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The evaluation of population structure in some alfalfa (*Medicago sativa*) ecotypes demonstrating distribution in Eastern Anatolia region

Doğu Anadolu bölgesinde yayılış gösteren bazı yonca (*Medicago sativa*) ekotiplerinde populasyon yapısı değerlendirmesi

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ABSTRACT

The legume forage alfalfa (*Medicago sativa* L.) is invaluable crop useful for animal husbandry and is grown worldwide and country-wide in Turkey. It is approved that origins of the first diversity regions are Caucasian, Northwest of Iranian, North-eastern of Turkey for alfalfa. Various ecotypes of alfalfa either are cultivated by a great number of breeders or grown as wild based on geographic and climatic conditions. With regard to population structure of alfalfa ecotypes, some separations are seen depending on genetic structure among the subspecies. In this study, population structure was evaluated for four different ecotypes tetraploid alfalfa populations demonstrating distribution in Eastern Anatolia Region using 31 SSR markers. Results showed that populations of 16 different genotypes clearly separated each other as three subspecies (*sativa*, *varia* and *falcata*). It is expected that this work will be an important reference both for the evaluation of more alfalfa populations and for the scientific classification of complex members.

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ÖZ

Baklagil yembitkisi olan yonca (*Medicago sativa* L.), hayvancılık için paha biçilemez bir üründür ve dünya çapında ve Türkiye genelinde yetiştirilmektedir. İlk çeşitlilik bölgelerinin kökeninin Kafkasya, Kuzey İran ve Türkiye'nin Kuzeydoğusu olduğu kabul edilmektedir. Yonca'nın değişik ekotipleri çok sayıda yetiştirici tarafından kültüre alınarak yetiştirilmekte ya da coğrafi ve iklim koşulları temelinde yabani olarak yetişmektedir. Yonca ekotiplerinin populasyon yapısı ile ilgili olarak alttürler arasındaki genetik yapı temelinde bazı ayrışmalar görülmektedir. Bu çalışmada, Doğu Anadolu Bölgesinde yayılış gösteren 4 farklı yonca ekotip populasyonunun 31 SSR markörü kullanılarak populasyon yapısı değerlendirilmiştir. Sonuçlar, toplam 16 farklı genotipten oluşan populasyonların 3 alttür (*sativa*, *varia* and *falcata*) şeklinde birbirinden açık bir şekilde ayrıldığını göstermiştir. Gerçekleştirilen bu çalışmanın hem daha fazla yonca populasyonunun değerlendirilmesinde hem de bilimsel açıdan kompleks üyelerinin sınıflandırılmasında önemli bir referans olabileceği düşünülmektedir.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a common legume crop which is grown across the world and in Turkey because of high nutritious value. The plant is included to a complex called as *M. sativa* species complex or *M. sativa-falcata* complex and this complex consists of diploid and tetraploid subspecies in terms of ploidy level and morphologic characters. There is a gene flow within the same and different ploidy levels in this complex (Quiros and Bauchan 1988). While diploid subspecies are *M. sativa* subsp. *caerulea*, *M. sativa* subsp. *falcata* and their hybrid *M. sativa* subsp. *hemicycla*, tetraploid subspecies are *M. sativa* subsp. *sativa*, *M. sativa* subsp. *falcata* and as most probably their hybride is *M. sativa* subsp. *varia* (İlhan et al. 2016).

The genus of *Medicago* has more than sixty various annual and perennial species and alfalfa also gets involved in this genus. It is known that primary gene centres of the genus are Caucasian, Northwestern Iran and Northeastern Anatolia Region of Turkey. (Hanson et al. 1988; Michaud et. al. 1988). Various ecotypes of alfalfa have improved depending on adaptations to climatic and geographic conditions. It is obvious that these ecotypes of alfalfa are separated from one another

according to their population structures or they tend to build up subpopulations for subspecies. Therefore, wild subspecies for different ecotypes are worthy to assess the populations (Sakiroglu et al. 2010). Although it is thought that *M. sativa* ssp. *falcata* is wild and worthless for agriculture, the genes, which are responsible from winter hardness, repent root pattern and resistance to some leave pathogens, have been transferred to modern cultivar alfalfa. The uses of this subspecies which adapted to cold habitats are invaluable for mostly in the northern regions. Accordingly, it is important to collect, identify and protect the species which are related to all systematic units within alfalfa species complex (Tanksley and McCouch 1997).

Molecular markers are widely used to designate the population structures, genetic diversities, molecular relations, genetic mapping, marker assisted selection and breeding efforts in plants. SSR (Simple Sequence Repeat) and SNP (Single Nucleotide Polymorphism) technologies are useful, affordable and efficient tools for genomic researches (Fischer et al. 2017).

