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Population Structure and Genetic Diversity of a Medicinal Plant Species *Retama raetam* in Southern Tunisia

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Abstract: *Retama raetam* is a stem-assimilating, C3, evergreen, medicinal plant species, desert legume common to arid ecosystems around the Mediterranean basin. This study addresses the genetic diversity and relationship among and within three populations collected from different habitats in southern Tunisia by Random Amplified Polymorphic DNA (RAPD). Estimates of the percentage of polymorphic bands, Shannon's diversity information index and Nei's gene diversity index were determined. Results showed that population from the Island Djerba has the lowest Nei's gene diversity; this also was for Shannon diversity index. An analysis of molecular variance indicated that the majority of variation existed within populations (68%) and that there was significant differentiation among populations ($\Phi PT = 0.316$, $p<0.001$). Genetic distance (ΦPT based values) between pair-wise populations ranged from 0.098 to 0.505 and the differentiation between pair-wise populations was significant when individual pairs of populations were compared. Based on the coefficient of gene differentiation (Gst), gene flow (Nm) was estimated and was found to vary from 0.490 to 4.609 between pair-wise populations and 1.42 among populations. The results of UPGMA cluster analysis and PCoA analysis indicated that most variation occurred within populations and that genetic differentiation had happened between populations. These findings are important for a better understanding of the adaptive strategy of *R. raetam* in southern Tunisia and will be useful for conservation managers to work out an effective strategy to protect this important species.

Key words: Genetic differentiation, population structure, gene flow, biodiversity conservation

INTRODUCTION

Response to environmental changes depends on the levels of genetic diversity that species have (Ayala and Keger, 1984). According to Qian and Ge (2001), lack of suitable amount of genetic diversity could make species sensitive to abiotic and biotic stress.

Plant conservation is extremely important when considering among population genetic differentiation (Gustafson *et al.*, 1999; Qiu *et al.*, 2005). Additionally, care should be taken when germplasm is collected from species for which within population genetic structure is poorly understood (Dyer and Rice, 1997; Zhao *et al.*, 2008). Therefore, investigations of population genetic diversity and genetic structure provide useful information and a better measure of the ecosystem's genetic health for biological conservation (Schaal *et al.*, 1991; Gustafson *et al.*, 1999).

Medicinal plants have been used for many years in folk medicine and nowadays they are used as natural sources for many modern drugs. In fact, many bioactive molecules are isolated from these plants and are used in pharmacology. In the recent decades, the abuse of its use and the increasing human disturbance has led to recent decline of some species (Schippmann *et al.*, 2002; Larsen and Olsen, 2007). Therefore, their conservation is most important for biodiversity manipulation (Kate and Laird, 1999). *Retama raetam* is a stem-assimilating, C3, evergreen, desert legume common to arid ecosystems around the Mediterranean basin, *Retama raetam* (Forsk.) Webb, belonging to the Fabaceae family, locally named as ''R'tam'', is a wild plant common to the North and East Mediterranean regions (Mittler *et al.*, 2001). It is used in folk medicine for treatment of many diseases such as hypertention, renal diseases, etc. (Kassem *et al.*, 2000; Bruits and Bucar, 2000). It was also used as an abortifacient, a purgative and a vermifuge in the traditional medicine (Al-Tubuly *et al.*, 2011). Earlier studies revealed that this plant possesses antibacterial, antifungal, antihypertensive, anti-oxidant, antiviral, diuretic and hypoglycemic properties and hepatoprotective, nephroprotective and cytotoxic effects

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Conservation is based on diversity of plant species and on genetic diversity of intra- and inter- population of these species belonging to different environment. Biotechnological tools make it easy to analyze genetic characteristics of a specific species in varying environmental conditions. Over the past decades, many molecular markers have been used in detecting species' polymorphism such as RAPD markers (Williams *et al*., 1990). This technique is widely used for studying the genetic diversity and the population structure of medicinal and aromatic plant populations (Messaoud *et al*., 2007; Solouki *et al*., 2008; Zheng *et al*., 2008).

