POPULATION GENETIC VARIATION IN SAINFOIN (FABACEAE) REVEALED BY RAPD MARKERS

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Abstract. Studies on plants show that populations growing on the stressful environments indicate higher levels of genetic diversity, and that in outcrossing species majority of total genetic variation allocated to within population rather than between populations. We compared the level of genetic variation between populations growing in stressful and normal environments, and measured levels of within- and between population genetic variations in *Onobrychis viciifolia* L. (Sainfoin, Fabaceae) based on RAPDs. Our results show that populations growing on the stressful environment i.e. saline soils indicated either the lowest (0.2466) or highest (0.3186) within-population genetic variation based on Nei's diversity. That disagrees with Niche-Width Variation Theory, which expects highest genetic diversity within stressful populations. Partitioning the total genetic variation by analysis of molecular variance (AMOVA) showed that 89.03% of total genetic diversity allocated to within populations while 10.97% of this variation dedicated to among populations, indicating predominantly outcrossing mode of pollination in sainfoin. The two population pairs growing under similar environmental stresses (cold climate and saline soil) showed higher genetic similarity. This may suggest that RAPDs patterns reflex selection rather than random drift.

Keywords: environmental stresses, genetic diversity, *Onobrychis viciifolia*, outcrossing, Sainfoin.

INTORDUCTION

The population genetic structure of the plant species can be influenced by several factors, including the breeding system (e.g. selfing/outcrossing rates and sexual/asexual reproduction), geography [16, 29] and environmental stresses such as those of climatic and edaphic [25, 26]. The breeding system affect on movement of genes in space and their transmission through time, so plays a fundamental role in determining the spatial and temporal patterns of genetic diversity within and between populations [15, 16]. Geographical factors impact on population genetic structure through gene flow and seed dispersal [15].

Studies on relationship between ecological factors and population genetic variation have become a central research in current plant biology [13, 22], and used to address the association between biological complexity and ecological factors [18]. In addition, assessment the levels and patterns of genetic variation among individuals and populations in plants is important for understanding the breeding systems and the population dynamics, as well as for decision on conservation programs [29].

Based on Niche Width Theory, it is expected that populations growing on the stressful environments show higher levels of genetic diversity within population than those populations growing on normal ones [33]. There have been many empirical studies supporting the theory by reporting higher genetic variation from populations growing in stressful environments [7, 26, 32]. Moreover, studying the patterns of the environmental effects on populations' genetic structure in plants could be used to understand the adaptation of species to the environmental changes [5, 30], the evolutionary processes and selective forces shaping the population genetic structure, which are difficult to reveal under less extreme conditions [25, 26]. Recent progresses in assessing relationship between ecological and molecular studies have mostly focused on edaphic and climatic adaptations [18].

This work aimed to study the impact of environmental (edaphic and climatic) stresses on the levels of genetic variation in populations growing on stressful and non-stressful environments, and also to estimate the levels of within- and between population genetic variations among different wild populations of *Onobrychis viciifolia* sampled from ecologically different regions using randomly amplified polymorphic DNAs (RAPDs).

MATERIALS AND METHODS

Study species and site

The species *Onobrychis viciifolia* Scop. (Sainfoin, Fabaceae) is a perennial forage legume and naturally distributed across Eurasia, and also widely cultivated for hundreds of years in Europe and Asia, and later in North America [35]. Sainfoin is a high quality forage legume and has excellent nutritional and palatability properties [34].

Five wild populations of *Onobrychis viciifolia* were studied from ecologically different regions in East-Azerbaijan, Iran. We included two populations from warm climate with saline soils (Amand and Bonab), and two populations from regions with high altitude and cold climate (Sarab and Heris). The fifth population (Khosrosha) was included in the study from an area situated in the middle of the other populations. The populations were separated from each other by minimum geographical distance of over 50 km, and isolated by high mountains and large city. Ten individual plants were randomly sampled from each population with minimum distance of 400 m between individuals in each population.

DNA extraction and PCR profile

Genomic DNA was extracted from seeds and/or seedlings according to Miller [21] previously used to

extract nuclear DNA from cress cotyledons, with minor modification of replacing silver sand by liquid nitrogen. The concentration of DNA samples was estimated by both the gel electrophoresis and spectrophotometry, and adjusted at 10ng/ml wherever necessary. Ten decamer arbitrary RAPD primers (CinnaGen, Iran) were examined, of which five primers produced the most polymorphic clear bands, and were therefore selected for further analysis (Table 1). PCR profile was carried out using Master Mix (CinnaGen PCR MasterKit, Cat. No. PR8251C) comprising of Taq DNA Polymrease (0.04 u/µl), $MgCl_2$ (3 mM), dNTP (0.4mM of each dATP,dCTP, dGTP, dTTP) being added 10 ng template DNA. Amplifications were performed in a Biometra thermal cycler for an initial 4 min denaturation at 94°C followed by 40 cycles of 1 min at 93°C (denaturation), 1 min at 40°C (annealing) and 1.5 min at 72°C (synthesis). All PCR products were separated by electrophoresis on 1.5% w/v agarose gels in 1X TBE buffer, stained with ethidium bromide (0.2 µg/µl), viewed under ultraviolet light and photographed using UV Transilluminator (UVP, USA). PCR reactions and electrophoresis were repeated at least twice in each case to ascertain the reproducibility of the bands.

