Microsatellite Marker-based Genetic Diversity Analysis and Developing Synthetic Varieties in Alfalfa (*Medicago sativa* L.)

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Cover illustration: Photo of the alfalfa variety Bereke grown in a field in Kyrgyzstan. Photo: Maksatbek Amanov

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ABSTRACT

Cultivated alfalfa (*Medicago sativa* L.) is an allogamous autotetraploid species with 2n = 4x = 32 chromosomes. Alfalfa is the most cultivated forage legume in the world mainly due to its wide adaptation to diverse environmental conditions and efficient nitrogen fixation ability. Kyrgyzstan is among the major alfalfa producing countries. The analysis of alfalfa genetic diversity helps to assess the future risk of genetic erosion and helps in the development of sustainable conservation strategies and wise use of the genetic variation in breeding programs. The aim of this Master thesis was to study the genetic diversity of alfalfa grown in Kyrgyzstan and in other countries and determine the genetic relationship using simple sequence repeat (SSR, microsatellite) molecular markers as well as to contribute to the development of synthetic alfalfa varieties for use as forage in Kyrgyzstan. A total of 78 cultivated alfalfa genotypes from 60 accessions representing 24 countries were used for the SSR analysis. *Medicago lupulina* and *M. scutellata* were represented by one sample each as outgroups. Ten SSR loci were analyzed in this study, nine of which were polymorphic. The locus *AGGT-004* was monomorphic across the 78 cultivated alfalfa individuals analyzed. A total of 53 alleles were recorded across the ten loci of cultivated alfalfa accessions, with an average of 5.3 alleles per locus. The number of alleles (N_a) for the nine polymorphic loci varied from two (locus *ACT-026*) to nine (locus *AGAT-012*). Of the ten SSR loci used, seven are highly recommended for alfalfa genetic diversity analysis, as high genetic variation was detected at these loci. The locus *AGAT-012* is highly interesting in that it had several alleles at low frequencies which might be linked to desirable traits. At this locus, 98.6% of the individuals investigated were heterozygotes. Analysis of variance (AMOVA) revealed that 95% of the total genetic variation was found within countries and the remaining 5% differentiated the accessions according to their countries of origin (F_{ST} = 0.13; P = 0.005). AMOVA revealed no significant differentiation between accessions from the Western and Eastern countries (P = 0.14). The Jaccard similarity coefficient and cluster analysis revealed a significant genetic variation between Kyrgyz alfalfa genotypes, which is significant enough for

alfalfa improvement, including the development of synthetic varieties. The use of Kyrgyz accessions, such as *BM-5* and *BM-9/4* for the development of synthetic varieties through crossing with genotypes from other Kyrgyz accessions is recommended, as the genotypes from these accessions were the most differentiated as compared to other genotypes originated from other Kyrgyz alfalfa accessions. The introduction of new alfalfa genetic material from countries such as Argentina, Oman, Australia, Iran, Iraq and Norway into Kyrgyzstan is a great opportunity to increase genetic variation in the alfalfa gene pool in the country.

Key words: Alfalfa, genetic diversity, Kyrgyzstan, microsatellite, SSR, synthetic variety

INTRODUCTION

Alfalfa (*Medicago sativa ssp sativa* L.*)* has a long and rich history, as it is one of the early domesticated crops. According to Putnam et al. (2001), the oldest written reference about alfalfa was found in Turkey, and was dated back to 1300 BC. The oldest archaeological evidence of alfalfa was found in Iran, and was estimated to be more than 6000 years old.

Alfalfa was highly associated with horses in several countries, and even the word "Alfalfa" means "best horse fodder or horse power" in Arabic. Later, during the colonization of Mexico, Peru and Chile by Spanish and Portuguese conquistadors, alfalfa was spread to the New World. Alfalfa was introduced to USA in the first half of the 19th century, during the gold rush (Putnam et al. 2001). The significance of alfalfa during the California Gold Rush in the 1850's was described by Coburn (1907) as "Gold could not always be found with pick and shovel, it could with well be found by alfalfa roots". Due to the significant importance of alfalfa as forage crop, the world alfalfa production area was as large as at 32 million hectares in the 1980s (Michaud et al. 1988b).

In his review of the most important inventions of the last 2000 years, Noblest Freeman Dyson chose alfalfa hay as the most important invention (Putnam et al. 2001). This is based on the fact that hay was essential for the expansion of human populations and old civilizations to areas with colder climates, as the only way for cattle and horses to survive during winter was by feeding on hay. According to Dyson, at some point in history some unknown genius invented hay which can be stored and, consequently, civilization moved north over the Alps. Alfalfa was also called the "Queen of forages" due to its high nutritional quality as animal feed. It contains approximately 17 to 21% of crude protein, and is a very important source of vitamins and minerals (Arinze et al. 2003). The vitamins commonly found in alfalfa are A, D, E, K, C, B_1, B_2, B_3, B_5, B_7 and B_9 (Norouzi and Khademi 2010). Alfalfa is also rich in minerals, such as phosphorus, calcium, potassium, sodium, chlorine, magnesium and copper (Mezni et al. 2010). On well adapted soils, alfalfa is the highest

yielding forage crop and plays an essential role in preventing soil erosion and in minimizing nitrate concentration (Huggins et al. 2001). Overall, the high vegetative yield and nutritional quality of alfalfa make it superior to other forage crops (Sumberg et al. 1983).

Alfalfa grows to a height of up to 1.5 meters and has a very strong, deep root system that sometimes stretches to over 12 meters (Putnam et al. 2001). This makes alfalfa very resilient, especially in drought affected areas. The dairy industry is the most important user of alfalfa, and considers it as the premier forage for dairy cows (Putnam et al. 2001). In addition to its use as excellent forage, alfalfa can also be used for human consumption in the form of alfalfa sprouts and as a component of health food products (Putnam et al. 2001). Alfalfa plays a significant role in honey production, as honey bees are frequent visitors of alfalfa fields during flowering. As a legume, alfalfa increases soil fertility through nitrogen fixation in association with the bacterial species *Sinorhizobium meliloti* (Putnam et al. 2001). It also suppresses weeds when incorporated in crop rotation scheme (Entz et al. 1995; Ominski et al. 1999).

Alfalfa is one of the most important cultivated legume species that is widely grown throughout the world, It has been considered a key component of sustainable agricultural system due to its high yield, quality and pest resistance, as well as its value for soil improvement through nitrogen fixation and crop rotation. It is considered as one of the world's most versatile crops, as it can grow in environments ranging from burning hot deserts to cool high mountain valleys (Leach and Clements 1984; Peterson et al. 1992; Prosperi et al. 2006). However, collecting data on the distribution, acreage and production of alfalfa is very difficult, as many countries report the area under alfalfa cultivation under "hay crops" and thus the reports are not accurate. Alfalfa is grown as an intensive cash crop under irrigation or as a low-intensity rainfed pasture crop (Putnam et al. 2001). It is better adapted to sandy loam soils than to silty-clay loam soils (Annicchiarico 2007) and does not tolerate acidic soil with a pH less than six (Sheaffer et al. 1988).

Alfalfa is a persistent perennial legume that can live 6-8 years (Jewett et al. 1996) through re-growing for up to four to ten times, depending on the environmental conditions, after the green mass is harvested for hay. Some unpublished sources indicated that it can survive for up to twelve years under favorable conditions. Although alfalfa is usually harvested as hay, it can also be made as silage, grazed or fed as a green chop.

Pollination in alfalfa

Alfalfa is a cross-pollinating species with well adapted flower structure for natural cross pollination. The common pollinating agents are insects, such as bees. According to (Elfattah 2010), honey bees (*Apis mellifera*), leaf cutter bees (*Megachile rotundata*), bumble bees (*Bombus spp*) and alkali bees (*Nomia melander*) are the most common and effective alfalfa pollinators. Rain and wind may also be natural pollinators in alfalfa (Bagavathiannan and Van Acker 2009).

Systematics and cytogenetics of alfalfa

The genus *Medicago* belongs to the Fabaceae family that consists of a number of domesticated species such as chickpea, clover, peas, beans and lentil (Doyle et al. 1996). *Medicago* species have a wide natural distribution that covers Asia, Europe and Africa (Lesins and Lesins 1979), and its traditional classification is based on seed characteristics as well as other important traits such as organ hairness, leaves, cotyledons, ploidy level and growth habit (Lesins and Lesins 1979). The genus includes about 83 species, of which a large number of species including alfalfa are being used as forage legumes (Barnes et al. 1988). Alfalfa belongs to the section *Falcago*, subsection Falcatae of the genus *Medicago*, which consists of diploid and tetraploid forms of alfalfa, *M. sativa* ssp. *sativa*, *M. sativa* ssp. *falcate*, and *M. sativa* ssp. *glutinosa* (Lesins and Gillies 1972). *M. sativa* ssp. *sativa* is the common cultivated alfalfa. The three subspecies of alfalfa are easily intercrossable, as they share the same karyotype (Irwin et al. 2001).

The basic chromosome number in most *Medicago* species is $x = 8$. However, the annual species *M. constricta, M. praecox, M. polymorpha, M. rigidula* and *M. murex* have a basic chromosome number of $x = 7$ (Quiros and Bauchan 1988). These species probably arose by chromosomal translocation involving two chromosomes and the subsequent loss of the resulting centric fragment (Gilles 1972). *Medicago* species are either diploids ($2n=2x=14$ or $2n=2x=16$), tetraploids ($2n=4x=32$) or hexaploids ($2n=6x=48$) (Quiros and Bauchan 1988). Common cultivated alfalfa is an allogamous autotetraploid species (Stanford 1951; Armstrong 1954; Quiros 1982).

