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Comparison of Tetraploid Alfalfa (*Medicago sativa* L.) Populations Collected from Turkey and Former Soviet Countries

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Abstract

Tetraploid *Medicago sativa* L. subspecies; *M. sativa* subsp. *sativa*, *M. sativa* subsp. *falcata* and *M. sativa* subsp. *varia* form the primary gene pool of the alfalfa. The center of diversity for this subspecies is seen as the Caucasus, Northwest Iran and Northeast Turkey, and its natural range is the former Soviet Union and the southern border of North Africa as the northern border. Genetic diversity among the primary gene pool and comparisons between regions of diversity provides a good reference for breeders when utilizing genetic resources. The United States Department of Agriculture Genetic Resources Information Network (USDA-GRIN) System provides reference data from its entire natural range, including Turkey and the Former Soviet region. In this study, seven populations collected from Turkey and thirteen alfalfa populations collected from Former Soviet Countries held in the USDA GRIN System were evaluated using 20 SSR markers. Within the scope of the study, the information between the locations of the subspecies was compared to reveal the hierarchical population structure. The results obtained from the STRUCTURE and PCA analyzes show that the populations are clustered in two main groups for both countries, but there is a high similarity in hybrid genome contents in the subspecies belonging to the Former Soviet countries. In addition, it was determined by AMOVA analysis that the variance within the populations was higher than that between the populations according to the subspecies analyzed from both countries. It is thought that the results will be effective in terms of using alfalfa genetic resources of these countries in breeding programs.

Key Words: Tetraploid alfalfa, Turkey, Former Soviet, Population, SSR markers

1. INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the commonly grown forage plants in the world and in Turkey. The tetraploid alfalfa is included in the *Medicago sativa* species complex or the *Medicago sativa-falcata* complex. The characteristic of this complex is that it consists of diploid and tetraploid subspecies with variations in ploidy level and morphological characters. There is also a gene flow between subspecies even at the same or different ploidy levels (Quiros and Bauchan 1988). Tetraploid subspecies are *Medicago sativa* subsp. *sativa*, *Medicago sativa* subsp. *falcata* and possibly their hybrid *Medicago sativa* subsp. *varia*. Diploid subspecies are *Medicago sativa* subsp. *caerulea*, *Medicago sativa* subsp. *falcata* and their hybrid *Medicago sativa* subsp. *hemicycla* (Şakiroğlu et al. 2011; İlhan et al. 2016).

The first gene centers of *Medicago sativa* L. are the Caucasus, Northwest Iran and the Northeast Anatolian Region of Turkey (Hanson et al. 1988; Michaud et al. 1988). As a result of the adaptation of alfalfa subspecies to different geographies and climatic conditions, it has been observed that they differentiate from each other on the basis of population or subpopulation over time (Şakiroğlu et al. 2015). Therefore, it is critical to evaluate and compare populations in terms of alfalfa populations grown in different geographies (İlhan, 2018a; Eren et al. 2022).

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Molecular markers, and especially next-generation sequencing technologies, serve as useful tools to determine population structures and divergences in alfalfa. SSR (Simple Sequence Repeat) marker technique, which is among these technologies, is frequently preferred due to its high polymorphism (Fischer et al. 2017).

In recent years, the population structure of tetraploid alfalfa has been elucidated and its place in the complex has been determined (İlhan et al. 2016). It is very useful to compare the populations of the germplasms of tetraploid alfalfa used in breeding research on the basis of gene flow mechanisms between subspecies (Şakiroğlu et al. 2010).

While there have been some attempts on tetraploid alfalfa populations, researchers have not had convincing results as they used chloroplast and mitochondrial DNA (Havananda et al. 2010). The use of nuclear DNA will be useful for understanding taxonomic relationships in the M. sativa species complex. The population structures of diploid subspecies are quite clear, but data on regional populations are not sufficient for tetraploids in particular. In line with this information, in this study, seven populations collected from Turkey and thirteen alfalfa populations collected from former Soviet countries, kept in the USDA GRIN System, were evaluated using 20 SSR markers.

