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Genetic Diversity Analysis of Red Clover (*Trifolium pratense* L.) in Iran Using Sequence Related Amplified Polymorphism (SRAP) Markers

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ABSTRACT

Progress in plant breeding requires a broad genetic basis. Knowledge of genetic diversity in cultivated species and their wild relatives is of critical importance for breeding purposes. The red clover, *T. pratense*, grows wildly in Iran in a vast range of habitats, mainly along the Zagros and Alborz Mountains. Despite being economically important in many other countries, information regarding the genetic diversity of this species in Iran is significantly lacking. In this study, the genetic diversity of 56 genotypes of red clover collected from Iran and one genotype of *T. diffusum*, used as outgroup, was evaluated using nine SRAP markers. The nine SRAP primer combinations created a total of 294 bands from DNA of 57 genotypes, from which 291 (98.9%) were polymorphic. All the measured parameters showed significantly high genetic diversity in the Iranian genepool of *T. pratense* with no clear geographic partitioning of genotypes. However, genotypes collected from around Tehran, Isfahan, and Kermanshah-Hamedan were loosely clustered with their co-regional genotypes. Based on the results of the STRUCTURE analysis, genotypes were genetically divided into two clusters, but these were not correlated with the eco-geographical groups. There was no correlation between genetic distance and geographic distance of genotypes. The result of this study showed value in sampling the Iranian genepool of the red clover, with the Western and the Northwestern genepools in more depth, for conservation and breeding purposes.

Keywords: Genetic distance, Genotypes, Molecular markers, Plant breeding, Systematic relationships.

INTRODUCTION

The clover genus, *Trifolium* L., comprises about 255 species (Ellison *et al.*, 2006; Zohary and Heller, 1984) worldwide, from which about 48 species occur in Iran (Heller, 1984). This genus originated, more likely, in Mediterranean region (Williams, 1987). At least 16 species of *Trifolium* are cultivated (Gillett and Taylor, 2001) as livestock forage and green manure crops. Many wild species are also heavily utilized by grazing animals (Crampton, 1985).

Red clover (*T. pratense* L.) is economically second to alfalfa among the forage legumes, and is grown for hay, silage, forage and as a soil conditioner (Greene *et al.*, 2004). The red clover is also an important component of temperate grasslands, which cover about eight percent of the global land area (Bundesamt fur Statistik, 2004). The center of origin of the red clover is thought to be the Mediterranean region (Southeastern Europe or Asia Minor) (Taylor and Quesenberry, 1996), from where

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the species distributed eastward through Iran to Afghanistan and Pakistan (Heller, 1984).

Trifolium pratense is a self-incompatible, insect-pollinated diploid species (Mukhina *et al.*, 1993) and its dispersal is highly facilitated by livestock, birds, and human activities (Semerikov *et al.*, 2002). Zohary and Heller (1984) identified more than 40 forms or varieties in the species, but no detailed taxonomy of this species has yet been established. Due to the obligate outcrossing, its populations are genetically very diverse insofar the cultivars remain heterogeneous (Dias *et al.*, 2008). However, this extensive variability is not well documented.

The genetic diversity of *T. pratense* in Caucasus (Mosjidis *et al.*, 2004 using isozyme; Greene *et al.*, 2004 using RAPD); USA (Dias *et al.*, 2008 using morphology and SSR; Mosjidis and Klingler, 2006 using isozyme and RAPD; Kongkiatngam *et al.*, 1995 using morphology, isozyme, and RAPD); Europe and Canada (Yu *et al.*, 2001 using isozyme), North America (Dias *et al.*, 2008 using SSR), Chile (Campos-de-Quiroz and Ortega-Klose, 2001 using RAPD), Italy (Pagnotta *et al.*, 2011 using AFLP), and cultivars from Europe (Herrmann *et al.*, 2005; Kolliker *et al.*, 2003 using AFLP) have been investigated. However, the red clover gene pool in Iran, which is very close to the center of diversity of the species, has been ignored and can be considered as the lost ring of the chain of investigations.

Sequence-Related Amplified Polymorphism (SRAP), which was first introduced by Li and Quiros (2001), has several advantages over other molecular markers such as simplicity, reproducibility, reasonable throughput rate, easy isolation of bands for sequencing, and it targets Open Reading Frames (ORFs) (Cai *et al.*, 2011). SRAPs have been successfully used to study genetic diversity and relationships in several species (Vandemark *et al.*, 2006; Ariss and Vandemark, 2007; Esposito *et al.*, 2007; Feng *et al.*, 2009; Run-fang *et al.*, 2010; Ahmad *et al.*, 2014; Aghaei *et al.*, 2015; Maghsoudi Kelardashti *et al.*, 2015). No attempts have still been made to evaluate *T. pratense* populations using SRAP markers.