Center of origin, diversity and distribution of alfalfa

Alfalfa has two separate centers of origin (Michaud et al. 1988; Sinskaya 1959). The first center is Transcucasia, Asia Minor and some parts of Iran whereas as the second center is Central Asia. Alfalfa from Transcucasia, Asia Minor and Iran is a good source of winter hardy genotypes. The Central Asian alfalfa is susceptible to leaf diseases, such as common leaf spot and downy mildew, and has a relatively low tolerance to drought. Most likely, alfalfa was domesticated near current Turkmenistan, Iran, Turkey and the Caucasus regions. This may be inferred from the work of Aristotle and Aristophanes who wrote about the importance of alfalfa to the early Babylonians, Persians, Greeks and Romans (Michaud et al. 1988b). Alfalfa has been distributed from its centers of origin to other part of the world where is currently grown.

Kyrgyzstan and alfalfa production

Kyrgyzstan is a landlocked country in Central Asia with a population of 5.2 million and a total surface area of 199 900 km², of which 70% is situated higher than 2000 m above sea level. The country borders Kazakhstan, Tajikistan, Uzbekistan and China (Fig. 1). The climate of Kyrgyzstan is continental and varies regionally with hot summer and cold winter. Agriculture is a very important sector in Kyrgyzstan as it contributes with 35% of the country's gross domestic product and provides

employment for over 50% of the population. Total arable land in the country is 1.3 million hectares (Bolch 2007; Ludi 2003).

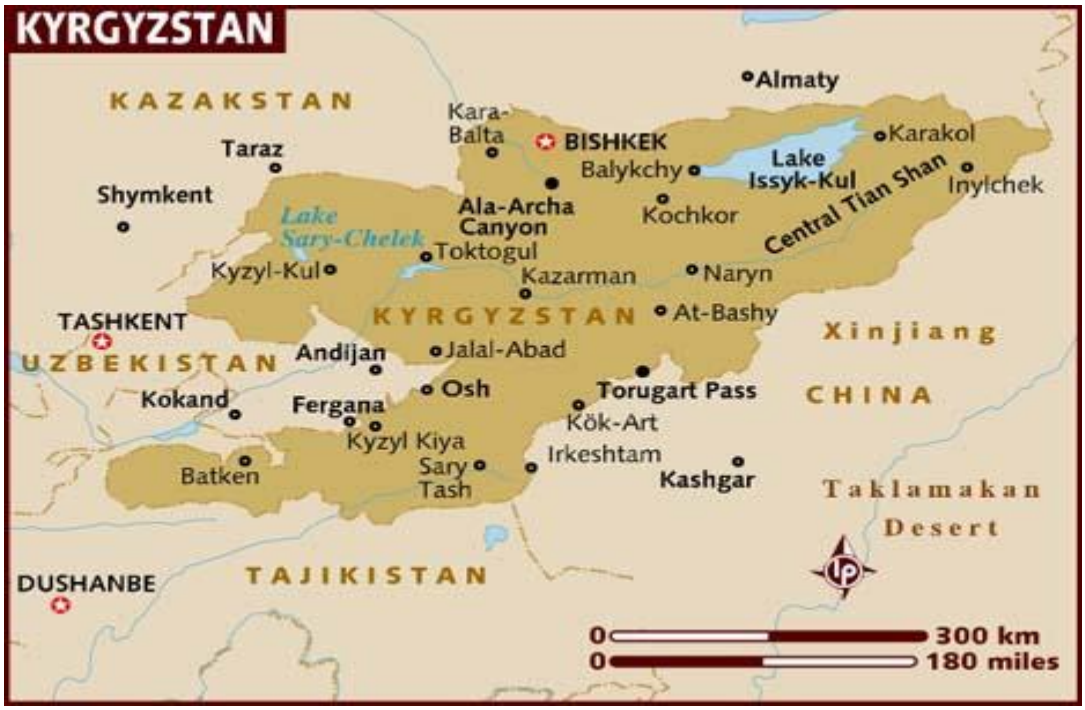

Figure 1. Map of Kyrgyzstan and its neighboring countries. Source: http://www.lonelyplanet.com/maps/asia/kyrgyzstan/

As part of the Vavilov's Central Asian Center of origin of crop species, Kyrgyzstan is rich in plant genetic resources including alfalfa and its wild relatives (source http://bio.su/dig_007_013.php). Kyrgyzstan was once a major producer of alfalfa for the former Soviet Union. In the 1980s, the country had regularly produced 7000 tonnes of seed annually, of which 5000 tonnes were exported to other former Soviet Union countries, mainly to Russia and the Baltic countries, as well as to the former GDR paragraph in the text): (source: http://www.agromarket.kg/index.php?option=com_content&view=article&id=174:2012-11-27-10-12-51&catid=17&Itemid=122)

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However, due to the collapse of the Soviet Union in 1990, the alfalfa production in Kyrgyzstan started declining and by 1999 the seed production had dropped to 313 tonnes (StatCom 1999). The present production of alfalfa for feed in Kyrgyzstan is about 163000 ha and the average hay yield is about 12 tonnes/ha (StatCom 2010. The potential to re-establish the alfalfa seed production for export to countries such as Kazakhstan, Russia, and Turkey as well as to Europe is high. In Kyrgystan, both the breeding program, and the *in situ* and *ex situ* conservation of alfalfa are conducted under the Kyrgyz Crop Research Institute, the Institute of Pastures and Forage Crops, the State Commission of Variety Testing Center, the Gene Bank and some seed farms.

Alfalfa breeding

Although the autotetraploid and the predominantly outcrossing nature of alfalfa present some difficulties in alfalfa breeding (Panella and Lorenzet.F 1966), breeders have been using selection to increase the frequency of desired agronomic characteristics (Flajoulot et al. 2005). Alfalfa breeding focused mainly on disease (e.g. Anthracnose, Downy Mildew) and pest resistance (e.g. Aphids), lodging as well as green mass quality and high forage yield (Irwin et al. 2001; Lonnet 1996; Volenec et al. 2002). Although alfalfa breeding is going on since 1919, its genetic improvement has been relatively low as compared to other major crops. For example, increasing seed yield through breeding has not been that successful and the yield is still very low (Casler 1997). This is regardless of the fact that breeders have made a great deal of efforts in collecting, assessing and comparing various alfalfa genetic resources worldwide. Alfalfa breeding in Kyrgyzstan is conducting by Kyrgyz Fodder & Pasture Institute. The institute has released the following varieties: Bereke, Buduchinya, Djulius, Jidurne, Manas, Mejotnenskaya, Tokmakskaya and Uzgenskaya. Nowadays, traits such as grazing resistance, trampling resistance, salt, drought and herbicide resistance are the focus of alfalfa breeders apart from yield and quality for efficient alfalfa germplasm utilization (Tentieva Batman, personal communication).

Synthetic varieties

Synthetic varieties are open pollinated populations developed through random mating of selected genotypes (Lonnquist 1961). Developing synthetic varieties through the use of full-sib and half-sib families or clones as parents is a commonly used breeding method in alfalfa (Flajoulot et al. 2005). Synthetic varieties have become increasingly favoured in alfalfa and other forage crops, mainly because it is cheaper than the development and use of hybrid varieties. Developing synthetic varieties also helps to minimize productivity loss with advancing generations of seed increase (Katepa-Mupondwa et al. 2002).

A synthetic variety is developed through intercrossing of several genotypes of known superior combining ability. Genotypes selected for synthetic variety development are those that are known to give superior hybrid performance when crossed in all combinations. Thus, properly selected male and female parents from diverse origin that can increase the possibility of heterosis when crossed are essential to successfully develop synthetic varieties. Therefore, before selecting clones for developing synthetic varieties, breeders should test the clonal progenies from polycross in yield trials, as indicated in (Evans et al. 1966; Hill et al. 1971; Busbice et al. 1974; Rowe and Gurgis 1982). Mass selection and phenotypic selection are the selection methods for synthetic variety development. Molecular markers have been widely used for genetic diversity studies and marker assisted selection for synthetic variety development (Maureira and Osborn 2005).

Detecting and quantifying genetic variation is an important step both for efficient conservation of the existing genetic resources of crop species and for selection of desirable materials for breeding. Highly reduced genetic diversity endangers the survival of the species under unfavorable environmental conditions, and thus promoting genetic diversity of useful plant species is very important (Gutierrez-Ozuna et al. 2009). DNA markers enable breeders to determine the presence of traits of interest in early stages of the development, unlike most agro-morphological and phenotypic markers (Tucak et al. 2008). Random amplified polymorphic DNA (RAPD), amplified fragment

length polymorphism (AFLP) and simple sequence repeats (SSR; also known as microsatellites) are among the commonly used DNA markers for genetic diversity analysis in various wild and cultivated species (Anthony et al. 2001; Sakai et al. 2001, Silvestrini et al. 2007, Moncada and McCouch, 2004). SSRs are often the markers of choice for genetic diversity studies mainly because of their amenability to high-throughput analysis, high polymorphism, abundance and co-dominant inheritance (Morgante and Olivieri 1993, Gupta and Varshney 2000; Suwabe et al. 2002). A large number of SSR markers have been developed and are being used for genetic mapping, marker assisted selection as well as for evaluation of the plant genetic diversity (Gupta and Varshney 2000).

Evaluation of alfalfa genetic diversity within and among populations plays a crucial role to minimize genetic erosion and to establish effective conservation strategies as well as for genetic improvement through breeding (Liu et al. 2007). Different molecular markers have been used to assess the genetic diversity in alfalfa (Flajoulot et al. 2005; Herrmann et al. 2010; Jenczewski et al. 1999; Sardaro et al. 2008). The application of SSR markers in tetraploid alfalfa for genetic diversity analysis has been proved suitable, as sufficiently high genetic variation has been detected within and among populations (Falahati-Anbaran et al. 2007; Diwan et al. 1997). However, there is little information regarding (1) the genetic diversity of alfalfa presently under cultivation in Kyrgyzstan and (2) the extent of genetic relationship between alfalfa grown in Kyrgyzstan and in other countries.