2. MATERIALS AND METHODS

2.1. Plant Materials

We selected 20 populations belonging to the tetraploid subspecies *Medicago sativa* subsp. *sativa*, *Medicago sativa* subsp. *varia* and *Medicago sativa* subsp. *falcata* as plant material. For each population, we sampled four individuals and totally selected 80 individuals (Table 1). These populations were obtained from the USDA GRIN NPGS System. The ploidy levels of tetraploid populations were detected with the flow cytometry method (Brummer et al. 1991). Plants were planted in soil under sterile conditions and grown in additional triplicates under greenhouse conditions (25±2 0C, 8/16-h photoperiod) at Kafkas University of Kars province.

2.2. Molecular Analyses

2.2.1. DNA Extraction and PCR Reactions

DNA extraction was performed based on the CTAB method for 80 individuals of tetraploid alfalfa subspecies (Doyle and Doyle 1990). We selected 20 SSR markers for alfalfa (Diwan et. al. 2000; Julier et. al. 2003; Robins et. al. 2007). PCR amplifications were conducted based on the M13 method (Schuelke 2000) and for each SSR markers independent amplifications were used (Julier et al. 2003; Sledge et. al. 2005). PCR products were genotyped using automated ABI3730 sequencer in The Samuel Robert Noble Foundation of US and then allele scoring was managed to GENEMARKER software (SoftGenetics, State College, PA).

2.2.2. Data Scoring and Analyses

Since the SSR marker system is the Codominant marker system, the scoring process was carried out using the 1-0 technique and data analyzes were made.

2.2.3. Population STRUCTURE analysis

In order to evaluate population structures, we used STRUCTURE software because of its reliable and handy properties. This program runs based on the Bayesian statistics and gives K groups for seperating populations or subpopulations. These analyses focus on finding the optimal K value which is

among ranged between 1 and 10 for 80 genotypes representing individuals. We used a mixed model and thought that allele frequencies were relevant to each other and then the number of optimal K with ad hoc (Pritchard et. al., 2000) and ΔK procedures (Evanno et. al., 2005) were investigated in Population STRUCTURE analysis.

Table 1. *Tetraploid alfalfa materials used in this study and their location information.*

PI	Replicates	Origin	Ploidy	Classification
PI 173733	4	Turkey	Tetraploid	Subsp. *sativa*
PI 179369	4	Turkey	Tetraploid	Subsp. *sativa*
PI 182240	4	Turkey	Tetraploid	Subsp. *sativa*
PI 206698	4	Turkey	Tetraploid	Subsp. *sativa*
PI 464801	4	Turkey	Tetraploid	Subsp. *varia*
PI 464813	4	Turkey	Tetraploid	Subsp. *varia*
PI 631582	4	Turkey	Tetraploid	Subsp. *falcata*
PI 299053	4	USSR	Tetraploid	Subsp. *sativa*
PI 315484	4	USSR	Tetraploid	Subsp. *sativa*
PI 440517	4	Kazakhistan	Tetraploid	Subsp. *sativa*
PI 476393	4	Ukrain	Tetraploid	Subsp. *varia*
PI 502441	4	Russia	Tetraploid	Subsp. *falcata*
PI 502446	4	Russia	Tetraploid	Subsp. *falcata*
PI 502459	4	Kazakhistan	Tetraploid	Subsp. *sativa*
PI 502474	4	Armenia	Tetraploid	Subsp. *sativa*
PI 502514	4	USSR	Tetraploid	Subsp. *varia*
PI 538983	4	Ukrayna	Tetraploid	Subsp. *falcata*
PI 641381	4	Russia	Tetraploid	Subsp. *falcata*
PI 641581	4	Kazakhistan	Tetraploid	Subsp. *falcata*
PI 641582	4	Kazakhistan	Tetraploid	Subsp. *falcata*

2.2.4. Principal Component Analysis (PCA)

As another approach, we also analyzed SSR marker variation by principal component analysis (PCA) to clarify the STRUCTURE analysis results. For this purpose, we plotted populations based on the first two major coordinates using the software GenAlEx (Peakall and Smouse, 2001).