RAPDs data analysis

The banding patterns were scored as 1 for present and 0 for absent of a band. Then, the data were entered in a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, ver. 2.02). The number and percentage of polymorphic RAPD bands were obtained for each population. Genetic diversity within each population was estimated based on Nei's [23] and Shanon's information index using Popgen (version 1.32). In order to compare relationship among the populations, the UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) dendrogram was generated based on matrix of Nei's distances among populations through the SHAN (sequential, hierarchical, agglomerative and nested clustering of the NTSYS-pc). Total genetic variation was partitioned into within and among the populations based on analysis of molecular variance (AMOVA) using Arlequin (version 3.11). The significance level for F-statistics analogous was determined using 1023 bootstrap replicates. The relationship between genetic and geographical distances was examined using Pearson Correlation test (SPSS, 11.3).

RESULTS

RAPD patterns

A total number of 34 clear, polymorphic and reproducible RAPD bands were obtained by using five out of 10 tested primers in five populations comprising of 50 individuals (Fig. 1).

Each primer produced on average 6.8 polymorphic bands (Table 1). Percentage of polymorphic RAPDs bands ranged from 66.67 to 84.62% (respectively in Bonab and Amand populations).

Genetic distance

The lowest (0.2466, Nei's; 0.3640, Shannon's diversity index) and highest (0.3186 Nei's; 0.4618, Shannon's) within-population genetic diversity were revealed respectively in Bonab and Amand populations both growing on stressful environments of saline soils (Table 2).

Partitioning of total genetic variation to within- and among-populations by analysis of molecular variance (AMOVA) indicated that 10.97% of total genetic variation belongs to among populations and 89.03% to within populations (Table 3).

Matrix of Nei's Distances showing genetic distance between pairs of populations indicated the shortest distances between Heris and Sarab (0.0635), and also between Bonab and Amand (0.0795) populations, while the largest distance was observed between Sarab and Bonab (0.1411) and also between Heris and Bonab (0.1654) (Table 4).

In the UPAGMA dendrogram based on matrix of Nei's distances (Table 4) the Bonab and Amand populations were closely nested on one branch, and Sarab and Heris populations were located on another branch, while the Khosrosha was separated from all other populations (Fig. 2).

Assessing the relationship between the geographical and genetic distances among the populations (Table 5) indicated that genetic distances between populations did not correlate with populations geographical distances in *O. viccifolia* in the study region (Fig. 3; $P > 0.3$, N=10, Pearson Correlation Test, SPSS, 11.3).

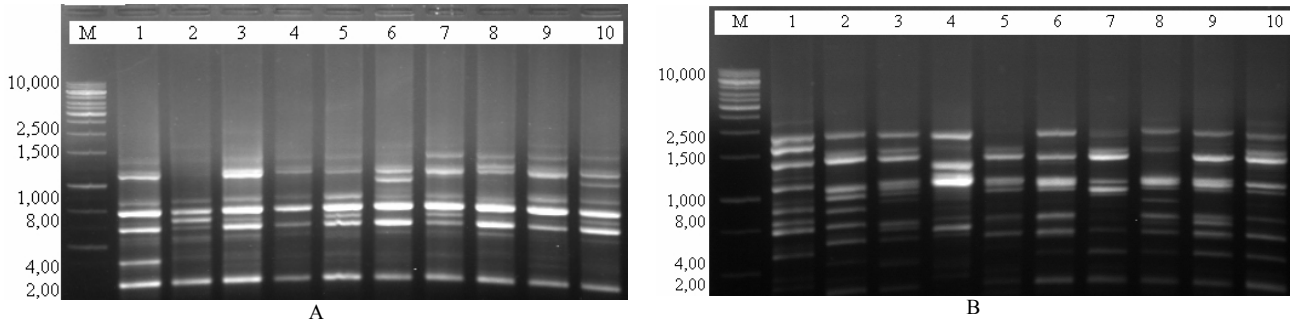

Figure 1. RAPD patterns of *Onobrychis viciifolia* populations produced by primer A in Bonab (A) and Heris populations (B). The first lane from the left is the standard size marker in base pair.

Table 1. Primers sequences and number of polymorphic bands produced in five different populations of *Onobrychis viciifolia*.