OBJECTIVES

The major objective of this Master thesis was to study the genetic diversity of alfalfa grown in Kyrgyzstan and determine its genetic relationship with alfalfa from other countries as well as to contribute to the development of synthetic alfalfa varieties for their use as forage in Kyrgyzstan; the ultimate goal of which is conservation and utilization of its genetic diversity. The following are the specific objectives of the study:

1. determine the genetic structure and the extent of genetic variation among Kyrgyz alfalfa accessions using SSR markers;
2. estimate the genetic relationship between alfalfa accessions from Kyrgyzstan and from other parts of the world using SSR markers;
3. characterize accessions of alfalfa originating from different countries for their desirable traits and for their use in the development of synthetic varieties;
4. develop synthetic-1 seeds based on genotypes with desirable traits

MATERIALS AND METHODS

Plant material

A total of seventy-eight cultivated alfalfa (*M. sativa*) samples representing sixty accessions from twenty-four countries were used in this study (Table 1). Of these accessions, six accessions were obtained from NordGen, twenty one accessions were obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA), thirty accessions were sampled from a field nursery in Kyrgyzstan where they were planted in two replications, and three accessions were collected from Sweden. Two samples obtained from Nordgen representing *M. lupulina* and *M. scutellata* were also included in this study for comparison.

DNA extraction

DNA from the thirty cultivated alfalfa accessions sampled from the field nursery in Kyrgyzstan was extracted from silica gel dried leaf tissue. For the remaining thirty cultivated alfalfa accessions and the two accessions of *M. lupulina* and *M. scutellata*, DNA was extracted from fresh leaf tissue after growing the seeds in pots in a greenhouse. In all cases, DNA was extracted individually from one to four plants per accession.

Individually sampled leaf tissue from the plants grown in the greenhouse was placed in 2 ml Eppendorf microcentrifuge tubes and immediately frozen in liquid nitrogen and then stored at -80°C until DNA extraction. The frozen samples and the silica gel dried leaf tissue were milled using a Retsch MM400 shaker (Haan, Germany) and DNA was extracted using a phenol-chloroform DNA extraction procedure: 600 µl of extraction buffer (pH 8.0) (consisting of 100 mM TrisHCl, 50 mM EDTA, 500 mM NaCl and 1% SDS) and 300 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added to the finely milled samples in the eppendorf tubes and the mixture was thoroughly vortexed. The samples were then centrifuged at 13400 rpm at room temperature for two minutes and the upper phase was transferred to new tubes. 300 µl phenol/chloroform/isoamyl alcohol was again added and mixed with the samples by gently flicking the tubes, and the samples were centrifuged for two minutes and the upper phase was transferred to new tubes. This was followed by the addition of 300 µl of chloroform to each sample, which was then centrifuged for two minutes and the upper phase transferred to new tubes. Ice-cold 99% ethanol was added to the samples in a 2:1 volume ethanol: sample ratio and the samples were left at room temperature for at least 15 minutes. Then, the samples were centrifuged for 10 minutes and the supernatant was removed by decanting. The pellet was washed twice with 500 µl 75% ethanol, and the samples were centrifuged for two minutes after each wash. Then, the tubes were left open in the fume-hood until the ethanol was completely evaporated and the samples were dry. The pellet was re-suspended in 50 µl of TE-buffer (pH 8) containing 20 µg/ml RNase. DNA quality and concentration was measured using a Nanodrop® ND-1000 spectrophotometer (Saveen Werner, Sweden).

PCR and electrophoresis

Twenty five SSR primer-pairs were initially screened for their good amplification and polymorphism as well as for their specificity to target loci. This led to the selection of ten primer-pairs for final analysis (Table 2).

The PCR reactions were carried out in a volume of 25 µl containing 25 ng genomic DNA, 0.3 µM forward and reverse primers, 0.3 mM dNTPs, 1 U Taq DNA polymerase (Saveen Werner AB), and 1×PCR buffer (20 mM Tris-HCl pH 8.55, 16 mM $(NH_4)SO_4$, 0.01% Tween®20 and 2 mM $MgCl_2$). The reactions were performed in a GeneAMP PCR system 9700 thermocycler using the following temperature profiles: initial denaturation at 95°C for 3 min, followed by 40 cycles of 30 sec denaturation at 94°C, 30 sec annealing at the optimized annealing temperature (*Ta*) for each primer-pair, and 45 sec primer extension at 72°C. This was followed by a 20 min final extension at

Table 1. List of *Medicago sativa* accessions used for the SSR-based genetic diversity analysis. The countries of origin and the supplying countries/institutions are given.

Accession name	Country of origin	Supplying countries/ institutions	Accession name	Country of origin	Supplying countries/ institutions
101-082	Afghanistan	ICARDA	90Z-5[2]	Kyrgyzstan	Kyrgyzstan
101-156	Afghanistan	ICARDA	Bereke	Kyrgyzstan	Kyrgyzstan
101-112	Algeria	ICARDA	BM-5	Kyrgyzstan	Kyrgyzstan
101-322	Argentina	ICARDA	BM-9/4	Kyrgyzstan	Kyrgyzstan
101-270	Australia	ICARDA	CIN-3[2]	Kyrgyzstan	Kyrgyzstan
101-186	Azerbaijan	ICARDA	G-712/5[2]	Kyrgyzstan	Kyrgyzstan
Hornet[3]	Canada	Kyrgyzstan	Manas	Kyrgyzstan	Kyrgyzstan
Longview[2]	Canada	Kyrgyzstan	T-206[4]	Kyrgyzstan	Kyrgyzstan
Rambler [3]	Canada	Kyrgyzstan	Uzgenskaya	Kyrgyzstan	Kyrgyzstan
101-406	China	ICARDA	Live	Norway	Nordgen
Veco	Denmark	Nordgen	113-378	Oman	ICARDA
JDL-5[2]	Denmark	Kyrgyzstan	101-242	Pakistan	ICARDA
101-456	Egypt	ICARDA	113-462	Pakistan	ICARDA
101-460	Egypt	ICARDA	Humlelucern[a]	Sweden	Nordgen
Jokioinen	Finland	Nordgen	Jenny	Sweden	Nordgen
Concerto[3]	France	Kyrgyzstan	Sverre	Sweden	Nordgen
Europe	France	Kyrgyzstan	SW Lesina	Sweden	Kyrgyzstan
Luzelle	France	Kyrgyzstan	SW Pondus	Sweden	Sweden
Meldor	France	Kyrgyzstan	101-027	Syria	ICARDA
Midi	France	Kyrgyzstan	131-385	Tajikistan	ICARDA
Symphonie[2]	France	Kyrgyzstan	137-866	Tajikistan	ICARDA
Timbale	France	Kyrgyzstan	Vakhskaya 233	Tajikistan	Kyrgyzstan
Provence	France	Nordgen	Vakhskaya 429	Tajikistan	Kyrgyzstan
Zenith	France	Kyrgyzstan	Vakhskaya 454	Tajikistan	Kyrgyzstan
Snail[b]	Germany	Nordgen	Vakhskaya 455[2]	Tajikistan	Kyrgyzstan
101-095	Iran	ICARDA	Vakhskaya 478[2]	Tajikistan	Kyrgyzstan
101-068	Iraq	ICARDA	100-985	Turkey	ICARDA

JDL-4	Japan	Kyrgyzstan	101-330	Turkey	ICARDA
101-191	Kazakhstan	ICARDA	Amer Stand 801S[2]	USA	Kyrgyzstan
137-664	Kazakhstan	ICARDA	Guardsman	USA	Sweden
90B-4	Kyrgyzstan	Kyrgyzstan	Seedway	USA	Sweden

[a] *Medicago lupulina*; [b] *Medicago scutellata*. Numbers given as superscripts refer to the number of plants analyzed from corresponding accessions using microsatellite markers. Accessions without superscripts were represented by only one sample.

72°C. The annealing temperature (T_a) was changed based on the melting temperature (T_m) of each primer-pair (Table 2).

Data scoring and statistical analysis

Prior to electrophoresis on polyacrylamide gels, amplification was confirmed by running 5 µl of the PCR products on 1.5% agarose gels and visualized using ethidium bromide. Confirmed amplified PCR products were separated on readymade high resolution polyacrylamide gels (ETC Electrophorase-technik, Germany) and the gels were stained using DNA silver Staining Kit (GE Healthcare Bio-Sciences AB, Sweden), as described in Geleta and Bryngelsson (2009). Allelic data was recorded for each locus as fragment size in comparison with a standard 100 bp DNA ladder. Alleles were also binary coded as present (1) or absent (0) in each genotype and these data was used for calculation of the Jaccard similarity coefficient and for cluster analyses. POPGENE version 1.31 (Yeh and Boyle 1997) was used for analysis of number and percentage of polymorphic loci, observed number of alleles, effective number of alleles (Kimura and Crow 1964), gene diversity (Nei 1973) and Shannon information index (Lewontin 1972). Mintab 15 statistical software was used for Pearson correlation analysis.

Genetic variation of alfalfa accessions grouped based on country of origin was estimated through analysis of molecular variance (AMOVA) using the Arlequin 3.0 program (Excoffier al. 2005). Cluster analysis based on unweighted pair group method with arithmetic average (UPGMA) using sequential agglomerative hierarchical nested (SAHN) was performed using the Jaccard similarity matrix (NTSYSpc; Rohlf 1998). The bootstrap values for Nei's standard genetic distance based cluster analysis, as an estimate of robustness of obtained trees, were obtained through the 1000

bootstrap resampling procedure using FreeTree–Freeware program (Pavlicek et al. 1999). TreeView (Win32) 1.6.6 program was used to view the trees (Page, 1996).