The objective of this study is to test the hypothesis that populations' genetic diversity is influenced by geographic isolation and to elucidate the genetic relationships between populations of *R. raetam*. Additionally, the efficient RAPD marker technique will be tested to develop efficient conservation strategies for this species.

MATERIALS AND METHODS

Surveyed populations and sampling: *Retama raetam*, known as white broom, is a perennial legume shrub of great ecological amplitude. This shrub can reach 3 m high (Fig. 1); it is characterized by tiny simple short-lived leaves (CRC Weed Management Guide, 2003). The flowers are composite and irregular. The fruit is a small ovoid pod with a beak containing one or two small kidney-shaped seeds (Spooner *et al*., 2008). The root system consists of a pivot which can reach several meters deep and adventitious roots that colonize the surface of the dunes.

Three Tunisian *Retama raetam* populations were analyzed (Table 1). The populations are located in lower arid and desert bioclimatic areas. The altitudes of the sites varied from 5 m (Djerba) to 316 m (Edhaher). Plants from each population were sampled at distances exceeding 20 m to avoid the sampling of closely related individuals. Samples were placed on ice in plastic bags and transported to the laboratory for molecular analyses.

Fig. 1: Dendrogram of UPGMA cluster analysis based on the RAPD data illustrating the genetic relationships based on SM similarity coefficient among the 24 individuals and 3 populations of *R. raetam* in different environments in southern Tunisia. A1-A10 were samples from Island Djerba, A11-A20 from Essaâden and A21-A24 from Edhaher

Table 1: Localities and sizes of the 3 *Retama raetam* populations sampled in this study

Sites	Population ID	Latitude (N)	Longitude (E)	Altitude (m)	Population size
Djerba	pop1	33°52'09''	010°55'23''	5	10
Essaâden	pop2	33°27'21''	010°36'40''	27	10
Edhaher	pop3	33°13'31''	009°55'42''	316	4

DNA extraction procedure and quantification: Total genomic DNA was extracted from green braches using modified CTAB (cetyltrimethyl ammonium bromide) method described by Abdellaoui *et al*. (2011). Approximately 200 mg of the youngest branch tissue was ground in liquid nitrogen (-196°C) in a sterile mortar to a fine powder then transferred to 3 mL of pre-heated (65°C) CTAB DNA extraction buffer (3% (w/v) CTAB; 50 mM EDTA, pH 8.0; 250 mM Tris-HCl, pH 8.0; 3 M NaCl; 1% PEG 6000; 0.5 M glucose; 3% PVP and 0.2% mercaptoethanol). The quantification of genomic DNA was achieved using a spectrophotometer (Jenway 6405 UV Visible). The yield was determined by measuring the absorbance at A260 and A280. The level of DNA purity was determined by the A260/A280 absorbance ratio (range 1.7-1.9). DNA purity was further tested by electrophoresing the extracted genomic DNA on 0.8% agarose gel at 80 V for 120 min in 0.5X TBE (Tris borate; 100 mM EDTA, pH 8) gel buffer in submerged agarose gel electrophoresis. The gel was stained with 0.25 μg mL^{-1} ethidium bromide for the visualization of genomic DNA. The gels were visualized and photographed using Gel Doc fitted with a 12-bit CCD camera and UV light (Bio-Rad). After spectrophotometric estimation, the genomic DNA solutions were stored at -20°C until further use.

Polymerase Chain Reactions (PCR) amplifications: The samples were screened for RAPD variation using the standard 10-base primers supplied by Operon Technologies. A 25 μL reaction cocktail was prepared as follows: 5 μL 5X buffer (Green Go Taq (Promega), 200 μM dNTPs (10 mM), 2.5 mM magnesium chloride (25 mM), 20 pM primer/25 μL (5 μM), 1U *Taq* polymerase, 4.0 μL sample DNA (10 ng μL^{-1}) and adjusted with sterile double-distilled water. A total of 40 RAPD primers were tested in this study, only 7 primers (OPA02, OPA03, OPC13, OPJ10, OPJ17, OPJ14 and OPG14) produced clear and polymorphic bands. The thermocycler (iCyclerTM, Bio-Rad) was programmed as follows: 2 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C; followed by a final 7-min extension at 72°C, then cooling to 4°C. The primers were checked twice for their reproducibility. The PCR products were loaded onto an agarose (1.5 % w/v; 5 μL of ethidium bromide) gel in 0.5X TBE (Tris-Borate-EDTA) buffer and electrophoresis was at 90V for 90 min. The bands were visualized and photographed using the Gel Doc fitted with 12 bit CCD camera and UV light (Bio-RAD).