Recently, population structure in alfalfa have trended tetraploid breeding populations. Because of complex nature of the subspecies, information about population structure of germplasms used in any alfalfa's breeding program is rather useful. Although there are some attempts relevant to tetraploid alfalfa populations, researchers have not convincing results because they used chloroplast and mitochondrial DNA (Havananda et al. 2010). Using the nuclear DNA would be useful for understanding the taxonomical relationships in *M. sativa* species complex. Population structures of diploid subspecies are obvious but not clear for tetraploids. It will be useful to reveal the population structure of the complex. In accordance with this information, we evaluated population structures of 4 different ecotypes demonstrating the distribution of tetraploid alfalfa in Eastern Anatolia region by using 31 SSR markers.

2. Materials and Methods

2.1. Plant Materials and Growing

We selected 4 populations belonging to tetraploid subspecies *M. sativa* subsp. *sativa*, *M. sativa* subsp. *varia* and *M. sativa* subsp. *falcata* of *M. sativa* species complex as plant material. For each population, we sampled four individual and totally selected 16 individuals (Table 1). These populations were obtained from the USDA GRIN NPGS System. The ploidy of tetraploid populations was detected with flow cytometry method (Brummer et al. 1991). Plants were planted in soil under sterile conditions. Plants were grown under greenhouse conditions (25±2 ˚C, 16-h photoperiod) and organized with 4 replicates at Kafkas University of Kars. Because some accessions have wild character, plants were transplanted to field using the randomized complete-block design with 3 replications in April and May months of the 2012, 2013 years. Field conditions are necessary to see growing and population structures of wild plants. We did not extra irrigation because Kars city is rainy in summer months.

2.2. DNA Extraction and PCR Reactions with SSR Markers

DNA extraction was carried out using CTAB method from young leaves for 16 individuals of subspecies (Doyle and Doyle 1990). We selected that 31 SSR markers are universal and useful for alfalfa (Diwan et al. 2000; Julier et al. 2003; Robins et al. 2007). M13 method was selected for PCR amplifications (Schuelke 2000) and for each SSR markers independent amplifications were used (Julier et al. 2003; Sledge et al. 2005). PCR products were genotyping using automated ABI3730 sequencer in The Samuel Robert Noble Foundation of US and then allele scoring was performed with GENEMARKER software (Soft Genetics, State College, PA).

2.3. Assessments of Population Structures

To evaluate population structures, we chose STRUCTURE software because of its reliable and handy properties. This program runs based on Bayesian statistics and gives K groups for separating populations or subpopulations. This analysis focus on finding optimal K value which is among ranged between 1 and 10 for 16 genotypes representing individuals. We used 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.

To approve the results from obtained STRUCTURE software, we conducted Principal Component Analysis (PCA) running GenAlEx (Peakall and Smouse 2001) program. With this program, genotypic values were plotted because of first two Principal Component estimates.

3. Results and Discussion

We performed STRUCTURE analysis to find out population dynamics. Within the scope of this study, optimal K value was found with respect to previously defined two methods (Figure 1 and 2). It is concluded that the optimal K value was 3 for each two procedures and this K value mean that there are 3 subspecies or 3 populations in *M. sativa-falcata* complex and these have separated each other in a consequence of STRUCTURE analyses. *Medicago sativa* subsp. *sativa* (the region of red colours) and *M. sativa* subsp. *falcata* (the region of green colours) were completely separated and *M. sativa* subsp. *varia* (the region of blue colours) was located between subsp. *sativa* and *falcata* as probably their hybrid (Figure 3 and 4). Whether *varia* was a hybrid of *sativa* and *falcata* subspecies or not and transitions between *falcata* and *sativa* showed that fertility barriers did not occur in full thus these units could not be admitted as species. Moreover, the individuals which also gave hybrid signals belong to *varia* genome were detected as

Table 1. The subspecies as used plant materials for alfalfa plants with origin regions, geographic information, ploidy levels and the numbers of populations and individuals.