The aims of this study were to evaluate the genetic diversity present in Iranian genepool of *T. pratense* and to elucidate the geographical pattern of genetic diversity using SRAP markers.

MATERIALS AND METHODS

Plant Materials

A total of 56 genotypes of *T. pratense* collected from Iran and one genotype of *T. diffusum* Ehrh. (which is the closest species to *T. pratense* growing in Iran, Heller, 1984; Zohary and Heller, 1984), used as outgroup, were studied (Table 1, provided as supplementary online material). Forty one genotypes were freshly collected by authors (Yousefi and Saeidi) during 2013-2014 and 15 genotypes were obtained from different herbaria [six from the Herbarium of the Islamic Azad University of North Tehran, eight from the Herbarium of the University of Isfahan (HUI), and one genotype from the Herbarium of the Research Institute of Forests and Rangelands (TARI)] (Table 1). Based on our knowledge of ecological and geographical conditions, the genotypes were divided into 5 geographic groups including genotypes collected from around Isfahan (Isf, 9 genotypes), around Tehran (Teh, 5 genotypes), Northwest of country (Nw, 21 genotypes), West (W, 10 genotypes) and the North of Iran (N, 11 genotypes).

DNA Extraction

Total genomic DNA was extracted from about 100 mg young dried leaves, using the CTAB extraction method of Gawel and Jarret (1991) with minor modifications. DNA quality and concentration was evaluated using 0.8% (w/v) agarose gel electrophoresis and spectrophotometry. DNA samples were diluted to 50 ng μL^{-1} and stored at $-20^{o}C$.

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Table 1. Genotypes code, variety, locality and collection site coordinates of *T. pratense* genotypes analysed in this study.

Genotype code	Geographic group	Variety	Locality	Alt. (m)	Latitude	Longitude
Wazr-1 [a]	Northwest	*pratense*	West Azerbaijan, Serow to Salmas	1805	N: 37, 72	E: 44, 65
Wazr-2 [a]	Northwest	*pratense*	West Azerbaijan, Urmia to Salmas	1580	N: 38, 18	E: 44, 76
Wazr-3 [a]	Northwest	*pratense*	West Azerbaijan, Salmas	1420	N: 38, 22	E: 44, 76
Wazr-4 [a]	Northwest	*pratense*	West Azerbaijan, Haftvan	1380	N: 38, 18	E: 44, 75
Wazr-5 [a]	Northwest	*sativum*	West Azerbaijan, Mamakan	1840	N: 38, 18	E: 44, 75
Wazr-6	Northwest	*sativum*	West Azerbaijan, Serow	1912	N: 37, 72	E: 44, 65
Wazr-7	Northwest	*pratense*	West Azerbaijan, Chaldoran to Candy Crush	1830	N: 39, 07	E: 44, 40
Wazr-8	Northwest	*maritimum*	West Azerbaijan, Bookan to Mahabad	1300	N: 36, 47	E: 45, 44
Wazr-9	Northwest	*pratense*	West Azerbaijan, Mahabad	1370	N: 36, 46	E: 45, 44
Wazr-10	Northwest	*sativum*	West Azerbaijan, Kitkeh to Sardasht	1519	N: 36, 23	E: 45, 39
Wazr-11	Northwest	*pratense*	West Azerbaijan, Piranshahr	1500	N: 36, 41	E: 45, 08
Wazr-12	Northwest	*sativum*	West Azerbaijan, Ziveh to Silvana	1580	N: 37, 42	E: 44, 85
Wazr-13	Northwest	*pratense*	West Azerbaijan, Tavalli	1550	N: 37, 30	E: 44, 47
Wazr-14	Northwest	*pratense*	West Azerbaijan, Khoy to Chaldoran	2078	N: 38, 41	E: 44, 40
Wazr-15	Northwest	*pratense*	West Azerbaijan, Siah Cheshmeh	1880	N: 39, 03	E: 44, 22
Wazr-16	Northwest	*pratense*	West Azerbaijan, Zaviyeh-ye Sofla	1850	N: 44, 22	E: 44, 20
Wazr-17	Northwest	*pratense*	West Azerbaijan, Zaviyeh-ye Sofla to Mako	2000	N: 39, 17	E: 44, 13
Wazr-18	Northwest	*sativum*	West Azerbaijan, Mako	1795	N: 39, 17	E: 44, 30
Wazr-19 [b]	Northwest	not determined	West Azerbaijan, Sardasht	1590	N: 38, 20	E: 44, 76
Eazr-20	Northwest	*pratense*	East Azerbaijan, Sabalan Mountain	1850	N: 38, 22	E: 47, 48
Eazr-21	Northwest	*pratense*	EastAzerbaijan, Arasbaran	1910	N: 38, 40	E: 46, 39
Gil-22 [c]	North	*villosum*	Gilan, Asalem-Khalkal Road	1800	N: 38, 90	E: 47, 85
Gil-23	North	*sativum*	Gilan, Sangar	29	N: 37, 28	E: 49, 59
Gil-24	North	*pratense*	Gilan, Rasht	7	N: 37, 15	E: 49, 48
Gil-25	North	*pratense*	Gilan, Rasht	48	N: 37, 15	E: 49, 48
Gil-26	North	*pratense*	Gilan, Rasht to Lahijan	-4	N: 37, 15	E: 49, 48
Gil-27	North	*pratense*	Gilan, Lahijan	-8	N: 37, 15	E: 49, 48
Maz-28	North	*pratense*	Mazandaran, Alamdeh	-7	N: 36, 56	E: 51, 99
Maz-29	North	*pratense*	Mazandaran, Noor	-20	N: 36, 55	E: 51, 95
Maz-30	North	*pratense*	Mazandaran, Royan to Chalus	-11	N: 36, 34	E: 51, 56