Data analysis: The positions of scorable RAPD bands were transformed into a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position) which was entered in the NTSYS-pc (The Numerical Taxonomy and multivariate Analysis System for Personnel Computer) computer program version 2.02j. These data were used for the calculation of pairwise genetic distances between genotypes using the Simple Matching (SM) coefficient in the SIMQUAL (Similarity for Qualitative Data Program) (Staub *et al*., 2000), using the formula:

$$SM = \frac{(a+d)}{n}$$

Where:
a = number of bands present in both genotypes
d = number of null alleles in both genotypes
n = total number of bands

The frequency of each band in a population was determined and the percentage of polymorphic bands (PPB) at a 95% criterion for any population was calculated. Shannon's diversity index and Nei (1973) gene diversity index were estimated with POPGENE32 version 1.31 (Yeh *et al*., 1999). Shannon's diversity index is defined as $H = \sum pi\log_2 pi$, where pi is the frequency of a given RAPD fragment. Shannon's diversity index is applicable to analysis of RAPD data as it is relatively insensitive to the bias produced by failures to detect heterozygous individuals (Dawson *et al*., 1995). Total genetic diversity (Ht), the within population genetic diversity (Hs) and the coefficient of genetic differentiation were calculated with POPGENE32 version 1.31 (Yeh *et al*., 1999).

The genetic variation within and among populations was estimated by AMOVA using the GenAlEx program, version 6.4 (Peakall and Smouse, 2006). The statistics PR (differentiation among populations), ΦRT (differentiation among regions) and ΦPT (differentiation within populations) were calculated. The significance of variance components and of the Φ statistics was estimated using permutation procedures. Furthermore, Gene flow (the number of migrants per generation, Nm) between pair-wise populations or among all the populations was calculated according to McDermott and McDonald (1993) formula:

$$Nm = 0.5\frac{(1 - Gst)}{Gst}$$

In order to estimate the relationships among individuals and populations, two methods were used in the present study: (1) dendrogram based on SM similarity coefficient between individuals was established using UPGMA of the NTSYS-pc program (Rohlf, 1994) and (2) PCA procedure was performed in GenAlEx 6.4 based on algorithm (Orloci, 1975). Relationships between the binary genetic distance matrix and the linear geographic distance matrix were estimated with the Mantel test (Mantel, 1967) in the GenAlEx 6.4 program (Peakall and Smouse, 2006) by 999 interactions. Also, relationship between cophonetic and SM matrices was performed with Mantel test (Mantel, 1967) using MXComp procedure of the of the NTSYS-pc program (Rohlf, 1994).

RESULTS

Seven of the forty primers which produced polymorphic, reproducible and clear bands were screened for RAPD-PCR amplification. The seven primers yielded a total of 41 reliable polymorphic loci (bands) (100%) from the 24 samples (3 populations) of *R. raetam* DNA amplified with a range from 350 to 3800 bp in length and a mean of 5 bands per primer. Among the seven RAPD primers, OPG14 gave the maximum number of 59 bands and the minimum number of 4 bands by OPC13.