2.2.5. Molecular Variance Analysis (AMOVA)

To reveal the state of molecular genetic variation within and between subspecies for Turkish alfalfa populations and Former Soviet populations, we performed molecular analysis of variance (AMOVA) using the software program GenAlEx 6.1 (Peakall and Smouse, 2001). To estimate within-population variance, we performed the analysis using all 80 populations and four genotypes for each population.

3. RESULTS and DISCUSSION

3.1. Population STRUCTURE

We analysed all data using STRUCTURE software for population seperations. We found that the optimal K value was 2 based on two methods (Fig. 1). The sativa and falcata subspecies are clearly separated from each other. However, when the K=3 value is examined, we also saw that varia, which is the third subspecies of tetraploid members, is positioned for hybridization between both, as a result of our analysis. It is seen that the samples taken from Turkey are generally classified into appropriate subspecies. However, it is understood that predominantly sativa subspecies show hybridization, especially in samples taken from the former Soviet countries. In the same way, a similar situation has arisen in *falcata* subspecies in these countries (Figure 2).

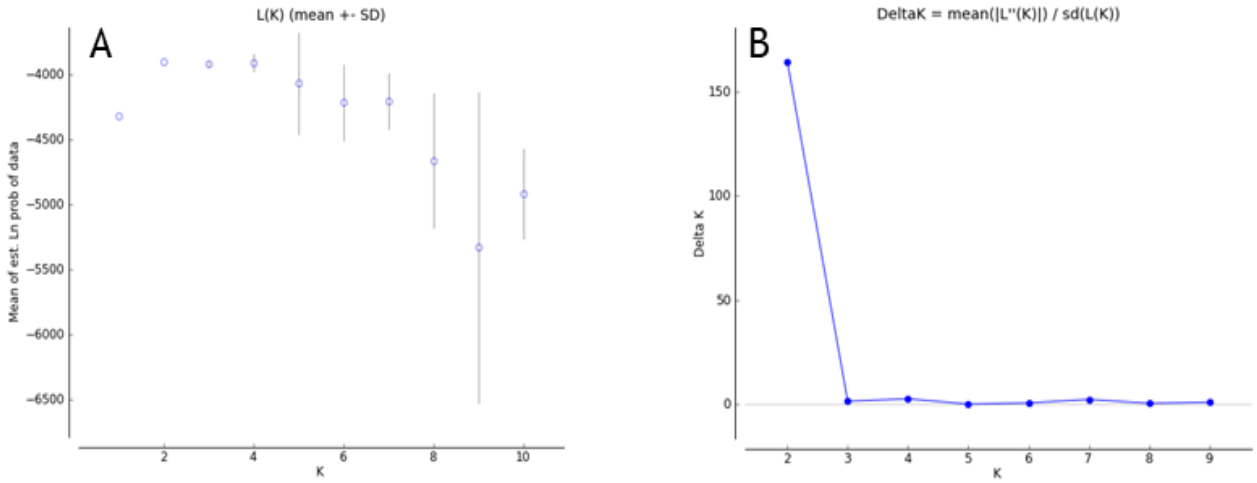

Figure 1. *(A) The ad hoc prodecure (Pritchard et al. 2000) and (B) The ΔK method (Evanno et al. 2005). These methods were used to determine the optimal K value.*