[a] Obtained from the Herbarium of the University of Isfahan (HUI). [b] Obtained from the Herbarium of the Islamic Azad University of North Tehran. [c] Obtained from the Herbarium of the Research Institute of Forests and Rangelands (TARI).

…Continued

Continued of Table 1.

Genotype code	Geographic group	Variety	Locality	Alt. (m)	Latitude	Longitude
Maz-31	North	*pratense*	Mazandaran, Chalus toward Karaj	80	N: 36, 38	E: 51, 28
Maz-32	North	*pratense*	Mazandaran, Kelardasht	1090	N: 36, 30	E: 51, 12
Isf-33	Isfahan	*pratense*	Isfahan, Falavarjan	1610	N: 32, 55	E: 51, 50
Isf-34	Isfahan	*pratense*	Isfahan, Falavarjan to Mobarakeh	1719	N: 32, 55	E: 51, 51
Isf-35	Isfahan	*pratense*	Isfahan, Mobarakeh	1736	N: 32, 55	E: 51, 51
Isf-36	Isfahan	*pratense*	Isfahan, Golpayegan to Khansar	1850	N: 33, 20	E: 50, 34
Isf-37	Isfahan	*pratense*	Isfahan, Khansar, Golestan Kooh	2350	N: 33, 19	E: 50, 34
Isf-38	Isfahan	*pratense*	Isfahan, Golpayegan	1813	N: 33, 20	E: 50, 33
Isf-39	Isfahan	*pratense*	Isfahan, Semirom, Dalankooh	1980	N: 31, 52	E: 51, 33
Isf-40	Isfahan	*pratense*	Isfahan,Vanak, Cheshme Naz	2090	N: 31, 52	E: 51, 33
Isf-41	Isfahan	*pratense*	Isfahan, Khansar	2079	N: 33, 15	E: 50, 18
Krm-42	West	*pratense*	Kermanshah, Kangavar	1487	N: 34, 29	E: 47, 56
Krm-43	West	*pratense*	Kermanshah, Gahvareh	1430	N: 34, 20	E: 46, 25
Krm-44	West	*maritimum*	Kermanshah, Dalaho	1529	N: 34, 16	E: 46, 14
Krm-45	West	*pratense*	Kermanshah, Kerend-e Gharb	1490	N: 34, 16	E: 46, 14
Krm-46	West	not determined	Kermanshah, Eslamabad-e Gharb to Kerend-e Gharb	1535	N: 34, 16	E: 46, 14
Ham-47 [a]	West	*pratense*	Hamadan, Serkan	1954	N: 34, 54	E: 48, 45
Ham-48 [a]	West	*pratense*	Hamadan, Tuyserkan to Malayer	2033	N: 34, 51	E: 48, 46
Ham-49 [a]	West	*pratense*	Hamadan, Tuyserkan	1870	N: 34, 55	E: 48, 48
Ham-50	West	*pratense*	Hamadan, Joraghan	1726	N: 34, 88	E: 48, 55
Ham-51	West	*sativum*	Hamadan, Darre Morad Beyg	2200	N: 34, 44	E: 48, 29
Teh-52 [b]	Tehran	*pratense*	Tehran, Abbas Abad hills	1350	N: 35, 74	E: 51, 43
Teh-53 [b]	Tehran	*sativum*	Tehran, Fasham	2145	N: 35, 93	E: 51, 52
Teh-54 [b]	Tehran	*sativum*	Tehran, Tochal	1837	N: 35, 88	E: 51, 41
Teh-55 [b]	Tehran	*sativum*	Tehran, Park-e Saee	1330	N: 35, 72	E: 51, 33
Teh-56 [b]	Tehran	*sativum*	Tehran, Lavasan	1700	N: 35, 82	E: 51, 62
T. diffusum			Kurdistan, Marivan	1320	N: 35, 52	E: 46, 17

[a] Obtained from the Herbarium of the University of Isfahan (HUI). [b] Obtained from the Herbarium of the Islamic Azad University of North Tehran. [c] Obtained from the Herbarium of the Research Institute of Forests and Rangelands (TARI).

