southeastern Asia has occurred within the past 500 years (Michaud et al., 1988). The initial populations were heterogeneous and intercrossing, and thus may not have undergone major selective changes, or, if they did, selections only affected a small subset of the loci before introduction into the U.S. In addition, the germplasm sources may not faithfully represent the original germplasm sources because of outcrossing, selection, and genetic drift during seed increase (Kidwell et al., 1994; Bauchan et al., 2003). After the germplasm was introduced into the U.S., it was maintained in different collections, many of which used open-pollination for seed increase and maintenance (Barnes et al., 1977). Thus, outcrossing would have occurred to homogenize the various germplasm sources.

In summary, we developed 78 new genomic SSRs obtained from alfalfa that have excellent utility for polymorphic assessment with potential application for phylogenetic and genetic mapping studies on alfalfa. However, their utility for distinguishing among accessions for diversity assessment will be highly influenced by the nature of the populations being analyzed.

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