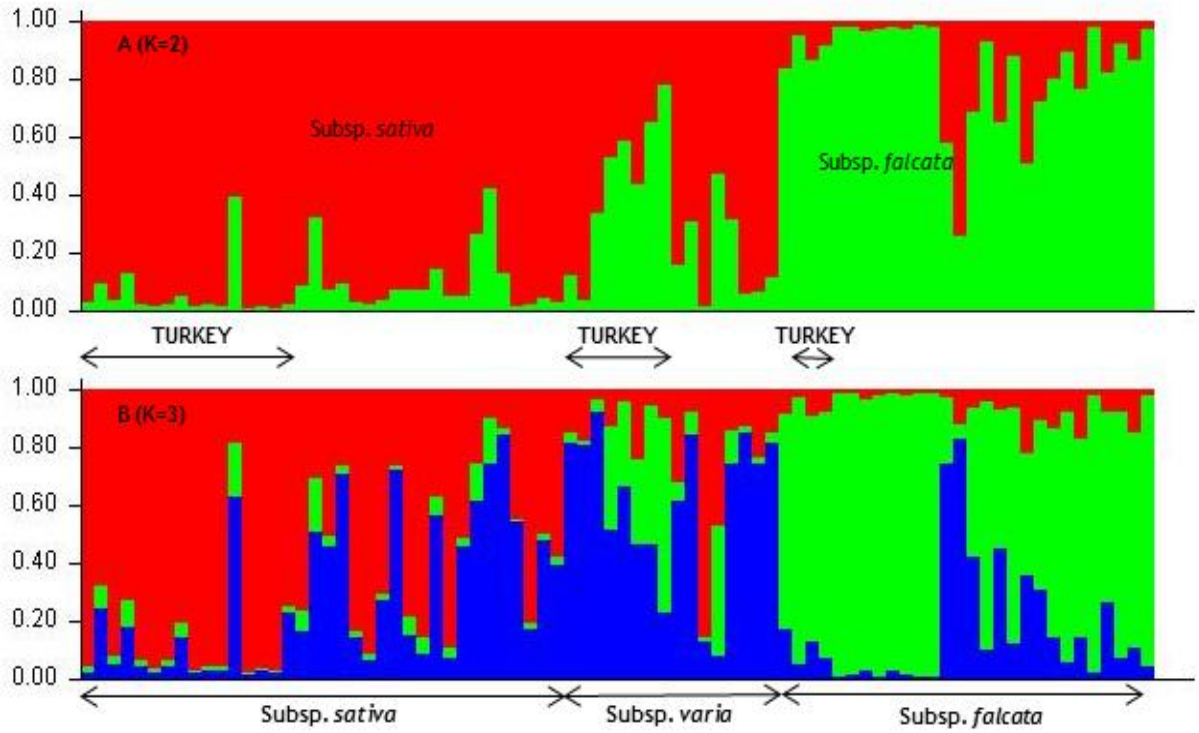

Figure 2. *(A) STRUCTURE analysis revealed that the K value was 2. (B) Considering K=3, subspecies giving possible hybridization signals (Blue colored genotypes).*

3.2. Principal Component Analysis (PCA)

As the second analysis, we preferred to SSR variations using Principal Component Analyses (PCA) to confirm STRUCTURE results. PCA results were coherent with those of the STRUCTURE results. With exceptions, in general, two main subspecies clusters were formed, but hybridizations were more common in the former Soviet countries. Based on the two principal coordinates, PC1 explained 41% and PC2 13% of the total genetic variance (53%) (Fig. 3).