Genetic diversity in the populations of *R. raetam*: The genetic diversity of 3 populations of *R. raetam* was analyzed using POPGENE32 version 1.31. The results in Table 2a showed that the genetic diversity has a larger differentiation among the populations when it was expressed by PPB, where the highest value was 87.80% (pop2) and the lowest value was 39.02% (pop1), each of them was lower than the total percentage of polymorphic bands (100%). The number of polymorphic loci was between 16 (pop1) and 36 (pop2) among these populations. The Shannon diversity index (I) was 0.36±0.11, ranging from 0.13±0.02 (pop1) to 0.39±0.020 (pop2) in each population. Nei's gene diversity (H) which represents the degree of genetic diversity is an indicator commonly used to assess the genetic differentiation among populations. It denotes the proportion occupied by genetic variation between populations in the total genetic variation (Sui *et al*., 2009). The results of this study showed that: pop2 has the highest Nei's genetic diversity while pop1 got the lowest value; this is similar to results revealed by Shannon diversity index (I). Pop2 presented the highest I with 0.39, while pop1 showed the lowest I (0.13). These results indicated that there was some difference in the genetic diversity of *R. raetam* among 3 populations.

Genetic differentiation and gene flow among populations of *R. raetam*: The assessment of the genetic structure for the 3 populations using POPGENE32 (Table 2b) showed that the total genetic diversity (Ht) was found to be 0.210 and the within-population genetic diversity (Hs) was 0.156, the mean expected heterozygosity (He) is 0.155, while the coefficient of gene differentiation (Gst) for all loci was 0.260 indicating that the variations among populations and those within populations contributed 26 and 74%, respectively.

The number of migrants per generation (Nm) is an estimated value from Gst to measure the gene flow, the higher its value, the less genetic differentiation among populations (Sui *et al*., 2009). According to Wright (1931) the differentiation among populations is caused by genetic drift if Nm>1. The amount of gene flow (Nm) among *R. raetam* populations was found to be 1.42 (>1) individuals per generation. AMOVA analysis (Table 3) further revealed highly significant ($p<0.05$) genetic

Table 2: (a) Genetic diversity indices within each of the three populations of *R. raetam*; (b): Genetic differentiation and gene flow of *R. raetam* populations

(a)	Na (±se)	Ne (±se)	H (±se)	I (±se)	NPB	PPB
Pop1	1.39±0.49	1.10±0.14	0.07±0.01	0.13±0.02	16	39.02
Pop2	1.88±0.33	1.39±0.29	0.25±0.10	0.39±0.02	36	87.80
Pop3	1.41±0.50	1.25±0.38	0.15±0.02	0.22±0.09	17	41.46
Total	2±0.00	1.30±0.25	0.21±0.13	0.36±0.11	41	100
(b)		Ht	Hs	He	Gst	Nm
Mean	24	0.21	0.156	0.155	0.26	1.42
SD		0.018	0.0079	0.015		

Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei (1973) gene diversity; I = Shannon's Information index; se: standard error

Table 3: Analysis of molecular variance (AMOVA) for 24 individuals of *R. raetam* using RAPD markers. Two AMOVAs including nested analysis (between regions, among populations within regions and within populations) and among population analysis (among populations and within) were used

Source of variance	df	SS	VC	% Total	Φ-statistics	p-value
Three populations						
Among populations	2	43.883	2.270	32	ΦPT= 0.316	0.001
Within populations	21	103.200	4.914	68		
Two regions						
Among regions	1	33.340	1.653	22	ΦRT= 0.219	0.004
Among populations within regions	1	10.543	0.985	13	ΦPR=0.167	0.034
Within populations	21	103.200	4.914	65	ΦPT=0.349	0.001

df: Degrees of freedom, SS: Sum of squared, VC: Variance component, p-value: Levels of significance were based on 999 random permutations

Table 4: (a) Genetic distance (ΦPT based values; below diagonal) and p-value (above diagonal); (b): Genetic identity (Above diagonal) and Nei's genetic distance (Below diagonal) among and between three populations of *R. raetam*, respectively based on RAPD data

(a)				(b)			
Population	Pop1	Pop2	Pop3	Population	Pop1	Pop2	Pop3
Pop1	***	0.001	0.003	Pop1	***	0.911	0.882
Pop2	0.334	***	0.091	Pop2	0.093	***	0.967
Pop3	0.505	0.098	***	Pop3	0.126	0.034	***