SRAP-PCR

Nine combinations of SRAP primers (Table 2) were used to amplify genomic DNA fragments. The PCRs were carried out in a volume of 25 µL, containing 11 µL sterile doubled-distilled water, 12 µL of the 2X Taq DNA polymerase master mix Red (Amplicon, Cat. No. 180301, 150 mM Tris–HCl pH 8.5, 40 mM (NH4)2SO4, 3.0 mM $MgCl_2$, 0.4 mM dNTPs, 0.05 units mL^{-1} Amplicon Taq DNA polymerase, inert red dye and a stabilizer), 0.5 µL of each primer (10 pmol mL^{-1}), and 1 µL of template DNA (50 ng μL^{-1}). PCR products were separated on 1.5% agarose gel electrophoresis in 1X TBE buffer along with 100 bp DNA ladder as size marker and stained with ethidium bromide (10 mg mL^{-1}). Gels were photographed under UV light for the following analysis.

Data Analysis

Each SRAP band with particular mobility on agarose gel was scored as present (1) or absent (0) across 57 genotypes. Genetic similarities were calculated based on the procedures of Jaccard (1908), simple matching and Dice (Nei and Li, 1979) similarity coefficients using the NTSYSpc ver. 2.02 (Rohlf, 2000) and PowerMarker ver. 3.25 (Liu and Muse, 2005) softwares. Dendrograms showing relationships between genotypes were then constructed using a similarity based Neighbor Joining (NJ) method and 100 bootstrap replicates were performed to assess branch support.

Analysis Of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) was also performed to assess the overall distribution of diversity within and between geographic groups, Mantel test (Mantel, 1967) to assess the correlation between genetic and geographic distance matrices, and Principal Coordinate Analysis (PCoA) to show the degree of genetic relatedness among groups using GenAlEx ver. 6.5 software (Peakall

Table 2. Sequences and other details regarding the nine SRAP primer combinations used in this study.

Primer combination	Primers Sequences (5' → 3')	Tb [a]	Pb [b]	Pp [c]	Ab [d]	Bs [e]
Me1+Em1	*TGAGTCCAAACCGGATA* *GACTGCGTACGAATTAAT*	21	21	100	20.1	100- 1750
Me1+Em2	*TGAGTCCAAACCGGATA* *GACTGCGTACGAATTTGC*	24	24	100	18.2	100- 1500
Me1+Em3	*TGAGTCCAAACCGGATA* *GACTGCGTACGAATTGAC*	21	21	100	19.6	230- 2500
Me3+Em1	*TGAGTCCAAACCGGAAT* *GACTGCGTACGAATTAAT*	26	26	100	18.9	100- 1400
Me3+Em2	*TGAGTCCAAACCGGAAT* *GACTGCGTACGAATTTGC*	23	22	95.6	20.2	120- 2900
Me3+Em3	*TGAGTCCAAACCGGAAT* *GACTGCGTACGAATTGAC*	26	26	100	22.4	100- 2500
Me4+Em1	*TGAGTCCAAACCGGACC* *GACTGCGTACGAATTAAT*	34	34	100	14.7	100- 1800
Me4+Em2	*TGAGTCCAAACCGGACC* *GACTGCGTACGAATTTGC*	22	20	90.9	26.3	100-1750
Me4+Em3	*TGAGTCCAAACCGGACC* *GACTGCGTACGAATTGAC*	31	30	96.7	17.4	100- 1800
Total		228	224			
Average		25.3	24.9	98.1		

[a] Total number of bands; [b] Number of Polymorphic bands; [c] Percentage of Polymorphism, [d] Average number of bands, [e] Band size range (bp).

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and Smouse, 2006). Moreover, Nei's (1973) gene diversity (H_e), and the Shannon diversity index (H) for each geographic group were measured using POPGENE32.

The genetic relationships among genotypes were reconstructed by Minimum Spanning Tree (MST) on the PCoA plot, based on the Dice similarity coefficient using NTSYSpc ver. 2.02 software.

Population genetic structure was assessed using a model based on Bayesian method implemented in STRUCTURE ver. 2.3.4 (Pritchard *et al.*, 2000; Falush *et al.*, 2003). The program was run with values of K ranging from 1 to 10, with 25,000 burn-in iterations and 75,000 MCMCs, with 15 independent runs for each *K*, using the admixture model with correlated allele frequencies. Graphical representations of these statistics were obtained using online program STRUCTURE HARVESTER ver. 0.6.94 (Earl and Vonholdt, 2012). The software CLUMPP 1.1 (Jakobsson and Rosenberg, 2007) was used to find optimal alignments of independent runs and the output was used directly as input into a program for cluster visualization DISTRUCT 1.1 (Rosenberg, 2004).

