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Genetic diversity of geographically isolated Iranian populations of *Betula pendula* Roth: implications for conservation

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Highlights

- The Iranian populations of birches exhibited high levels of genetic diversity, population differentiation, and the presence of unique haplotypes.
- The high genetic differentiation amongst the populations may contribute to the local geographical structure and poor gene flow amongst individuals.
- The results can potentially be used to adopt appropriate strategies for the conservation and management of isolated tree populations.

Abstract

The effects of long-term habitat fragmentations on genetic and population differentiation of *Betula pendula* Roth were investigated using chloroplast DNA (cpDNA) variations. Leaf samples were collected from four small remnant populations across the north of Iran. Three pairs of universal primers were used to amplify cpDNA, large single copy regions of *trn*C-*trn*D, trnK1-*trn*K2 and *trn*D-*trn*T. A total of 18 of the cpDNA haplotypes in the four populations were identified, however, no clear phylogeographic structuring of haplotypes could be detected. The total genetic diversity (H_T) for all populations was high (0.932). Average intra-population genetic diversity was estimated as $H_S = 0.729$ and average differentiation of populations $G_{ST} = 0.218$. Mantel tests of isolation by distance revealed a significant relationship between Wright's inbreeding coefficient (Fst) and geographical distances for the four populations in Iran ($r = 0.77$, $p < 0.05$). The results of the hierarchical analysis of molecular variance (AMOVA) indicated that a 66% variation was partitioned within populations, whilst the variance amongst the four populations was only 34%. We suggest that significant genetic differentiation amongst populations can likely be attributed to reduced gene flow as a result of habitat fragmentation.

Keywords habitat isolation; genetic differentiation; haplotype diversity; genetic drift; long-term isolation
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1 Introduction

Recurrent climatic oscillations associated with glacial cycles throughout the late Tertiary and the Quaternary period have been associated with extreme changes in the patterns of species distributions and population structures in the northern hemisphere (Liu et al. 2002; Petit et al. 2003; Hewitt 2004). Aitken et al. (2008) expressed that there are three possible fates for forest tree populations in a rapidly changing environment: persistence through migration to track ecological niches spatially; persistence through adaptation to new conditions in current locations; and extirpation. Other factors, such as limited connectivity due to habitat fragmentation, may also influence the genetic structure of populations by restricting gene flow and pollinator activity (Hewitt 2000).

During recent years, chloroplast DNA (cpDNA) has traditionally been the marker of choice due to its ease of amplification and uniparental inheritance in determining the phylogeography or migratory footprints of species (Wang et al. 2012; Fan et al. 2013; Meng and Zhang 2011; Yu and Nason 2011). The chloroplast DNA is generally maternally inherited in most angiosperms (Rajora and Dancik 1992; Dumolin et al. 1995) including *Betula pendula* Roth.

This uniparental mode of inheritance also means that cpDNA haplotypes (as haploid markers) are only dispersed by seeds. On the other hand, in comparison to the nuclear genome, cpDNA has a smaller effective population size (1/4 in dioecious species and 1/2 in monoecious species, such as silver birch), therefore revealing a clearer picture of the genealogical history of a species (Vettori et al. 2004).

In plant populations seed flow is typically considered to be more limited than pollen flow, therefore, cpDNA based markers are extremely useful for studying the route of seed migration and to identify the locations of major refugia during the Last Glacial Maximum (LGM). Mutation rates are also typically low in chloroplast-DNA, and it is not influenced by recombination (Palme et al. 2003; Heuertz et al. 2004). As a result these features make cpDNA a useful marker for molecular phylogeographic studies (Petit et al. 1993; Newton et al. 1999). Many reports have successfully applied cpDNA markers to identify the locations of glacial refugia and postglacial colonization routes in various plant species including *Alnus glutinosa* (L.) Gaertn. (King and Ferris 1998), *Betula pendula* (Palme et al. 2003; Maliouchenko et al. 2007), *Quercus alba* L. (Csaikl et al. 2002) and *Populus nigra* L. (Cottrell et al. 2005; Fussi et al. 2010) .

The birch family, *Betulaceae*, contains trees and shrubs that are an ecologically and economically important component of temperate and boreal forests of the northern Hemisphere (Schenk et al. 2008). The silver birch, *Betula pendula*, is widely distributed in Europe and Asia Minor, including eastern Turkey, northern Iraq and northern Iran (Krussmann 1984). *Betula pendula* is a diploid (2n = 28), monoecious, wind-pollinated tree with small seeds that rapidly recolonizes open areas disturbed by forest fire or logging (Fischer et al. 2002; Jonsell 2000). Birches are an important source of timber production and are also important in the pharmacological industry (Martin et al. 2008). In Iran, the natural distribution of *B. pendula* is limited to the northwestern part of the country in Marmisho valley, Shahrestanak, valley of Lar and Shahrood and the easternmost part of the southern slopes of the Elburz Mountains. Currently the scattered and low-density distribution of *B. pendula* in Iran has led to its listing as an endangered species in the Red List of plants of Iran (Jalil and Jamzad 1999).