PI	[a]Subsp.	Origin City	Lattitude	Longitude	[b]Pop. No	[c]Indiv. No	Germplasm
173733	*sativa*	Van	37 deg. 16 min. 0 sec. N (37.26666667)	38 deg. 49 min. 0 sec. E (38.81666667)	1	4	Uncertain
179369	*sativa*	Van	39 deg. 0 min. 0 sec. N (39)	43 deg. 21 min. 0 sec. E (43.35)	1	4	Uncertain
464801	*varia*	Elazığ	-	-	1	4	Wild
631582	*falcata*	Sivas	39 deg. 45 min. 0 sec. N (39.75)	37 deg. 2 min. E (37.03333333)	1	4	Wild

[a]**Subsp.** Subspecies, [b]**Pop. No.** The number of used populations, [c]**Indiv. No.** The number of used individuals.

hybrid subspecies. Moreover, while some individuals of *M. sativa* subsp. *sativa* had especially subsp. *varia* genome at lower levels as well as subsp. *falcata* genome, an individual of *M. sativa* subsp. *falcata* had especially subsp. *sativa* genome (Figure 3 and 4).

On the other hand, we conducted Principal Component Analyses (PCA) to approve STRUCTURE analysis results. Results were coherent with those of the STRUCTURE analysis. In other words, it showed that there were different 3 subspecies and these subspecies were divided into 3 different clusters. In addition, clusters represent subsp. *sativa* (red colours), subsp. *falcata* (green colours) and subsp. *varia* (blue colours) (Figure 5).

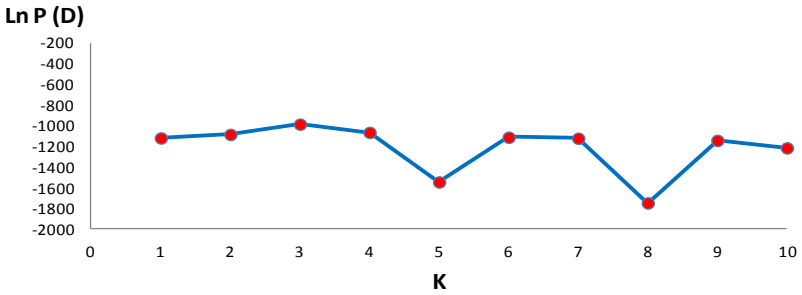

Figure 1. Result of K value (3) based on ad hoc procedure (Pritchard et al. 2000).

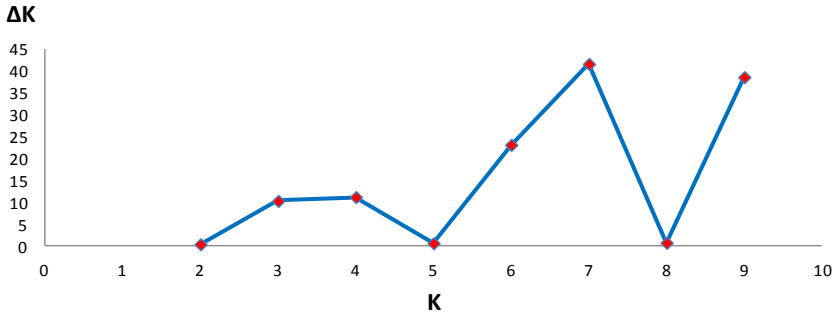

Figure 2. Result of K value (3) based on ΔK procedure (Evanno et al. 2005).

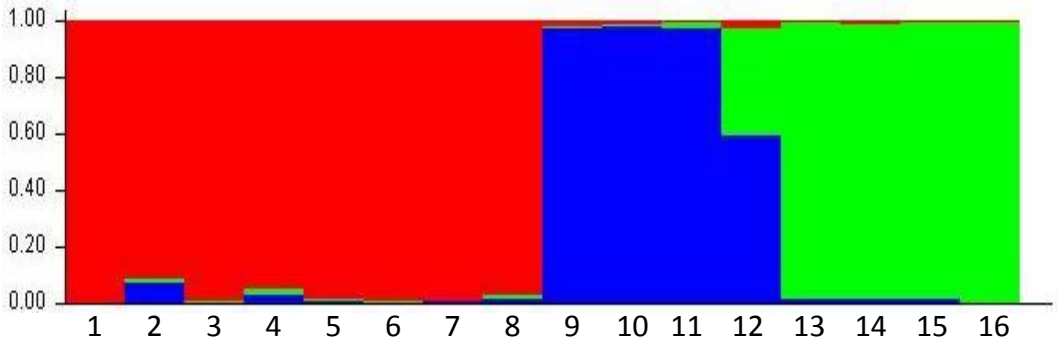

Figure 3. Result of K value (3) based on ΔK procedure (Evanno et al. 2005).