RESULTS

The nine SRAP primer combinations created a total of 294 bands from DNA of 57 genotypes, from which 291 (98.9%) were polymorphic. Sixty six bands with high missing data were not entered in raw data matrix and the analyses were made based on 228 bands. The number of polymorphic bands detected with each primer combination ranged from 21 (Me1, Em1 and Me1, Em3) to 34 (Me4, Em1) with an average of 25.3 (Table 2). The sizes of the amplified products ranged from 100 to 2,900 bp.

Genotypes collected from the Northwest of the country showed the highest percentage of polymorphism (85.96%) and the genotypes collected from around Tehran showed the lowest percentage of polymorphism (26.32%), but these were correlated with the number of genotypes (Table 3).

Based on the topology of the dendrogram (Figure 1), no robust clustering of genotypes related to the geographic region was observed. Only genotypes collected from around Tehran (Teh in dendrogram), Isfahan (Isf in dendrogram), and genotypes collected from the neighboring regions, Hamedan (Ham) and Kermanshah (Krm), were mainly grouped with their co-regional genotypes. Pattern of relationships in the Minimum Spanning Tree (MST) and PCoA plot (Figure 2) was generally concordant with grouping of genotypes as shown in NJ dendrogram.

The AMOVA attributed 88% of the total genetic diversity to the within-geographic groups and 12% to the between groups. The genetic differences (PhiPT) among groups were also estimated using the AMOVA procedure to investigate the hierarchical partitioning of genetic variation among groups, it amounted to 0.12. The gene flow among groups, or genetically effective migration rate (Nm), was indirectly calculated from PhiPT as the $Nm= 0.25 [(1/PhiPT)–1]= 1.83$.

Results of the Mantel test showed that there was no significant correlation between genetic distance and geographical distance of genotypes of *T. pratense* ($r^2= 0.0251$, P= 0.02).

For all groups, the mean Nei`s diversity index (H_e) was lower than the mean Shannon index (H_o). The lowest mean Shannon index (0.139) was measured in Tehran group, while the highest value (0.382) was found in the Northwest group.

For the SRAPs, the model-based approach of STRUCTURE indicated $K = 2$ as the most probable number of genetic clusters. These clusters were not correlated with the geographic regions (Figure 3).

In a preliminary evaluation of morphological variations (data not shown), the genotypes were highly diverse and the morphological diversity was distributed all

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Table 3. Genetic diversity parameters measured within populations (geographic regions) of *T. pratense.*[a]

Population	Ng [a]	Pp [b]	Shannon index (H)	Nei`s diversity index (H_e)	Ea [c]
Northwest	21	85.96	0.382 (0.015)	0.254 (0.012)	3
North	11	79.82	0.374 (0.016)	0.259 (0.013)	2
Isfahan	9	58.33	0.309 (0.019)	0.221 (0.014)	1
West	10	62.28	0.313 (0.019)	0.221 (0.014)	3
Tehran	5	26.32	0.139 (0.016)	0.104 (0.012)	0
Total	56				
Average		62.54 (10.43)	0.304 (0.008)	0.212 (0.006)	

[a] Number of genotypes; [b] Percentage of Polymorphism, [c] Number of Exclusive alleles.