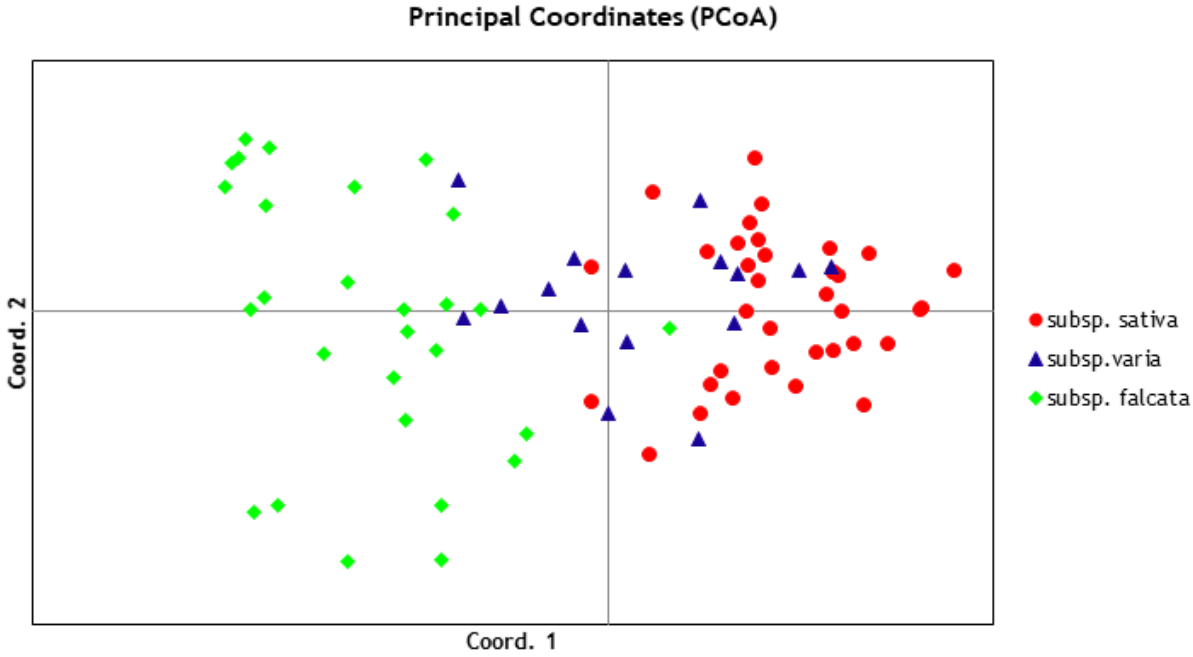

Figure 3. *Clustering of tetraploid subspecies depending on Principal Component Analysis.*

3.3. Molecular Variance Analysis (AMOVA)

AMOVA analyzes were carried out participation in tetraploid subspecies based on STRUCTURE data. AMOVA showed that 17% of the total genetic variance was explained by the three subspecies, 13% was confined among populations, and the remaining 70% was present within populations (Table 2).

Table 2. *Molecular variances among subspecies, among populations and within populations based on AMOVA analysis.*

Source	df	SS	MS	Est. Var.	%
Among Regions	2	217,690	108,845	3,326	17%
Among Pops	17	414,072	24,357	2,576	13%
Within Pops	60	843,250	14,054	14,054	70%
Total	79	1475,013		19,956	100%

df: degree of freedom, SS: Sums of Square, MS: Means of Square

Many studies investigating the alfalfa plant included in a systematic complex are already available in the literature. While some of these studies focused on chloroplast and mitochondrial DNA (Havananda et al., 2010; Vysniauskiene et al., 2015), some focused on genomic DNA (Şakiroğlu et al., 2010; İlhan et al. 2016). Due to the successful results obtained from genomic DNAs, this study is thought to support previous studies. The results we found were reported by Şakiroğlu (2010) and Havananda et al. al., (2010) supports that, with 89 SSR markers simultaneously, diploid *falcata* and *caerulea* subspecies are generally differentiated into 2 main groups according to their geographical distribution and

ecogeographic structures (Şakiroğlu, 2010). Likewise, in the study conducted by İlhan et al. (2016), it is consistent with the result that tetraploid subspecies also diverge from each other in the form of *sativa* and *falcata* with a K=2 value, but just like in this study, it is clear that the 3rd subspecies, *varia*, shows hybridization signals.

4. CONCLUSION

In this study, population structures were compared with 20 SSR markers in tetraploid alfalfa populations obtained from Turkey and Former Soviet countries. It is predicted that studies evaluating population structures including alfalfa subspecies in *Medicago sativa* - *falcata* complex may be useful in alfalfa breeding programs. It is also understood that SSR markers are useful tools in population dynamics studies in tetraploids as well as diploids. However, it is clear that studies using more markers and populations can yield more productive results. We think that this study will be a useful study for the discovery of alfalfa genetic resources in the world.

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