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Table 2. The sequences of the SSR primers used in the present study, the fragment size range previously reported and the number and size of alleles revealed in the present study

Primer name	Repeat motif	Forward primer (5'—3')	Reverse primer(5'—3')	T_m (°C)	ASR^{prs}	ASR^{pst}	N_a
MTIC-189	TC	CAAACCCTTTTCAATTTCAACC	ATGTTGGTGGATCCTTCTGC	56	133-173[a]	110-165	8
FMT-13	(GA)GG(GA)	GATGAGAAAATGAAAAGAAC	CAAAAACTCACTCTAACACAC	54	162-204[a]	133-165	6
B14B03	CA	GCTTGTTCTTCTTCAAGCTCAC	CTGACTTGTGTTTTATGC	54	163-215[a]	140-160	3
AFCA-11	CA	CTTGAGGGAACTATTGTTGAGT	AACGTTTCCCAAAACATACTT	56	136-160 [b]	130-145	5
AFCT-45	(CT)AT(CT)	TAAAAAACGGAAAGAGTTGGTTAG	GCCATCTTTTCTTTTGCTTC	58	123-145[b]	140-180	7
AAC-008	(AC)(TA)	ACTCTTAGGAGCAGGATCAC	GCAGGAGCTCTAGTGGTATG	58	231[c]	150-190	5
ACT-009	CT	AAGCAACCGAACAACGATTT	AGTGACAGTTATGGGGGTGG	58	203[c]	180-240	7
AGGT-004	(GGT)(CAA)(TCC)	AAATGATATTGTAGGAAGATCGTGC	AGTTATGAGGGAGCACCGAG	58	164[c]	175	1
AGAT-012	GAT	GATAGTCCGTACCTTGGCTCTG	TGTTCAGCTCTTCATCTACATCTTC	58	106[c]	65-80	9
ACT-026	CT	AAACCACCCTTCCATCTTCC	AGGGTGGAGAAGAAGCATGA	56	222[c]	215-245	2
mean							5.3

ASR^{prs} = Allele size range previously reported: [a]Flajoulot et al. (2005), [b]Diwan et al. (1997), [c]He et al. (2009). ASR^{pst} = Allele size range in the present study. N_a = number of alleles. The number of polymorphic loci is 9 and the overall percentage of polymorphic loci is 90%.

Field trial and data scoring

Field trial for the development of synthetic varieties was conducted at the nursery of Forage and Pasture Institute (Ministry of Agriculture of Kyrgyzstan), which was isolated from other alfalfa cultivations in March 2008. The trial site was located in the northern part of Bishkek in the middle of the Chui Oblast valley, where irrigation is available. The field trial site was located in the agro-climatic zone characterized by grey-brownish and light chestnut soils, with the altitude ranging from 800 m to 1000 m (42°58'06N and 74°28'40E). The field size was 5000 square meter (100 m X 50 m). The nursery contained 54 accessions (Table 3) in two replications and each variety was represented by 30 plants in each replication. Seeds from each accession were planted in three rows with ten plants in each row at a distance of 60 cm between plants.

Table 3. List of alfalfa varieties planted at the nursery of Forage and Pasture Institute and used for the selection of desirable genotypes for the development of synthetic varieties

Variety name	Country of origin	Variety name	Country of origin	Variety name	Country of origin
Bereke	Kyrgyzstan	Vakhskaya 478	Tajikistan	JDL 4	Japan
Manas	Kyrgyzstan	Vakhskaya 454	Tajikistan	JDL 5	Denmark
Europe	France	Approved	Canada	JDL 6	USA
T – 206	Kyrgyzstan	Hornet	Canada	JDL 7	USA
BM – 5	Kyrgyzstan	Longview	Canada	JDL 8	Argentina
MB – 5	Kyrgyzstan	Rambler	Canada	JDL 9	Argentina
G - 712/5	Kyrgyzstan	Concerto	France	JDL 10	Spain
90 B - 4	Kyrgyzstan	CIN - 3	Kyrgyzstan	Coussouls	France
90 Z- 5	Kyrgyzstan	Symphonie	France	Apica	USA
BM- 9/4	Kyrgyzstan	Zenith	France	Live	Norway
Vakhskaya 233	Tajikistan	Luzelle	France	SW Jenny	Sweden
Vakhskaya 300b	Tajikistan	Meldor	France	SW Julus	Sweden
Vakhskaya 300c	Tajikistan	Melissa	France	SW Lesina	Sweden
Vakhskaya 416	Tajikistan	Midi	France	SW Nexus	Sweden
Vakhskaya 429	Tajikistan	Timbale	France	DS 9705	USA
Vakhskaya 430	Tajikistan	JDL 1	Poland	DS 9706	USA
Vakhskaya 455	Tajikistan	JDL 2	Italy	Amer Stand 801 s	USA
Vakhskaya 474	Tajikistan	JDL 3	Italy	FG ST 62T035/5074 - 5	USA

Vakhskaya 300**b =basic seed;** Vakhskaya 300**c = commercial seed**

Notes were taken on flower color, height, leaf size, stem stiffness, plant healthiness, vigor and plant type, as these traits were used in official variety descriptions of alfalfa. The height was measured in cm, and the other characters were scored on a 1 – 9 scale where 9 is the best or biggest. Plant type was scored from 1 = prostrate to 9 = erect plant. Flower color was scored as 1= purple (light purple, purple, dark purple) and 2 = blue (light blue, blue, dark blue).

RESULTS

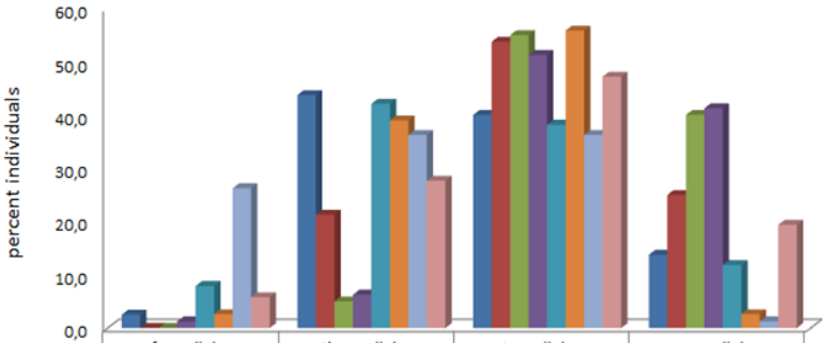
SSR markers

Out of the 10 loci analyzed, nine loci were polymorphic whereas only one allele was detected across all the 78 cultivated alfalfa individual plants at the locus *AGGT-004* (Table 2). Thus, the overall percentage of polymorphic loci was 90%. Three of the ten loci produced less than four alleles (Table 2) across the 78 individual plants. The average number of individuals with one, two, three and four alleles per locus for the remaining seven loci was 19.4%, 47.2%, 27.7% and 5.8%, respectively (Figure 2). A total of 53 alleles were recorded across the ten loci, with an average of 5.3 alleles per locus (Table 2). The number of alleles (N_a) for the nine polymorphic loci varied from two (locus *ACT-026*) to nine (locus *AGAT-012*). The number of alleles detected at the loci *B14B03*, *AAC-008*, *AFCA-11*, *FMT-13*, *ACT-009*, *AFCT-45* and *MTIC-189* was 3, 5, 5, 6, 7, 7 and 8, in that order.

At locus *ACT-026*, the frequency of the dominant allele was 88%. At locus *B14B03*, the dominant allele accounted for 78% of the total number of alleles, but also a rare allele with the frequency of l% was detected at this locus. In *AGAT-012*, only three of the nine alleles had a frequency of more than 10%, the highest being 33% (Table 4). Four of the alleles at this locus had a frequency of only 1%.

Table 4. Allele frequencies of the ten SSR loci across the 78 alfalfa individual plants

Alleles	AAGT-004	ACT-026	B14B03	AAC-008	AFCA-11	FMT-13	ACT-009	AFCAT-45	MTIC-189	AGAT-012
Allele1	1.00	0.88	0.01	0.33	0.02	0.03	0.12	0.04	0.14	0.25
Allele2	-	0.12	0.21	0.35	0.46	0.07	0.05	0.10	0.11	0.01
Allele3	-	-	0.78	0.12	0.34	0.43	0.26	0.47	0.19	0.26
Allele4	-	-	-	0.10	0.13	0.26	0.19	0.08	0.11	0.06
Allele5	-	-	-	0.10	0.05	0.13	0.30	0.06	0.10	0.06
Allele6	-	-	-	-	-	0.08	0.05	0.18	0.13	0.33
Allele7	-	-	-	-	-	-	0.03	0.07	0.15	0.01
Allele8	-	-	-	-	-	-	-	-	0.07	0.01
Allele9	-	-	-	-	-	-	-	-	-	0.01



	four alleles	three alleles	two alleles	one allele
AAC-008	2.5	43.8	40.0	13.8
AFCA-11	0.0	21.3	53.8	25.0
AFCAT-45	0.0	5.0	55.0	40.0
FMT-13	1.3	6.3	51.3	41.3
MTIC-189	7.9	42.1	38.2	11.8
ACT-009	2.6	39.0	55.8	2.6
AGAT-12	26.3	36.3	36.3	1.3
Mean	5.8	27.7	47.2	19.4

Figure 2. A graph showing the percentage of individuals with different number of alleles per locus for the seven SSR loci at which more than four alleles were detected across the samples studied.