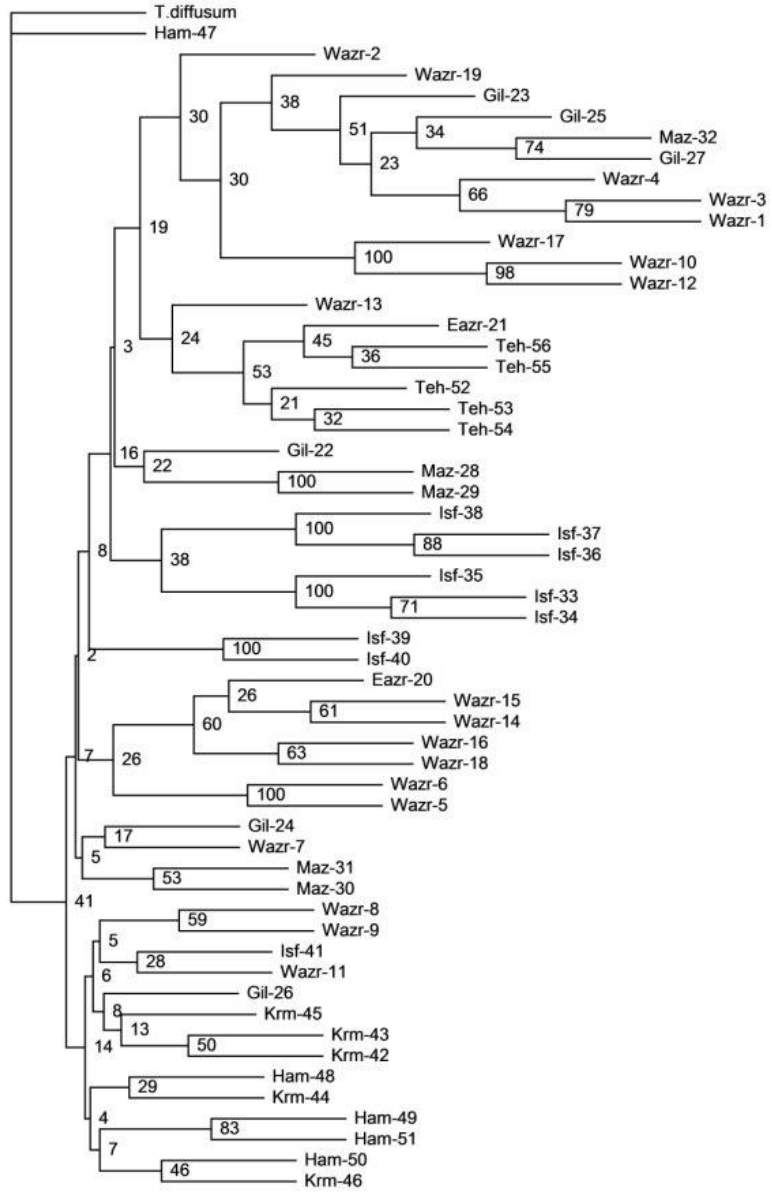

Figure 1. The rooted SRAP dendrogram showing molecular relationships among 56 Iranian genotypes of *T. pratense* generated using Neighbor-Joining method and Jaccard's similarity coefficient. The relevant geographic group of each genotype is shown in Table 1.

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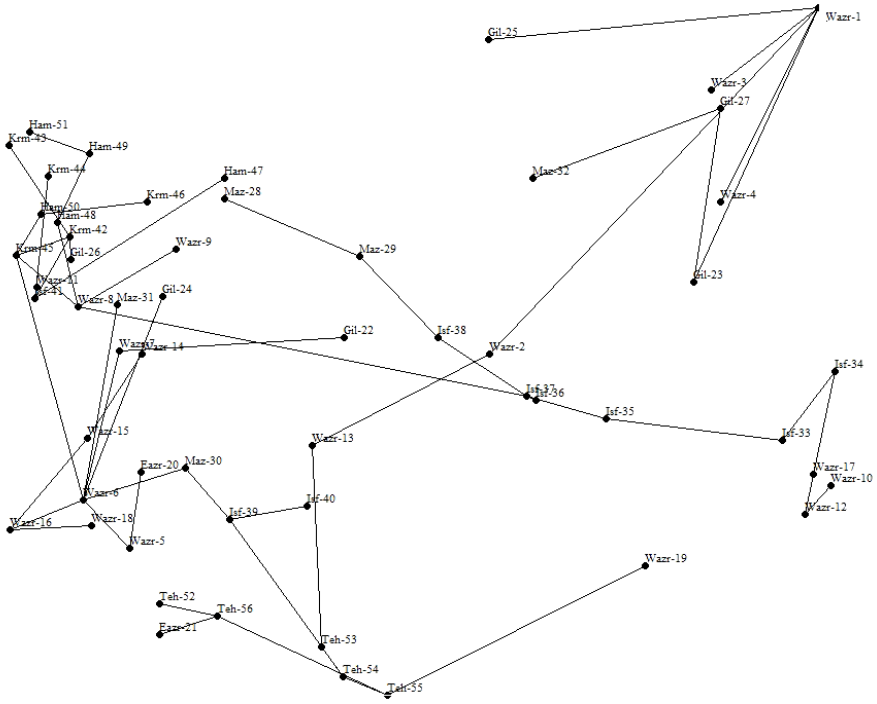

Figure 2. Minimum Spanning Tree (MST) and 2D plot generated by principal coordinate analysis and Dice similarity coefficient based on SRAPs of 56 *T. pratense* genotypes (Table 1).