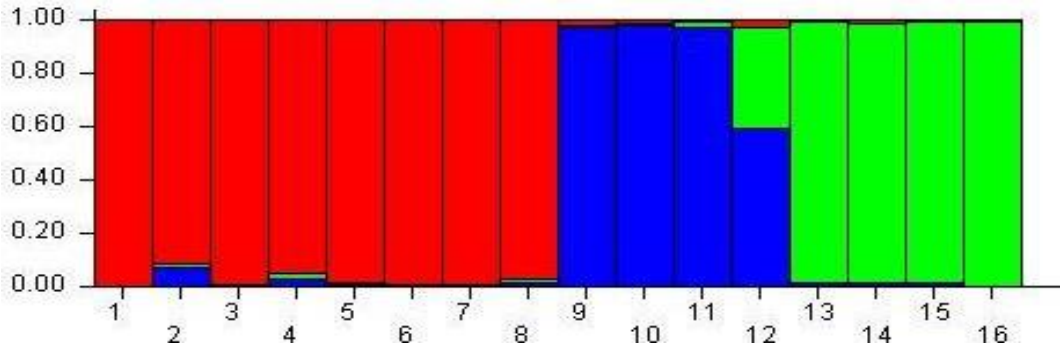

Figure 4. Result of K value (3) based on ΔK procedure (Evanno et al. 2005).

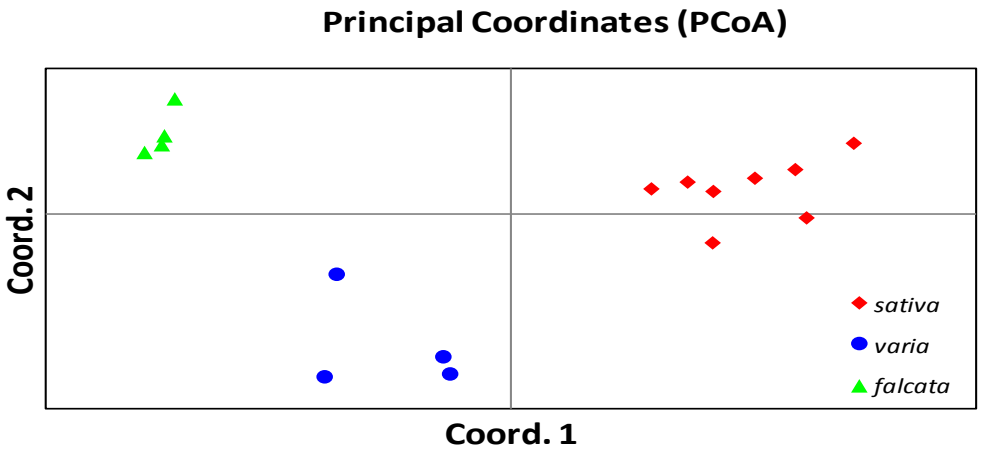

Figure 5. Three subspecies (*sativa*, *varia* and *falcata*) separated each other based on two principal components as 3 of clusters.

There are many researches about alfalfa in the *M. sativa-falcata* complex, but while some of them focused on mitochondrial or chloroplast DNA (Schumann and Hancock 1989; Johnson and Palmer 1989; Mengoni et al. 2000; Muller et al. 2003; Havananda et al. 2010; Vysniauskiene et. al. 2015) the others used genomic DNAs (Brummer et al. 1991; Yu and Pauls 1993; Kalo et al. 2000; Segovia et al. 2003; Sakiroglu et al. 2010; Sakiroglu and Kaya 2012) due to efficient results like this study. It is concluded that this study supported previous researches with satisfactory results. These results are coherent with Sakiroglu et al. (2010) and Havananda et al. (2010) study results which were carried out simultaneous but in diploid subspecies which were used with 89 SSR markers diploid *falcata* and *caerulea* were separated according to geographical distributions and ecogeographical structure respectively as well as hybrid subspecies *hemicycla* Sakiroglu et al. (2010). İlhan et al. (2016) have founded that subsp. *sativa* and *falcata* clearly separated each other but *M. sativa* subsp. *varia* was suspicious about whether it was hybrid or not. However, sharp distinctions were observed in these subspecies in this study.

4. Conclusions

In this research we tried to clarify population structures of some alfalfa ecotypes demonstrating distribution in Eastern Anatolia Region populations using SSR markers. It is seen that subspecies of alfalfa in the *M. sativa-falcata* complex can be classified depending on their populations, and evaluating their population structures will pave the way for alfalfa breeding programs. Moreover, it is proved that SSR markers, which are described previously in alfalfa, are useful tools for population dynamics researches in the other organisms as well as alfalfa but the comprehensive studies need to be done to elucidate alfalfa populations. We expected that this research will be an important reference for creating and using alfalfa germplasms in the world.

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