Table 5. Sample size, observed and effective number of alleles, Nei's gene diversity (h), Shannon information index (I) and the proportion of observed heterozygotes for the ten SSR loci studied

Locus	sampe size	*na*	*ne*	*h*	*I*	*%H*
AAGT-004	80	1.0	1.0	0.0	0.0	0.00
ACT-026	78	2.0	1.3	0.2	0.2	0.42
B14B03	77	3.0	1.5	0.3	0.5	0.32
AAC-008	80	5.0	2.8	0.6	1.1	0.89
AFCA-11	80	5.0	2.5	0.6	1.0	0.75
FMT-13	80	6.0	2.8	0.6	1.2	0.59
ACT-009	77	7.0	2.9	0.6	1.3	0.97
AFCAT-45	80	7.0	2.9	0.6	1.3	0.60
MTIC-189	76	8.0	4.8	0.8	1.7	0.88
AGAT-012	80	9.0	2.0	0.4	0.8	0.99
Mean	79,0	5.3	2.4	0.47	0.90	0.64

na = observed number of alleles, *ne* = effective number of alleles (Kimura and Crow 1964) , *h* = gene diversity (Nei 1973); *I* = Shannon information index (Lewontin 1972).

Two alleles at *AFCA-11*, three alleles at *FMT-13*, three alleles at *ACT-009*, four alleles at *AFCAT-45* and one allele at *MTIC-189* have allele frequency of less than 10%. All alleles at the locus *AAC-008* have a frequency of more than 10%. In *AFCA-11*, three of the five alleles, have a frequency of more than 10%. Of the seven alleles detected at the locus *AFCAT-45*, the dominant allele has a frequency of 47%. At locus *FMT-13*, the highest allele frequency was 43%. The analyses of Nei's gene diversity and the Shannon information index revealed the highest diversity at the locus *MTIC-189* (h = 0.8; I = 1.7), which also had the highest number of effective alleles (ne = 4.8) (Table 5). Among the polymorphic loci, the lowest diversity was detected at the locus *ACT-026* with both gene diversity and Shannon information index of 0.2 (Table 5). Although *AGAT-012* had the highest number of alleles (na = 9), the estimates of both the gene diversity and the Shannon information index was about half of those of *MTIC-189* (na = 8) due to the fact that most of the alleles at *AGAT-012* were present at low frequencies (Table 4). The Pearson's correlation coefficients between *na* and *h* (r = 0.77; P = 0.005), *na* and *I* (r = 0.82; P = 0.02), *na* and *%H* (r = 0.86; P = 0.001), *h* and *I* (r =

0.98; P < 0.001), *h* and *%H* (r = 0.79; P = 0.004) and *I* and *%H* (r = 0.76 = 0.006) were highly significant.

Genetic variation within and among groups of accessions grouped according to countries of origin

The genetic differentiation of the accessions grouped according to countries of origin was estimated based on the analysis of molecular variance (AMOVA; Excoffier et al. 2005). When all sixty accessions were included, five percent of the total genetic variation was attributed to the between-countries of origin-component (F_{ST} =0.05; P = 0.005) (Table 6). The remaining 95% of the total genetic variation was attributed to the within-countries-component. When 12 countries represented by a single accessions were excluded, the differentiation among the remaining 12 countries was not significant (F_{ST} = 0.01; P = 0.246). AMOVA was also conducted by grouping the plants into accessions from Western and Eastern countries. In this analysis, the variation among the groups was only 0.08%, which was not statistically significant (P = 0.14; Table 6). Other groupings according to geographic areas, such as continents also did not result in a significant differentiation among groups.

Genetic distance between accessions grouped according to countries of origin

The Nei's standard genetic distance (Nei, 1972) was calculated for 24 groups of accessions that were grouped according to countries of origin. The genetic distance between countries ranged from 0.03 (France vs Kyrgyzstan) to 1.00 (Syria vs Argentina) which is more than a 30-fold variation (Table 7). Only 5.4% of the 276 pairs have a genetic distance of less than 0.1. In general, the accessions from France and Kyrgyzstan were the least differentiated with an average genetic distance of 0.18 from all other accessions. On the other hand, the accession from Argentina (accession *101-322*) was the most differentiated with an average genetic distance of 0.67 from other accessions. The mean Nei's genetic distance between *M. lupulina* and *M. sativa*, and between *M. scutellata* and *M. sativa* was 0.76 and 0.73, respectively. The mean genetic distance between *M. lupulina* and *M. scutellata* was 0.59. The result shows a high differentiation of accession *101-322* from the remaining *M. sativa*

accessions, as its average genetic distance to the rest is only slightly lower (0.67) than the average genetic distance between *M. sativa* and *M. lupulina* (0.76) and between *M. sativa and M. scutellata* (0.73).

Table 6. Partitioning of SSR-based total genetic variation of alfalfa accessions into within and among countries of origin based on AMOVA (a) when all accessions were included; (b) when only countries represented by more than one accession were included; (c) when accessions were grouped into Western and Eastern accessions.

Groups	Sources of variation	Degrees of freedom	Variance components	Percentage of variation	Fixation index	P-value
By grouping the accessions according to countries of origin[a]	AC	24	Va = 0.35	5.00	F_{ST} = 0.05	Va and F_{ST} = 0.005
	WC	55	Vb = 6.73	95.00		
	Total	79	7.09			
By grouping the accessions according to countries of origin[b]	AC	5	Va = 0.05	1.12	F_{ST} = 0.01	Va and F_{ST} = 0.246
	WC	49	Vb = 4.24	98.88		
	Total	54	4.29			
By grouping the accessions according to countries of origin and then into eastern and western countries	AG	1	Va = 0.06	0.08	F_{SC} = 0.0495 F_{ST} = 0.0503 F_{CT} = 0.0008	Vc and F_{ST} = 0.008 Vb and F_{SC} = 0.01 Va and F_{CT} = 0.14
	ACWG	23	Vb = 0.35	4.95		
	WC	25	Vc = 6.73	94.97		
	Total	49	7.14			

[a] when all accessions were included; [b] when only accessions from countries represented by more than one accession were included. AC = among countries; WC = within countries; AG = among groups from eastern and western countries; ACWG = Among countries within groups. Note: Other groupings according to geographic areas, such as continents did not result in a significant differentiation among groups.

Table 7. Nei's standard genetic distance between alfalfa accessions from 24 countries around the world

	JAP	FIN	ALG	IRQ	ARG	SYR	SWE	EGY	IRN	USA	AUS	CAN	DEN	CHN	AZE	OMA	AFG	PAK	KYR	FRA	NOR	TAJ	TUR
FIN	0.30																						
ALG	0.51	0.23																					
IRQ	0.44	0.54	0.60																				
ARG	0.83	0.88	0.83	0.57																			
SYR	0.18	0.18	0.27	0.50	1.00																		
SWE	0.11	0.33	0.40	0.32	0.55	0.21																	
EGY	0.18	0.47	0.44	0.43	0.46	0.30	0.09																
IRN	0.39	0.45	0.43	0.30	0.66	0.43	0.23	0.35															
USA	0.20	0.14	0.22	0.42	0.62	0.09	0.17	0.21	0.30														
AUS	0.25	0.47	0.59	0.40	0.51	0.40	0.26	0.21	0.57	0.27													
CAN	0.19	0.28	0.35	0.21	0.62	0.17	0.10	0.19	0.20	0.11	0.32												
DEN	0.16	0.24	0.34	0.44	0.57	0.13	0.14	0.13	0.39	0.08	0.20	0.18											
CHN	0.52	0.14	0.24	0.49	0.85	0.27	0.46	0.49	0.45	0.19	0.60	0.34	0.26										
AZE	0.35	0.28	0.28	0.51	0.59	0.20	0.30	0.27	0.49	0.15	0.39	0.24	0.22	0.31									
OMA	0.37	0.71	0.91	0.62	0.51	0.49	0.38	0.39	0.76	0.42	0.41	0.49	0.30	0.77	0.41								
AFG	0.12	0.33	0.45	0.30	0.86	0.13	0.15	0.19	0.34	0.18	0.26	0.15	0.16	0.35	0.29	0.45							
PAK	0.39	0.17	0.22	0.40	0.72	0.23	0.32	0.40	0.44	0.17	0.53	0.24	0.25	0.23	0.22	0.45	0.38						
KYR	0.14	0.21	0.26	0.29	0.44	0.11	0.08	0.10	0.23	0.06	0.21	0.07	0.08	0.27	0.15	0.33	0.13	0.23					
FRA	0.11	0.19	0.27	0.29	0.57	0.12	0.07	0.12	0.19	0.06	0.25	0.09	0.06	0.24	0.21	0.33	0.13	0.21	0.03				
NOR	0.42	0.30	0.45	0.25	0.68	0.47	0.26	0.51	0.23	0.33	0.57	0.20	0.49	0.40	0.56	0.92	0.41	0.38	0.31	0.29			
TAJ	0.19	0.22	0.26	0.36	0.60	0.11	0.14	0.15	0.31	0.11	0.29	0.17	0.08	0.24	0.17	0.33	0.13	0.21	0.07	0.07	0.45		
TUR	0.17	0.30	0.40	0.40	0.95	0.08	0.16	0.22	0.35	0.15	0.37	0.17	0.11	0.30	0.33	0.44	0.09	0.33	0.13	0.10	0.46	0.09	
KAZ	0.26	0.22	0.28	0.34	0.64	0.15	0.21	0.26	0.41	0.13	0.31	0.15	0.17	0.31	0.15	0.40	0.23	0.13	0.12	0.14	0.40	0.19	0.27

Note: The mean Nei's standard genetic distance between *M. lupulina* and *M. sativa* and between *M. scutellata* and *M. sativa* included in this analysis was 0.76 and 0.73 respectively. The mean genetic distance between *Medicago lupulina* and *M. scutellata* was 0.59.