***Probability values based on 999 permutations are shown above diagonal

differences among the three populations. Of the total genetic diversity without considering regions, 32% was attributable to between-populations diversities and 68% to diversity within populations. Thus, AMOVA (ΦPT = 0.316) also supported the results of Nei's gene diversity statistics and Shannon's information measure that there was a relatively high level of genetic differentiation among populations. When considering regions' effect, 13% was attributable to among-population diversities, 22% among regions and the rest (65%) to differences within populations. The AMOVA of the proportion of diversity among two regions indicated that an important amount of variation (22%) occurred among the two regions, with 13 and 65% between populations within regions and within a population, respectively (Table 4a). In addition, genetic distance (ΦPT based values) between pair-wise populations was calculated based on the binary data using AMOVA software. The values of ΦPT genetic distance ranged from 0.098 ($p>0.05$) between pop2 and pop3 to 0.505 between pop1 and pop3 and the Nm between pair-wise populations ranged from 0.490 between pop1 and pop3 to 4.609 between pop2 and pop3 (Table 4a). These results indicate the important gene flow between pop2 and 3 resulting in less genetic differentiation (0.098). However, pop1 originating from the Island Djerba showed significant genetic distances (0.334 and 0.505; $p<0.01$) with pop2 and pop3, respectively.

Genetic relationships among populations and construction of dendrogram: Nei's unbiased genetic distances varied from 0.034 to 0.126 between the three *R. raetam* populations estimated using POPGENE 32. The nearest distance (0.034) was between pop2 and pop3 (Table 4b). An UPGMA dendrogram based on Nei's genetic distance matrix showed that the three populations were clustered into two groups, i.e., pop2 and pop3 in one group and pop1 in the other group. The UPGMA dendrogram based on SM's similarity coefficients between all individuals studied was shown in Fig. 1. The dendrogram indicated four clusters. The first one gathered almost the individuals (A1-A10) from pop1 (Djerba). However, some individuals from pop 2 (A11, A12 and A15) existed also in the cluster one. The second, the third and the last clusters grouped individuals from pop2 and pop 3. This could be explained by the important gene flow (4.609) between these two populations. These supported the result of AMOVA.

The Mantel test revealed a significant cophenetic correlation ($r = 0.85$; $p<0.01$); thus, the dendrogram provides a good fit to the SM's similarity matrix. Principal coordinates analysis (PCoA) binary genetic distance computed from RAPD data collected from 24 individuals was studied (Fig. 2). The proportion of total variance comprising each axis was 53.42% for axis 1 and 35.22% for axis 2. PCoA showed three groups. The first one gathered two sub-groups represented essentially by individuals from pop1 and some others from pop2 and only one individual (A23) from pop3. The second group is composed by the individual A16 from pop2. However, the third group is formed by individuals from pop2 and pop3. This result support with that showed by UPGMA dendrogram.

DISCUSSION

Morphological and molecular markers are important to genetic analysis of related plant species and populations (Helm *et al*., 2009). DNA markers are the most powerful techniques because they are not influenced by environmental factors and developmental stages of plant (Karimi *et al*., 2009). RAPD marker is one of the powerful techniques to analyze genetic diversity in different species. This method needs no plants sequence information prior to application. The significant differentiation in *R. raetam* between populations from island Djerba and those sampled from Essaâden and Edhahar can be explained by the geographic isolation between these populations that can limit gene flow and result in significant differentiation between them. According to Pluess and Stocklin (2004), clonality and spatial isolation of populations may decrease genetic diversity within and increase genetic differentiation between populations. The closest geographic distance between the sampled *R. raetam* populations in this study was over 55 km. Pop1 was sampled from the Island "Djerba" causing rarity of both seed and pollen dispersals of this species. Therefore, it is reasonable that a proportion of genetic diversity resides among populations of *R. raetam*. Also, significant differentiation is expected