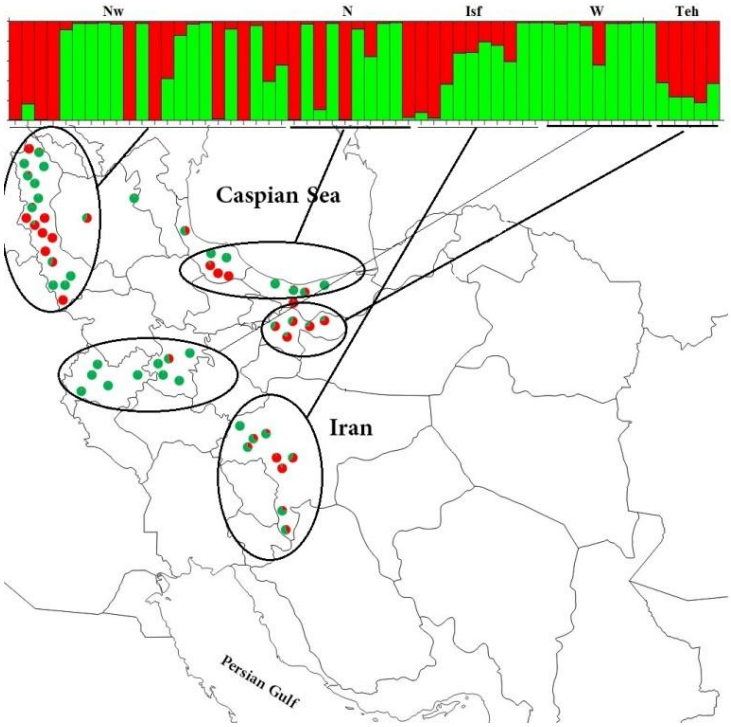

Figure 3. Map of the collection site and population structure of the 56 genotypes of *T. pratense* grouped in 5 geographic groups (Nw= Northwest, N= North, Isf = Isfahan, W= West, Teh = Tehran) analyzed using SRAP markers. Structure analysis results revealed two clusters (Green: cluster 1 and Red: cluster 2) for five regions for the wild Iranian *T. pratense* gene pool. Each vertical column represents one genotype.

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over the regions, inasmuch as we couldn`t exactly assign any genotype to any previously defined varieties or subspecies. Anyway, as shown in Table 1, we assigned some genotypes to their closest variety based on the morphological characters, solely for providing an overview of infraspecific taxonomy of the species in Iran. There was no geographic gap between the distribution ranges of the varieties.

DISCUSSION

This study provided a view of the genetic diversity of *T. pratense* in Iran. The red clover populations in Iran occur mainly along the Zagros Mountain chain (with a Northwest-Southeast orientation) and Alborz Mountain chain (with a Northwest-Northeast orientation) in different habitats with different elevation and ecological conditions. They grow as wild and weedy plants and there are no cultivation reports of this species in Iran. However, *T. pratense* along with other clover species constitute the suave elements of pastures in this region.

This species occurs sparsely in Iran, hence, the range of each population is unclear, therefore, low number of individuals (56 genotypes) of this species are collected and analyzed in this study. Also, for the same reason, we couldn`t recognize true natural populations and, therefore, genotypes were divided into geographic groups.

The high level of genetic diversity measured in this study was comparable with the level of morphological diversity in some red clover collection reported by Kouame and Quesenberry (1993), Dias *et al.* (2008) and Pagnotta *et al*. (2011). Since the center of diversity of the red clover is considered to be the Mediterranean region (Taylor and Quesenberry, 1996), the Iranian populations of this species are considered as peripheral populations and therefore expected to have lower diversity. The high genetic diversity in Iranian germplasm revealed in this study can be regarded as a result of high ecological adaptations reinforced by outcrossing and high gene flow among populations.

Regarding the clustering pattern in dendrogram (Figure 1), the genotypes collected from Kermanshah (Krm) and Hamedan (Ham) in the west, particularly Ham-47, appeared in the base of the dendrogram, close to the outgroup. The western region of Iran, where is close to the Mediterranean region, is more likely receptive of the early migrants of the species. Therefore, the western region may contain more diverse populations, as they appeared in this study. Of course, subsequent distribution of different genotypes via birds, livestock, and human activities probably defaced the migration pathways of the species in Iran.

The result of Mantel test, showed no correlation between genetic and geographic distance. Also, the STRUCTURE diagram, showed 2 genotypic clusters of *T. pratense* in Iran (Figure 3), both distributed all over the regions. However, regarding the clustering revealed in dendrogram, there are some tendencies in genotypes to be compartmented in local populations such as those growing around Tehran, around Isfahan and from Kermanshah to Hamedan. Factors such as adaptation, natural selection, and gene flow usually shape the pattern of diversity within a species. In this study, the amounts of PhiPT, Nm and AMOVA clearly justified the occurrence of high gene flow among populations. Most studies in species with pollination mechanisms similar to the red clover showed that most of the gene flow occurs at shorter distances from the source (see for example: Woodfield *et al*., 1995; St Amand *et al*., 2000; Hüsken and Dietz-Pfeilstetter, 2007; Van Deynze *et al*., 2008; Lucas *et al.*, 2012). Regarding the geographical distance between groups of red clover evaluated in this study, pollen transmission by pollinators could not be an effective tool for this level of gene flow. Probably other mechanisms such as seed dispersal by human, migratory birds, or livestock facilitated the gene flow over the long distances. The location of most of the