Cluster analysis

A cluster analysis was conducted on the 78 samples of *M. sativa*. The two wild species *M. lupulina* and *M. scutellata* were included as out-groups. In this analysis, 13 out of the 60 accessions were represented by more than one individual. In most cases, individual plants from the same accessions were clustered together. The four samples of *T-206*, the two samples of *G-712/5* and the two samples of *90Z-5, all* from Kyrgyzstan, the three samples of *Concerto* and the two samples of *Symphonie* from France, the two samples of *Vakhskaya-455* from Tajikistan, and the two samples of *Amer-Stand 801S* from USA were all placed within Cluster I (Figure 3). Among this group, the samples of *Vakhskaya-455*, *G-712/5* and *90Z-5* were clustered closely together according to their corresponding accessions. The two samples of accession *Longview* from Canada and the two samples of *Cin-3* from Kyrgyzstan were also clustered closely together within Cluster III. On the other hand, the two samples of *Vakhskaya-478* from Tajikistan (Clusters I and V) and the three samples of *Hornet* (Clusters I and IV) and the three samples of *Rambler* (Clusters II and IV) were each placed under two different clusters. Generally, the cluster analysis revealed a poor clustering pattern of accessions according to countries of origin. Several cases in which accessions from different countries clustered closely together and accessions from the same country were placed in different clusters were observed (Figure 3).

The average Jaccard similarity coefficients between (1) the 20 samples of *M. sativa* from Nordgen and Sweden representing nine accessions; (2) the 21 samples of *M. sativa* from ICARDA representing 21 accessions; (3) the 37 samples of *M. sativa* from Kyrgyzstan representing 30 accessions were 0.50, 0.44 and 0.48, respectively. The average Jaccard similarity coefficients between (1) the Nordgen and ICARDA accessions; (2) Nordgen and Kyrgyzstan; and (3) ICARDA and Kyrgyzstan accessions were 0.42, 0.43 and 0.44, respectively. The Jaccard similarity between the four individual plants of accession *T-206* varied from 0.39 to 0.62. On the other hand, the Jaccard

similarity coefficient between the two individuals from accession *Vakhskaya-455* was 0.68. The average similarity between individuals of the accession *T-206* (0.49) was only slightly higher than the overall average for the seventy eight individuals investigated (0.45).

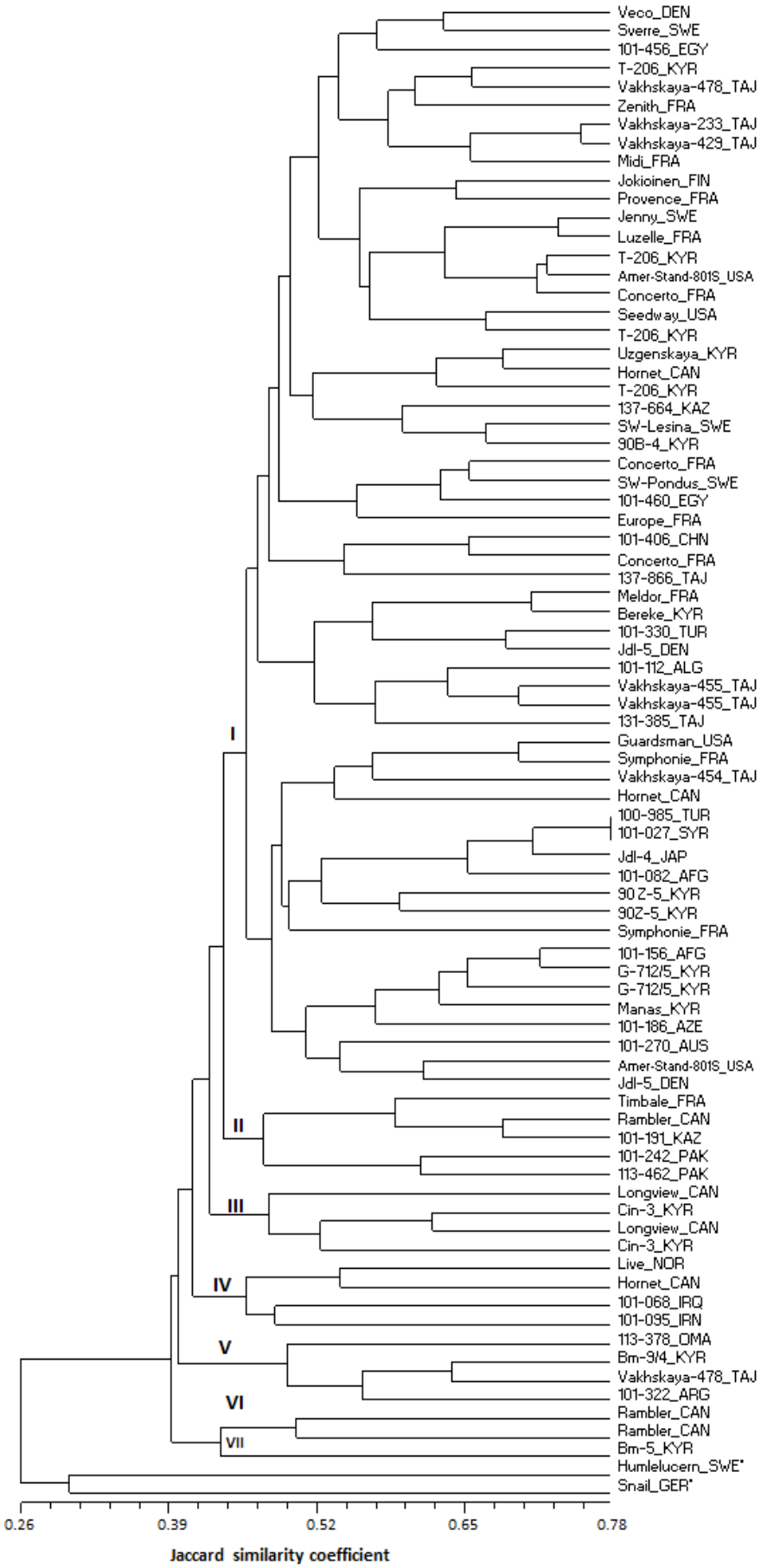


Figure 3. UPGMA phenogram of 78 *M. sativa* samples representing 24 countries based on Jaccard similarity coefficient. GER* and SWE* are *M. lupulina* and *M. scutellata* samples, originally from Germany and Sweden, included as outgroups.

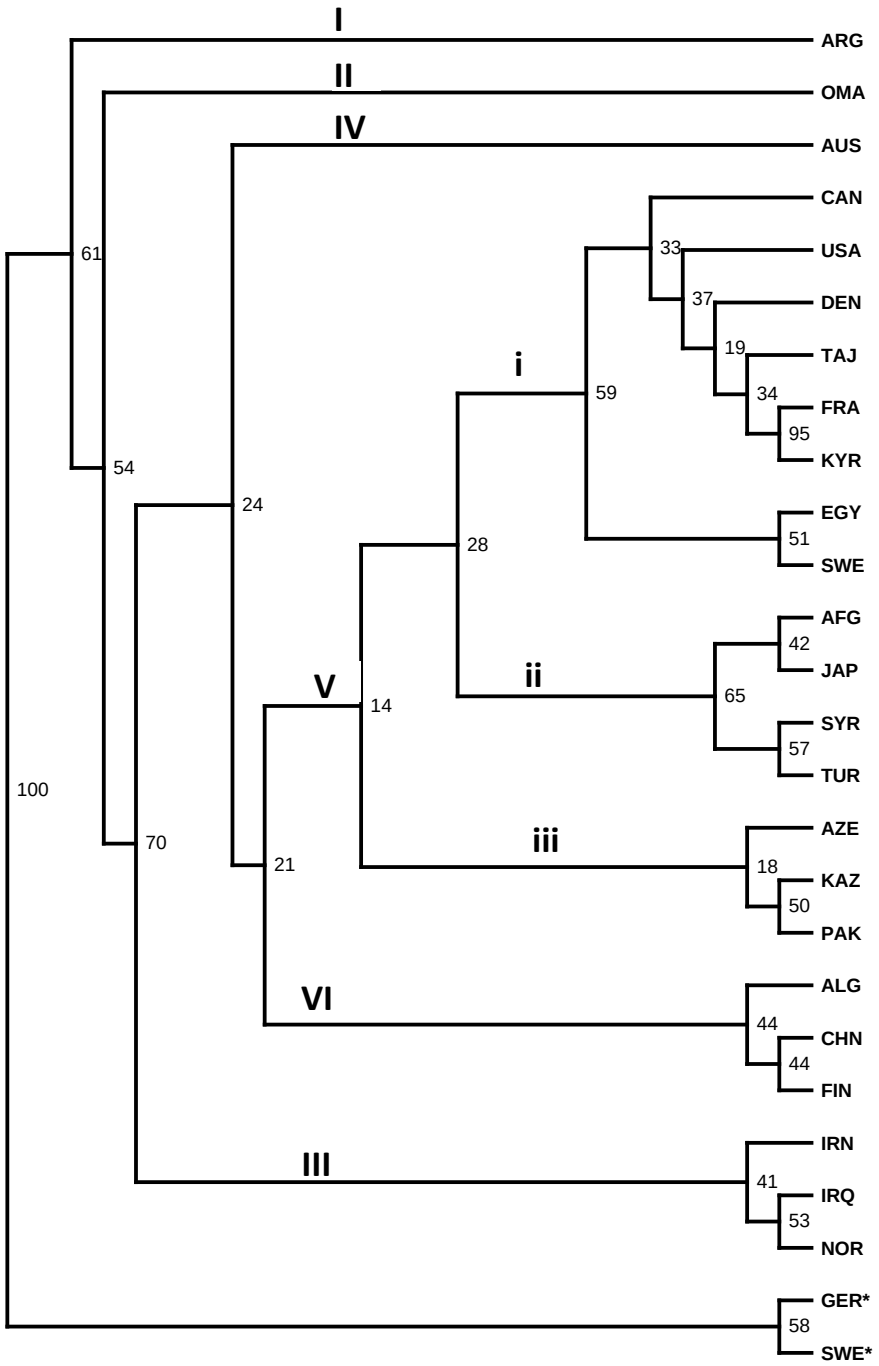


Figure 4. UPGMA phenogram for *M. sativa* accessions representing 24 countries based on Nei's standard genetic distance. Numbers in front of the branches are bootstrap values. GER* and SWE* are *M. lupulina* and *M. scutellata* samples, originally from Germany and Sweden, included as outgroups. Note: Countries' names were abbreviated by taking the first three letters except in the case of IRN, IRQ and CHN that stand for Iran, Iraq and China (See Table 1 for full names of countries).