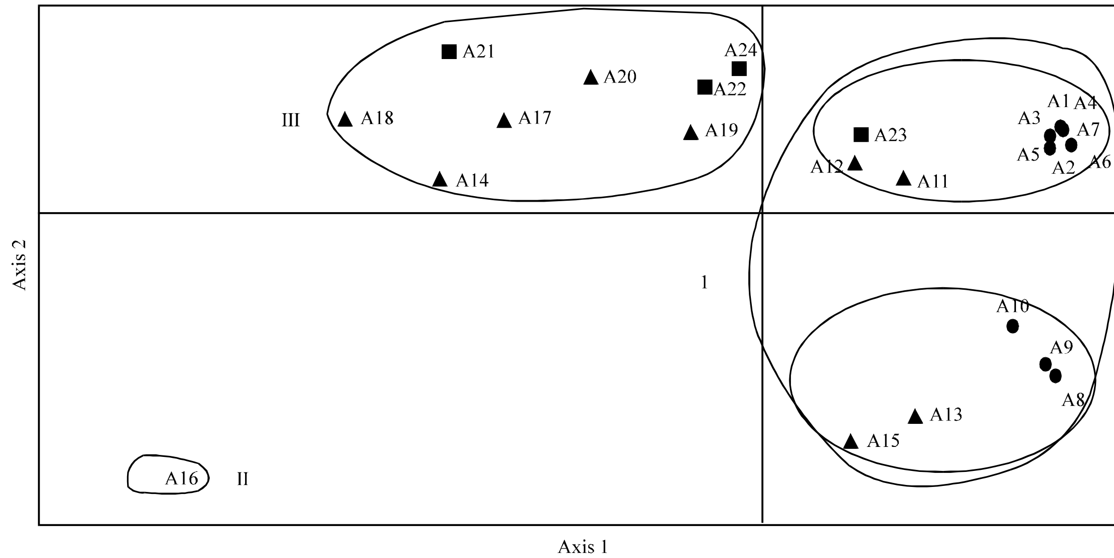

Fig. 2: Principal Coordinates Analysis (PcoA) based on the RAPD data illustrating the genetic relationships based on binary genetic distance among the 24 individuals and 3 populations of *R. raetam* in different environments in southern Tunisia. A1-A10 were samples from Island Djerba, A11-A20 from Essaâden and A21-A24 from Edhaher

from the heterogeneous environment existing between Djerba microclimate and the other two stations. The heterogeneity of environmental factors, such as the rainfall periodicity and quantity and the microclimate which may influence flowering time, probably block the genetic exchange between populations. This suggests that annual mean precipitation and soil characteristics could be important factors affecting genetic diversity of *R. raetam* populations. *R. raetam* showed relatively low levels of genetic diversity (H = 0.07) within populations sampled from the Island. This result corroborate with that showed by Pluess and Stocklin (2004) indicating that spatial isolation of populations lead to low genetic diversity within the population. This low genetic diversity in *R. reatam* could be explained by the heterogeneous and fragmented environment by natural and human disturbance that may block gene flow between different populations. As was shown by Lewis and Crawford (1995), Wang *et al*. (2006) and Zhang *et al*. (2010) low gene flow among small, isolated populations is proposed to result in inbreeding, loss of heterozygosity because of genetic drift and genetic differentiation among populations. Ouji *et al*. (2011) showed a low gene flow (Nm = 0.814), in a population of *Vicia faba* which was not sufficient to counterbalance genetic drift. The high level of gene flow (Nm = 4.609) between population Essaâden and population Edhaher shows that geographical barriers could not significantly have affected the gene flow. However, the geographical distance might have some effects on genetic relationships, but it was not the key factor. Djerba and Essaâden are short in distance (68 km), but the first is far away from Edhaher (117 km).

CONCLUSION

In this investigation the use of RAPD markers to characterize and determine the genetic structure of the populations has proved to be a useful tool in the study of southern *Retama raetam*. We provide information on genetic relatedness within species for conservation. An understanding of the level and partitioning of genetic variation within the species would provide an important input into determining appropriate management strategies. However, further studies should be done taking into account different localities and populations and also a big number of individuals.

REFERENCES

Abdellaoui, R., H. Gouja, A. Sayah and M. Neffati, 2011. An efficient DNA extraction method for desert *Calligonum* species. Biochem. Genet., 49: 695-703.