Primers code	Sequences (5' to 3')	No. polymorphic Bands
A	TGGTCGCAGA	9
B	GGACACCACT	8
C	CCACACTACC	6
D	TGAGCCTCAC	6
E	CCGAACACGG	5

Table 2. Percentage polymorphic RAPD bands and levels of genetic diversity in five different populations of *Onobrychis viciifolia*. Both the lowest and highest genetic variation were detected in two populations both growing on saline soils

Population	%Polymorphic RAPD bands	Nei's diversity (H)	Shannon's index (I)
Bonab	66.67	0.2466	0.3640
Sarab	71.79	0.2507	0.3777
Khosrosha	76.92	0.2821	0.4175
Heris	76.92	0.3112	0.4617
Amand	84.62	0.3186	0.4618

Table 3. Partitioning the total genetic variation to within and between populations in *Onobrychis viciifolia* by AMOVA (Significance test conducted using nonparametric method randomly sampling of 1023 pretribulations times; F_{ST} =0.10971)

Mode of variation	df	Sum of square	Variance	%Variance	*P* value
Among populations	4	53.1	0.7328	10.97	0.00 >
Within populations	45	267.6	5.9466	89.03	0.00 >

Table 4. Matrix of genetic distance between pairs of *Onobrychis viciifolia* populations based on Nei's distances

Population	Amand	Bonab	Sarab	Khosrosha	Heris
Amand	0.0	-	-	-	-
Bonab	0.0795	0.0	-	-	-
Sarab	0.1386	0.1411	0.0	-	-
Khosrosha	0.0882	0.0854	0.0895	0.0	-
Heris	0.1268	0.1654	0.0635	0.1218	0.0

DISCUSSION

In the current study the two populations of *Onobrychis viccifolia* both growing on stressful environment of saline soils indicated either the lowest or highest within-population genetic variation. This is, in part, in disagreement with the niche-width variation theory [33], which expects higher levels of genetic diversity in populations growing on the stressful environment. The reasons for lower genetic variation within population of *O. viccifolia* in stressful environments could be due to the fact that these populations have either recently established owing to the founder effect, or derived from a bottleneck effect. This could likely be resulted from implementing small number of primers. However, using ISSRs markers on this subject the obtained results were confirmed (un-published data).

Several comparative studies on plant populations have reported the highest amount of genetic variation in population growing under environmental stresses. The highest levels of RAPDs diversity were revealed in wild populations of *Hordeum spontaneum* from areas under aridcold or aridhot stresses [26]. Similarly, the populations of *Triticum dicoccoides* L. growing under environmental stresses of the high-altitude and arid-cold showed highest RAPDs diversity compared to those populations from normal environments [21]. Among different populations of *Aster tripolium* a salt dumps population showed the highest RAPDs variability despite having small size [7]. Positive relationship has also been reported between genetic diversity and ecological factors using other molecular markers. SSRs and ISSR-based higher genetic diversity were detected in populations of *Hordeum spontaneum* growing under arid-hot stress [32], and of *Viola tricolor* growing on the contaminated soils with heavy metals e.g. zinc, lead, and cadmium compared to those populations growing on non-contaminated soils [31]. In contrast, some reports have shown that levels of genetic variation in plant populations growing on stressful environments are equal to or lesser than those values obtained for populations growing under normal conditions. The equal levels of within-population genetic diversity were reported among copper tolerant

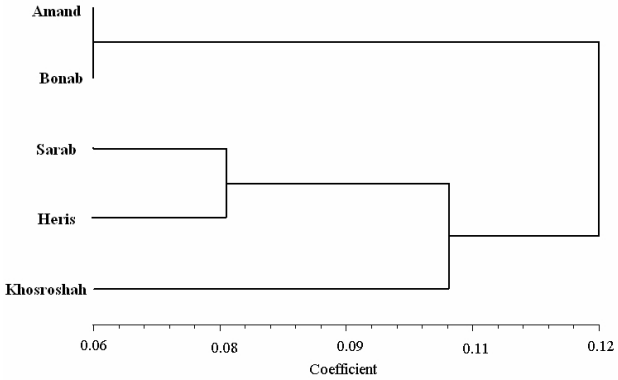

Figure 2. UPGMA dendrogram based on Nei's distance matrix showing genetic similarity among five populations of *Onobrychis viccifolia*

Table 5. Ggeographical (Km) and genetic distances (Nei's distance) between population pairs of *Onobrychis viccifolia*

Population pair	Geographical distance	Genetic distance
Amand - Bonab	150	0.0795
Amand - Sarab	150	0.1386
Amand - Khosrosha	50	0.0882
Amand - Heris	70	0.1268
Bonab - Sarab	80	0.1411
Bonab - Khosrosha	110	0.0854
Bonab - Heris	160	0.1654
Sarab - Khosrosha	130	0.0895
Sarab - Heris	40	0.0635
Khosrosha - Heris	70	0.1218