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genotypes near the road sides, which is easily exposed to the seed dispersal by human, reinforced this possibility. These factors probably resulted in a pattern of genetic diversity which is not clearly consistent with the 5 supposed eco-geographical groups. Greene *et al.* (2004) showed that the pattern of diversity in Caucasus populations of red clover is correlated with ecological condition. Regarding the mosaic pattern of ecological conditions in Iran that change even in a short distance, adaptation could be another important factor that resulted in a mosaic pattern of diversity in *T. pratense.*

The results of AMOVA revealed that the main proportion of diversity resided within the geographic groups. This situation could have resulted from the outcrossing nature of the species and ecological adaptations. Since the effect of selection imposed by environmental heterogeneity increases with the spatial scale of populations (Ward, 2006), the high within group diversity of red clover revealed in this study can be related to the spatial distance of genotypes, because the genotypes of each group in this study were collected from relatively wide regions.

There is no information on the genetic diversity of other clover species in Iran to be compared with this results. But, in the pattern of genetic diversity in inbreeding species such as *Aegilops tauschii* Coss. (Saeidi *et al.*, 2006), diploid species of *Triticum* L. (Mousavifard *et al.*, 2015) in Iran, despite being highly diverse, some eco-geographical grouping was evident, which probably resulted from their inbreeding nature. Comparison of genetic diversity of species with different breeding systems in same area could elucidate the influence of breeding mechanism of the species on the pattern of genetic diversity.

The highest polymorphism and Shannon information index (H) were measured among the Northwestern genotypes and the lowest ones among the genotypes collected around Tehran; however, these were correlated with the number of genotypes of each region. Therefore, it can be said that these differences have resulted from sample size and the genetic diversity is evenly distributed over the regions.

In this study, the morphological and physiological traits that are important in the breeding point of view, and for finding populations with higher priority for conservation, were not evaluated. Many studies showed that there were no correlation between patterns of diversity revealed by morphological traits and molecular markers (see for instance Mansour *et al*., 2015). Regarding the importance of the wild gene pool of red clover as a gene source for crop improvement, we strongly recommend that the future studies should be focused on examining the morpho-physiological traits and adaptation-based diversity among Iranian gene pool of this species. A more detailed analysis of genetic diversity at a smaller spatial scale among local populations using more genotypes would be helpful to elucidate the patterns of diversity related to adaptation and short distances gene flow.

The result of this study showed high genetic diversity in the Iranian germplasm of the red clover and the value in probing this genepool for useful alleles for breeding purposes. There is value in sampling the populations all around Iran, with the Western and the Northwestern populations more intensively, for conservation and breeding purposes.

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تحلیل گوناگونی وراثتی شبدر قرمز (*Trifolium pratense* L.) در ایران با استفاده از نشانگر SRAP

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چکیده

موفقیت یک برنامه اصلاح نبات مستلزم داشتن اطلاعات وسیع از مبانی وراثتی است. شناخت تنوع وراثتی یک گونه زراعی و خویشاوندان خودروی آن برای اهداف اصلاح نبات اهمیت حیاتی دارد. شبدر قرمز، *T. pratense*، در ایران عمدتاً در دامنه های البرز و زاگرس و در زیستگاه های مختلف با شرایط گوناگون بوم شناختی می روید. علی رغم اهمیت اقتصادی این گونه در برخی کشورهای دیگر، اطلاعاتی در مورد تنوع وراثتی این گونه در ایران وجود ندارد. در این تحقیق تنوع وراثتی ۵۶ ژنوتیپ شبدر قرمز از نقاط مختلف ایران و یک ژنوتیپ از گونه *T. diffusum* که به عنوان برون گروه استفاده شده بود، با استفاده از ۹ نشانگر SRAP ارزیابی شد. در واکنش زنجیره ای پلیمراز مجموعاً ۲۹۴ باند از ۹ نشانگر SRAP در کل نمونه ها ایجاد شد که ۲۹۱ (۹۸/۹٪) باند آن پلی مورف بودند. تمام پارامترهای اندازه گیری شده نشان دهنده تنوع وراثتی بالای این گونه در ایران بود وگروهبندی مشهودی از ژنوتیپ ها مرتبط با مناطق مختلف جغرافیایی مشاهده نشد. با این وجود،

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ژنوتیپ های جمع آوری شده از اطراف تهران، اصفهان و کرمانشاه-همدان تا حدودی شباهت های وراثتی با نمونه های همان مناطق نشان دادند. در تحلیل داده های STRUCTURE ژنوتیپ ها به دو گروه ژنتیکی تقسیم شدند که هر دو گروه در تمام مناطق پخش شده بودند. همبستگی معنی داری بین فواصل ژنتیکی ژنوتیپ ها و فواصل جغرافیایی آنها مشاهده نشد. نتایج این مطالعه نشان داد که خزانه وراثتی شبدر قرمز در ایران، بویژه جمعیت های غربی و شمالغربی آن، برای اهداف حفاظت و اصلاح نبات ارزشمند است.

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