Al-Tubuly, R.A., A. Auzi, A.A. Al-Etri-Endi, N. Lutfun and S.D. Sarker, 2011. Effects of *Retama reatam* (Forssk.) Webb and Berthel. (Fabaceae) on the central nervous system in experimental animals. Arch. Biol. Sci. Belgrade, 63: 1015-1021.

Ayala, F.J. and J.A. Keger, 1984. Modern Genetics. 2nd Edn., Benjamin/Cummings Pub. Co., Menlo Park, USA., Pages: 923.

Bruits, M. and F. Bucar, 2000. Antioxidant activity of *Nigella sativa* essential oil. Phytother. Res., 14: 323-328.

CRC Weed Management Guide, 2003. Weed management guide white weeping broom-*Retama raetam*. http://www.environment.gov.au/biodiversity/invasive/weeds/publications/guidelines/alert/pubs/r-raetam.pdf.

Dawson, I.K., A.J. Simons, R. Waugh and W. Powell, 1995. Diversity and genetic differentiation among subpopulations of *Gliricidia sepium* revealed by PCR-based assays. Heredity, 74: 10-18.

Dyer, A.R. and K.J. Rice, 1997. Evidence of spatial genetic structure in a California bunchgrass population. Oecologia, 112: 333-339.

Gustafson, D.J., J. Gibson and D.L. Nickrent, 1999. Random amplified polymorphic DNA variation among remnant big bluestem (*Andropogon gerardii* Vitman) populations from Arkansas Grand Prairie. Mol. Ecol., 8: 1693-1701.

Helm, A., T. Oja, L. Saar, K. Takkis, T. Talve and M. Partel, 2009. Human influence lowers plant genetic diversity in communities with extinction debt. J. Ecol., 97: 1329-1336.

Karimi, H.R., S. Kafkas, Z. Zamani, A. Ebadi and R. Fatahi, 2009. Genetic relationships among *Pistacia* species using AFLP markers. Plant Syst. Evol., 279: 21-28.

Kassem, M., S.A. Mosharrafa, N.AM. Saleh and S.M. Abdel-Wahab, 2000. Two new flavonoids from *Retama raetam*. Fitoterapia, 71: 649-654.

Kate, K.T. and S.A. Laird, 1999. The Commercial Use of Biodiversity: Access to Genetic Resources and Benefit Sharing. Earthscan Publications, London, ISBN: 9781853839412, Pages: 398.

Larsen, H.O. and C.S. Olsen, 2007. Unsustainable collection and unfair trade? uncovering and assessing assumptions regarding central Himalayan medicinal plant conservation. Biodivers. Conserv., 16: 1679-1697.

Lewis, P.O. and D.J. Crawford, 1995. *Pleistocene refugium* endemics exhibit greater allozyme diversity than wide spread congeners in the genus *Polygonella* (Polygonaceae). Am. J. Bot., 82: 141-149.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res., 27: 209-220.

McDermott, J.M. and B.A. McDonald, 1993. Gene flow in plant pathosystems. Annu. Rev. Phytopathol., 31: 353-373.

Messaoud, C., M. Afif, A. Boulila, M.N. Rejeb and M. Boussaid, 2007. Genetic variation of *Tunisian Myrtus communis* L. (Myrtaceae) populations assessed by isozymes and RAPDs. Ann. Forest Sci., 64: 845-853.

Mittler, R., E. Merquiol, E. Hallak-Herr, S. Rachmilevitch, A. Kaplan and M. Cohen, 2001. Living under a dormant canopy: A molecular acclimation mechanism of thedesert plant *Retama raetam*. Plant J., 25: 407-416.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. USA., 70: 3321-3323.

Orloci, L., 1975. Multivariate Analysis in Vegetation Research. Junk, Hague.

Ouji, A., M. Jose Suso, M. Rouaissi, R. Abdellaoui and M. El Gazzah, 2011. Genetic diversity of nine faba bean (*Vicia faba* L.) populations revealed by isozyme markers. Genes Genomics, 33: 31-38.