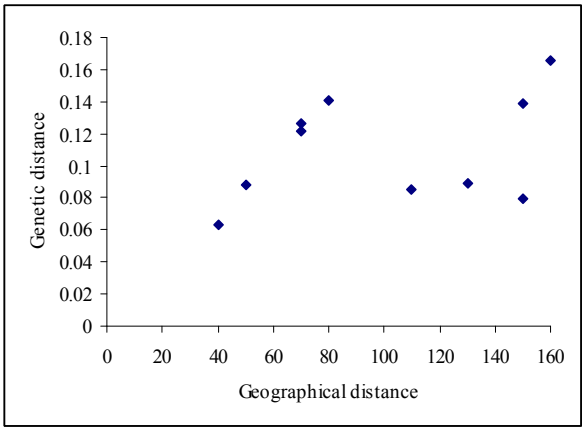

Figure 3. Lack of correlation between geographical (Km) and genetic distances (Nei's) among populations of *Onobrychis viccifolia*

and non-tolerant populations of *Agrostis stolonifera* [36] and *Arrhenatherum elatius* [11]. Significant lower levels of RAPD diversity were detected in populations of *Sedum alfredii* growing on soils with high concentration of heavy metals [10], and of *Larix gmelinii* growing on fluoride polluted area [19]. These lower genetic variations detected in stressful populations have been attributed to either the founder effect, bottleneck effect and/or gene flow among normal and stressful populations. All these data indicate that patterns of genetic variation among populations of plant taxa growing under environmental stresses are more diverse than pattern previously proposed.

Our results on genetic variation of different populations of *Onobrychis viciifolia* showed that most of the total genetic variation allocated to within (89.03%), rather than among populations (10.97%). This indicates that *O. viciifolia* is mostly an outcrossing species. This is consistent with values reported for other outcrossing plant species using RAPDs, in which within-populations genetic variation accounts for the majority of total variation e.g. 73% to 80.5% in *Buchloe dactyloides* [17], 74% to 95% in *Eucalyptus globulus* [24], 63% in *Vicia dumetorum* [6], 86% in *Fitzroya cupressoides* [2], 74% in *Ranunculus reptans* [14], 93% in *Podocarpus salignus* [3], 81.4% in *Pilgerodendron uviferum* [4] and 88.7% in *Astragalus oniciformis* [1], although cases of equal or lower levels of within-populations genetic variation were reported for outcrossing legumes, for example, 50% in *Medicago sativa* [9] and 40.1% in obligate outbreeder *Gliricidia sepium* [8].

Narrow range of polymorphic RAPD bands percentage (66.7 - 84.6%) and the within-population genetic variation (0.25- 0.32, Nei's) were detected across the Sainfoin populations. This low genetic variation from population to population is a general pattern in outcrossing species, because predominantly outcrossing species have higher levels of genetic variability within populations but a lower degree of differentiation among populations compared to selfing species [16].

The genetic closeness between the two geographically distant but ecologically similar populations of Bonab and Amand (both growing on saline soils), and Heris and Sarab (both distributed in regions with high altitude and cold climate) may reflect the ecological impact, rather than geography. Due to small number of populations included in this study, it was not possible to assess statistically the relationship between ecology and population similarity. In general, the association of genetic variation with environmental conditions was considered due to either natural selection or local gene dispersal [15, 16]. Regarding natural barriers e.g. high geographical distances (over 50Km) and large cities between the five populations studied, it is unlikely that gene flow occurs between these five populations. This might suggest a positive relationship between RAPD patterns and ecological similarities among populations of Sainfoin studied. A partial ecological relationship between RAPDs patterns was revealed among *O. viccifolia* populations using bulked DNA [28]. However, many other studies have interpreted the association between environmental conditions and RAPD patterns among populations of plant species due to natural selection (see below). Correlation of RAPD differentiation with the divergence of climatic factors and lack of that with geographical distances was reported among different populations of *Stipa grandis* [27]. Similarly, association of RAPD genetic similarity with environmental stresses such as hot or cold steppes and deserts, but not geography was observed among populations of wild barley *Hordeum spontaneum* [26]. Significant correlations of RAPDs similarities with both climatic and soil factors, and lack of any these correlations with geography, were reported among different populations of wild *Triticum dicoccoides* [12]. In addition, similar association between genetic similarity and the environmental stresses has been reported in plant populations using allozyme markers and SSRs [20]. All these studies along with our data may support the idea that there is a selection-based cause for association between genetic and ecological similarities in the plant populations. However, many more studies by including greater numbers of populations are required to establish the general pattern of association between ecological factors and RAPD markers.

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