The cluster analysis revealed a slightly lower genetic similarity among different groups of accessions as compared to the level of genetic similarity within groups of accessions. However, the difference

was not that high enough to show a clear clustering pattern of accessions according to their groups (Figure 3). Cluster analysis was also conducted by grouping the accessions into countries of origin (Figure 4). *Medicago lupulina* (SWE*) and *M. scutellata* (GER*) were used as out-groups also in this analysis (Figure 3). The accession from Argentina was separated from the rest of the accessions first, with a bootstrap support of 61% (Cluster I), followed by the accession from Oman, with a bootstrap support of 54% (Cluster II). Accessions from Iran, Iraq and Norway were clustered in Cluster III with a bootstrap support of 70% whereas the accession from Australia was placed separately under Cluster IV although the bootstrap support is low (24%). Cluster V was the largest cluster, with low bootstrap support, and comprised a group of accessions from 15 countries. This cluster was further grouped into three sub-clusters, but with low bootstrap support. Sub-clusters i, ii and iii comprised groups of accessions from 8, 4 and 3 countries, in that order. The last cluster, Cluster VI comprised accessions from Algeria, China and Finland. All countries represented by more than one accession were placed under Cluster V.

Genotype selection for the development of synthetic varieties

Table 8 shows the variation in eight traits of interest scored in June 2009 among the nursery plants. Data collected in June 2008 showed that plant establishment ranged from 18% (in Meldor) to 77% (in Zenith) with an average establishment rate of 48%. There was also a large variation among the plots in terms of establishment that ranged from 7 to 97%. The score for vigor, which was the most important trait, increased from 4.62 in June 2008, the planting year, to 7.46 in June 2009 when the first selection of entries for the syn-1 plantation took place (in a 1-9 scale). Data on the vigor before each cut was recorded instead of weighing the green matter. The number of superior plants from each variety was recorded in order to identify the over-all number of superior plants at the end. Plants from different entries having similar phenotypes and a good performance over the years were propagated by stem cuttings and planted in isolation for seed set by cross pollination. Figures 5-7 represent some of the field activities conducted for the development of synthetic varieties.

Table 8. Variation among the nursery plants in eight traits on interest when scored in June 2009

Trait	Score range
Flower color	Light blue - dark violet
Plant height	22 – 135 cm
Leaf size	2 – 9
Stem stiffness	3 – 9
Resistance to diseases	2 – 9
Resistance to pest	4 – 9
Plant vigor	1 – 9
Plant type	1 - 9

Note: the score for resistance to disease and pest is based on general assessment of plant health and presence/absence of pests

On September 29, 2009 extreme plants with small (9 plants with an average score of 2.9) and large leaves (12 plants with an average score of 8.8) were identified. At the same time a preliminary selection of 41 plants to be used in the final synthetic was done in several steps. In the first step plants were selected with a vigor note of 8 or 9. The number of selected plants was 204. Then, in the second step plants with a height taller than 80 cm were selected. The third step selection was for medium sized leaves (score 5-6). Small leaves gave a lower green matter yield, and a large leaf size tends to be in connection with weak winter hardiness. The fourth step was to select for healthy plants with best possible disease and pest resistance (score 7-9). This selection further reduced the number of target plants to forty-one. However, some of these plants did not survive the winter and 26 were available for stem cuttings in spring 2010, and in some cases other plants had to be selected for the synthetic plantation. Overall, 26 plants were selected and their stem cuttings were taken in May 2010.

For making a stem cutting, an alfalfa plant in vigorous growth is used. The best season is in spring with approx. 50 cm tall stems. An internode from the stem is taken by cutting it off just under a leaf

and cutting it off above the next leaf. The lower leaf is removed, and the upper leaf is reduced to just one or two leaflets. Then the stem cutting is planted 2-3 cm deep in a sand-soil mixture. It should preferably be covered by a plastic sheet to reduce evaporation. Generally, roots develop after five weeks and the stem cutting can be transplanted into the field.

Table 9. List of accessions from which the 26 plants were selected for the development of synthetic varieties and the corresponding number of selected and survived plants from each variety

Variety	No of plants selected for stem cuttings	Number of plants with surviving stem cuttings	Total number of stem cuttings that survived
90 Z - 5	3	1	3
Approved	1	0	0
Bereke	2	2	6
BM - 5	1	0	0
BM - 9/4	1	0	0
Coussouls	1	1	2
DS 9706	2	2	3
G - 712/5	1	0	0
Hornet	1	1	1
JDL 4	2	1	8
JDL 6	1	0	0
JDL 8	1	0	0
Live	1	0	0
Manas	2	1	5
MB - 5	1	0	0
T 206	1	1	6
Vakhskaya 416	1	0	0
Vakhskaya 429	1	0	0
Vakhskaya 454	1	0	0
Vakhskaya 478	1	0	0
Total	26	10	34

Note: the 34 plants grown from stem cuttings were transplanted 5 weeks after stem cuttings.

Out of the 26 selected samples genotypes, 12 were from Kyrgyz alfalfa accessions suggesting that these alfalfa accessions are well adapted to the environmental conditions in Kyrgyzstan. The remaining plants were from France (1), Tajikistan (4), Canada (2), Japan (2), USA (3), Argentina (1) and Norway (1). 24 stem cuttings were taken from each of the 26 selected plants. Unfortunately, the survival of these stem cuttings were quite low (only about 20 %).

Figure 5. The Bishkek nursery planted in March 2008; the photo was taken in September 2009

Due to the low survival rate of the stem cuttings, only ten selected plants had surviving stem cuttings in 2011 and 2012 (Table 9). However, for creating a synthetic variety, 10 entries are enough, especially when taking into consideration the diverse origin of these 10 plants. Five of them were from Kyrgyzstan, two from USA, and one each from Japan, France and Canada. The average vigor score of the surviving plants was 8.5.

Figure 6. Regenerated stem cuttings growing in plastic cups for root development.

Figure 7. Alfalfa clones from stem cuttings that were transferred to a plot after being grown in plastic cups for five weeks. The plants were covered by plantain's leaves to protect them from direct sun. The photo was taken on the same day of planting

DISCUSSION

Alfalfa is the most cultivated forage legume due to its wide adaptation to diverse environmental conditions and efficient nitrogen fixation in symbiosis with *Rhizobium meliloti* (Kalo et al. 2000; Liu et al. 2007). The analysis of its genetic diversity helps to assess future risk of genetic erosion and to develop sustainable conservation strategies and wise use of its diversity in breeding programs. The results of the SSR based genetic diversity analysis of 60 alfalfa accessions from 24 countries is discussed in the following sections.

The SSR loci and alleles

Since cultivated alfalfa is an allogamous autotetraploid species (2n = 4x = 32; Quiros, 1982), a maximum of four alleles are expected per individual plant at a single copy microsatellite loci. In this study, three of the ten loci produced less than four alleles (Table 2) across the 78 individual plants. The higher the number of alleles detected in a given population the higher the proportion of individuals having four alleles per locus, in allogamous tetraploid species such as alfalfa. In this study, the maximum number of alleles detected per locus was nine (*AGAT-012*). At this locus, 26.3% of the individual plants investigated had four alleles, which is higher than the proportion detected in the other six loci. On the other hand, *AFCA-11* and *AFCAT-45* had no individuals with four alleles regardless of the fact that a total of five and seven alleles, in that order, were detected at these loci. The percentage of individual plants with four alleles per locus was lower than that reported in Mengoni et al. (2000). For example, there were no individuals with four alleles at locus *AFCA-11* in the present study whereas Mengoni et al. (2000) reported 26%. In our study both AFCA-11 and AFCAT-45 had a dominant allele with a relatively high frequency of more than 45%, which may be the reason for no individuals having four alleles. Overall, this study suggests that, in average, the number of plants with two alleles per locus was greater than those with one, three and four alleles, at multi-alleleic (four or more) SSR loci, which is expected for randomly outcrossing species.

Out of the ten SSR loci studied, five (*MTIC-189, B14B03, AFCA-11, ACT-009, ACT-026*) are simple dinucleotide repeat loci whereas three (*FMT-13, AFCT-45* and *AAC-008*) are dinucleotide repeat loci composed of two types of repeat motifs (Table 2). *AGAT-012* is a simple trinucleotide repeat locus whereas *AGGT-004* is a trinucleotide repeat SSR locus composed of three types of repeat motifs. The average number of alleles per locus at the five simple dinucleotide repeat SSR loci was 5.0 whereas that of the three complex dinucleotide repeat SSR loci was 6.0. In this study, the highest number of alleles was revealed in a trinucleotide locus (*AGAT-012*), which suggests that this locus is interesting for genetic diversity analysis and for genetic linkage mapping. Although the number of alleles observed at *AGAT-012* locus was nine, its effective number of alleles was only two due to the fact that six of the nine alleles had a frequency of less than 10%, and four of these alleles had a frequency of 1%. In terms of effective number of alleles per locus, *MTIC-189* had the highest value (4.8), which is attributable to its relatively proportional frequencies among its eight alleles.