Peakall, R. and P.E. Smouse, 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teachingand research. Mol. Ecol. Notes, 6: 288-295.

Pluess, A.R. and J. Stocklin, 2004. Population genetic diversity of the clonal plant Geum reptans (Rosaceae) in the Swiss Alps. Am. J. Bot., 91: 2013-2021.

Qian, W. and S. Ge, 2001. Analyses of population genetic structure by using dominant markers. Acta Genet. Sin., 28: 244-255.

Qiu, Y.X., X.W. Zhou, C.X. Fu and Y.S.G. Chan, 2005. A preliminary study of genetic variation in the endangered, Chinese endemic species *Dysosma versipellis* (Berberidaceae). Bot. Bull. Acad. Sin., 46: 61-69.

Rohlf, F.C., 1994. NTSYS-PC Numerical Taxonomy and Multivariate Analysis System. Setauket, Exeter Publishing, New York.

Schaal, B.A., W.J. Leverich and S.H. Rogstad, 1991. Comparison of Methods for Assessing Genetic Variation in Plant Conservation Biology. In: Genetics and Conservation of Rare Plants, Falk, D.A. and K.E. Holsinger (Eds.). Oxford University Press, New York, ISBN: 0-19-506429-1, pp: 123-134.

Schippmann, U., D.J. Leaman and A.B. Cunningham, 2002. Impact of cultivation and gathering of medicinal plants on biodiversity: Global trends and issues. FAO. Biodiversity and the Ecosystem Approach in Agriculture, Forestry and Fisheries. October 12-13, Rome.

Solouki, M., H. Mehdikhani, H. Zeinali and A.A. Emamjomeh, 2008. Study of genetic diversity in chamomile (*Matricaria chamomilla*) based on morphological traits and molecular markers. Sci. Hortic., 117: 281-287.

Spooner, A., J. Carpenter, G. Smith and K. Spence, 2008. *Retama raetam* (Forssk.) wbb. Flora Base the Australian Western Flora.

Staub, J.E., Y. Danin-Poleg, G. Fazio, T. Horejsi, N. Reis and N. Katzir, 2000. Comparative analysis of cultivated melon groups *Cucumis melo* L. using random amplified polymorphic DNA and simple sequence repeat markers. Euphytica, 115: 225-241.

Sui, X.Q., K. Wang, S.H. Zheng, L. Li and S.Z. An, 2009. Effects of landscape fragmentation on genetic diversity of *Stipa krylovii* Roshev (*Stipa* L.) in agro-pastoral ecotone in Northern China. Afr. J. Biotechnol., 8: 3431-3439.

Wang, J.L., Y.B. Gao, N.X. Zhao, A.Z. Ren and W.B. Ruan *et al*., 2006. Morphological and RAPD analysis of the dominant species *Stipa krylovii* Roshev. in Inner Mongolia steppe. Bot Stud., 47: 23-35.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18: 6531-6535.

Wright, S., 1931. Evolution in Mendelian populations. Genetics, 16: 97-159.

Yeh, F.C., R. Yang and T. Boyle, 1999. POPGENE, Microsoft Window-based Freeware for Population Genetic Analysis. Version 1.31, University of Alberta, Edmonton, Canada.

Zhang, D.Q., L.M. Gao and Y.P. Yang, 2010. Genetic diversity and structure of a traditional Chinese medicinal plant species, *Fritillaria cirrhosa* (Liliaceae) in southwest China and implications for its conservation. Biochem. Biophys. Res. Commun., 38: 236-242.

Zhao, N.X., Y.B. Gao, J.L. Wang and A.Z. Ren, 2008. Population structure and genetic diversity of *Stipa grandis* P. Smirn, a dominant species in the typical steppe of northern China. Biochem. Syst. Ecol., 36: 1-10.

Zheng, W., L. Wang, L. Meng and J. Liu, 2008. Genetic variation in the endangered *Anisodus tanguticus* (Solanaceae), an alpine perennial endemic to the Qinghai-Tibetan Plateau. Genetica, 132: 123-129.