The total number of alleles obtained per polymorphic loci in the present study is lower than that reported in a number of previous studies (e.g. Falahati-Anbaran et al., 2007; He et al. (2009). For example, the number of alleles obtained in this study at the loci *AFCA-11* (5 alleles) and *B14B03* (3 alleles) is lower than that reported by Liu (2007), which were 14 and 9 alleles, respectively. Similarly, the number of alleles detected in the present study at the loci *AAC-008* (5 alleles), *AAT-009* (7 alleles), *AGGT-004* (1 allele) and *ACT-026* (2 alleles) was lower than the number of alleles for each of these loci reported in He et al. (2009). The number of alleles detected in the present study at loci *FMT-13* (6 alleles) and *B14B03* (3 alleles) was also lower than the number of alleles for each of these loci reported in Flajoulot et al. (2005). The difference might be due to few individuals (1 to 4) analyzed per accession in the present study. Herrmann et al. (2010) suggested 20 to 40 individuals as a reasonable number of individuals per population for reliable genetic diversity study in alfalfa populations. Hence, the results from this study should be interpreted with caution.

All of the ten SSR loci used in this study, except *AAGT-004*, *ACT-026* and *B14B03*, are highly recommended for alfalfa genetic diversity studies, as they reveal high genetic variation among the populations studied. The locus *AGAT-012* is highly interesting, as it has several alleles at low frequencies which might be linked to desirable traits, such as disease resistance and should be further studied for its application in genetic linkage mapping. The use of molecular markers for efficient selection of genotypes with desirable traits is well-established (e.g. Dudley 1993; Edwards et al. 1992; Obert et al., 2000). This approach can increase the efficiency of the breeding process by allowing simultaneous selection of various desirable traits with high precision in one generation. The large number of alleles detected in some loci in this study suggests the suitability of microsatellite markers for developing molecular markers linked to desirable traits. Obert et al. (2000) developed AFLP markers linked to downy mildew resistance in alfalfa. The presence of rare alleles at SSR loci such as *AGAT-012*, is a good opportunity to evaluate alfalfa genetic material for the association between traits of interest and these rare alleles. At the locus *AGAT-012*, 99% of the individuals investigated were heterozygotes (see Table 5). This means that two or more rounds of selfing are required to obtain sufficient amounts of homozygotes provided that the loci are linked to desirable traits.

Genetic variation among accessions and countries

Previous studies have shown that cultivated tetraploid alfalfa has a high genetic variation within populations (Falahati-Anbaran et al. 2007). This is in line with the general understanding that outcrossing species tend to be more diverse within populations, with less genetic differentiation among populations (Hamrick and Godt 1996; Nybom 2004). The level of genetic variation among the 78 samples analyzed in the present study varied significantly among the polymorphic SSR loci with the highest genetic variation obtained at the locus *MTIC-189* (h = 0.8; I = 1.7; Table 5). The result suggests a significant variation in polymorphism information content of alfalfa polymorphic SSR loci. Thus, a thorough screening procedure should be applied to identify highly variable

polymorphic loci that are suitable to group alfalfa genetic resources into certain classes for efficient conservation and breeding. In this regard, the top three SSR loci are *MTIC-189*, *ACT-009* and *AFCAT-45*.

The gene diversity index, which is the same as expected heterozygosity, for *FMT-13* and *B14B03* in the present study was 0.6 and 0.3, respectively. In their analysis of seven cultivars of alfalfa, Flajoulot et al. (2005) reported a gene diversity of 0.8 and 0.7 for these loci, in that order. The relatively low gene diversity in our study as compared to that of Flajoulot et al. (2005) is expected, as larger number of alleles was reported for these loci in Flajoulot et al. (2005). The overall mean gene diversity obtained in the present study (h = 0.47) was also lower than the gene diversity of 0.70 reported in Flajoulot et al. (2005) and of 0.76 reported in Bagavathiannan et al. (2010). The mean observed heterozygosity in the present study of 0.64 (Table 5) is slightly lower than the observed heterozygosity reported in Bagavathiannan et al. (2010) (0.72) for global collection of alfalfa analyzed using SSR markers. This suggests the level of genetic diversity that the studied populations have might be higher than the level revealed in the present study. In other words, an increase in number of individuals per population may result in higher values of genetic diversity parameters.

Falahati-Anbaran et al. (2007) reported significant differences in the level of genetic variation between different populations of Iranian alfalfa. The variation in the level of Jaccard similarity coefficient between individuals within accessions obtained in the present study (data not shown) suggests different levels of genetic variation among accessions, in line with the report by Falahati-Anbaran et al. (2007). For example, the Jaccard similarity between the four individual plants of accession *T-206* varied from 0.39 to 0.62. On the other hand, the Jaccard similarity coefficient between the two individuals from accession *Vakhskaya-455* was 0.68. The average similarity between individuals of the accession *T-206* (0.49) was only slightly higher than the overall average for the seventy eight individuals investigated (0.45).

Genetic differentiation, genetic distance and cluster analysis

Analysis of molecular variance (AMOVA) revealed that the differentiation of accessions according to countries of origin was 5% of the total variation (F_{ST} = 0.05), which was statistically significant (P = 0.005). However, this variation was only 1% when some of the accessions were excluded (P = 0.25). The differentiation among blocks of countries (Western vs. Eastern) was also insignificant. The present study showed that the genetic distance between accessions from the same regions versus different regions was quite similar. The morphological and RAPD based studies of alfalfa by Tucak et al. (2010) produced similar results. The results suggest continuous gene flow between countries, resulting in genetic overlap, in agreement with the report of Muller et al. (2001) and Falahati-Anbaran et al. (2007). This is supported by the distribution of individuals from each country across the different branches of the dendrogram generated based on the SSR data of the 78 individuals (Figure 3). No clear clustering pattern of accessions according to countries was observed. For example, accessions from Kyrgyzstan were distributed across most of the clusters (Figure 3).

The results from the present study clearly showed a high genetic variation of alfalfa within accessions and countries but low differentiation among accessions from the same country as well as among accessions from different countries. Falahati-Anbaran et al. (2007) also reported a higher level of genetic variation among individuals within populations as compared to variation among populations in Iranian alfalfa. Similarly, Flajoulot et al. (2005) reported that less than 1% of the total genetic variation differentiated populations of alfalfa.

The cluster analysis of accessions grouped according to countries of origin revealed that the extent of genetic similarity between alfalfa accessions was not in agreement with geographic proximities of countries, except in a few cases. For example, the Swedish accessions were closely clustered together with one of the Egyptian accessions in Cluster V sub-cluster ii. Similarly, the Norwegian accession was clustered with the Iranian and Iraqi accessions in Cluster III, which strengthen the suggestion of strong gene flow between accessions through the exchange of seeds between countries.

Furthermore, gene flow might have also occurred through pollen during characterization and evaluation of accessions from different countries by ICARDA and NordGen and in the farmers' field in Kyrgyzstan. The Kyrgyz accessions were clustered together with accessions from a number of countries in Cluster V Sub-cluster i. The most closely related accessions to Kyrgyz accessions were from France (Figure 4). Since the number of individuals per accession and the number of accessions per country used in this study is small, it would be interesting to conduct similar study with optimum number of individuals per accession and accessions per country to determine if similar clustering pattern can be obtained.

Developing an alfalfa synthetic variety

The goal of the field trial was to develop high yielding (seed and green mass) synthetic varieties that are tolerant/resistant to drought, pests and diseases. The objective was also to propose improvements of the whole seed chain from plant breeding to seed processing in order to develop a sustainable industry based on commercial opportunities for alfalfa. However, this MSc study covers only the first part of the steps required for the development of synthetic varieties due to time constraints. It was known from the beginning of the project that the time was limited, and the method used to breed a synthetic variety of alfalfa in Kyrgyzstan was a simplified and time-saving variant. There was no time to evaluate the selected plant clones before they were entered into a synthetic variety. The hot weather resulted in low survival of the selected 26 plants, and in 2011 only 10 plants remained after the winter. Low survival of alfalfa stem cuttings was also reported by (Tesar and Jackobs 1972) who reported of 40% or less survival rate.

The ten plants with survived stem cuttings in this experiment should be enough to develop a synthetic variety. (Fotiadis 1988) indicated that 4-10 clones are optimal for producing a synthetic variety. In 2011, seeds were harvested from these 10 clones, but the yield was very low and we decided to keep the seed until the following harvest season. The seed yield during these two (?) years will be bulked and planted, and will constitute the synthetic-1 (syn-1) generation. To develop a

synthetic variety, the parent clones should be grown together in an isolated plot and allowed to intercross ((Poehlman 1977). Then, each clone should be harvested separately, and equal amounts of seed from each clone mixed and used as seed for sowing the next generation, which gives a syn-2 generation.

In conclusion, this study revealed a significant level of genetic variation in alfalafa in Kyrgyzstan, which is significant enough for alfalfa improvement through breeding including the development of synthetic varieties. The syn-1 seeds from the 34 plants that correspond to ten clones would lead to the development of an interesting synthetic variety. The determination of the relatedness among genotypes and populations serves as basis for selecting genetically distant parents in breeding programs. The accessions *BM-5* and *BM-9/4* appeared to be the most differentiated among Kyrgyz accessions, as revealed by SSR-based cluster analysis (Figure 3). Thus, individual genotypes from these accessions might be interesting for the development of synthetic varieties through crossing with genotypes from other Kyrgyz accessions. However, it is highly likely that new genetic variation can be introduced to the country through introduction of new accessions/populations from other countries. The cluster analysis (Figure 4) showed that accessions from countries such as Argentina, Oman, Australia, Iran, Iraq and Norway are worth considering for the development of synthetic varieties through crossing with Kyrgyz populations.

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