Knowledge of the genetic diversity and population structure of threatened or endangered plant species is critical to their conservation and management (Holsinger and Gottlieb 1991). Currently there are no existing studies of the genetic diversity of *B. pendula* in Iran. The main objective of this study was to investigate the effect of long-term geographical isolation on the level of genetic variation and to quantify the extent of genetic population differentiation and phylogeographical structure of Iranian populations of *B. pendula.*

Table 1. The geographical characteristics of four isolated populations of *Betula pendula* in Iran and the sample size (N) in each population.

Populations	N	Population area (Hectare)	Latitude (°N)	Longitude (°E)	Altitude (Meter Above sea level)
Syahmarzkouh	6	5	36° 38′	55° 10′	2344
Sangedeh	5	100	36° 58′	53° 10′	2579
Shahrestanak	6	5	35° 44′	51° 23′	2404
Marmisho	5	1	37° 34′	44° 35′	1741

2 Material and methods

2.1 Sampling, DNA extraction and PCR-RFLP analysis

All leaf materials were collected from four out of the five *B. pendula* populations in the northern part of Iran. The names of populations, geographical positions, size of populations and the number of individuals sampled per population are given in Table 1. In order to avoid investigating clones or close relatives, sampled individuals within a population were separated by at least 100 m.

The leaves were frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. Total genomic DNA was isolated from the ground powder using a protocol adapted from Porebski et al. (1997). Three pairs of universal primers that were reported by Demesure et al. 1995 and Grivet et al. 2001, were used for the amplification of *trn*C-*trn*D (CD), *trn*K1-*trn*K2 (K1K2*),* and *trn*D-*trn*T (DT) the chloroplast regions. The names and sequences of these six primers included: *trn*C-forward (5'-CCAGTTCAAATCTGGGTGTC) and *trn*D- reverse (5'-GGGATTGTAGTTCAATTGGT); *trn*K1-forward (5'-GGGTTGCCCGGGACTCGAAC) and *trn*K2- reverse (5'- CAACGGTAGAGTACTCGGCTTTTA); and *trn*D-forward (5'-ACCAATT-GAACTACAATCCC) and *trn*T- reverse (5'-CTACCACTGAGTTAAAAGGG). They were used to amplify CD, K1K2, and DT chloroplast regions respectively. PCR reactions were carried out in a total volume of 25 µl consisting: 2.5 µl of 10 X PCR buffer; 0.2 mM dNTPs; 2 mM $MgCl_2$; 0.1 mg. ml^{-1} Bovine serum albumin (BSA) per double-distilled water filtrated by 0.2 Millipore; 0.2 mM of each of the forward and reverse primers; 1 unit of *Taq* DNA polymerase and 1.5 µl of genomic DNA. A touch-down PCR program was used for the amplification: 4 min at 94 °C, 14–20 cycles of 45 s at 94 °C, 45 s at 60–48 °C decreasing 0.5 °C with each cycle of 53–40 °C, 3–6 min at 68–72 °C, then 15–20 cycles of 45 s at 94 °C, 45 s at 53–40 °C, 3–6 min at 68–72 °C and finally 10 min at 68–72 °C. Annealing temperatures and cycling time were dependent on the fragment of interest and were obtained from Palme et al. (2003).

For the PCR-RFLP analysis, each of the PCR products were digested with three restriction enzymes: *Taq*I, *Hin*fI and *Eco*RI, except the CD amplification products which were digested using only *Hinf*I. The restriction reactions were carried out in a total volume of 10 µl containing: 5.5 µl PCR product (~100 ng); 2.5µl H_2O; 1 µl 10X buffer and 1 µl (~3 units) of the restriction enzymes. After mixing these components, the tubes were incubated overnight at temperatures optimal for each restriction enzyme (37 °C for *Hinf*I and *Eco*RI and 65° C for *Taq* I).

Restriction fragments were run on an 8% polyacrylamide gel electrophoresis (PAGE) using general Tris-Borate-EDTA buffer at 225 V for 150 min, visualized by silver ($AgNo_3$) staining (Green and Sambrook 2012) and then scanned. Bands were scored as presence (1) vs. absence (0) of the band (Table 2). In order to establish the size of the individual fragments, digested fragments were run alongside a 50 or 100-bp DNA size standard.

Agarose and polyacrylamide gel electrophoresis and gel stain performed by Green and Sambrook (2012) methods. All of the PCR and restriction enzyme reactants were prepared from Fermentas Co. (Leon-Rot, Germany).

2.2 Data analysis

Haplotype diversity and population differentiation was investigated using PCR-RFLP haplotype frequencies obtained from combined analysis of the three chloroplast regions. The mean intra-population gene diversity (H_S), the total genetic diversity (H_T) and the genetic differentiation amongst populations (G_{ST} or Wright's F_{ST}) were calculated. The analysis of molecular variance (AMOVA) was carried out to evaluate variance components and their significance in the levels of variation amongst populations and the individuals within those populations of *B. pendula* using the GenAlEx v6.5 software (Peakall and Smouse 2006). In order to identify the presence of a phylogeographic structure, comparisons of G_{ST} and N_{ST} were tested according to Pons and Petit (1996) using PERMUT software. To assess isolation-by-distance (IBD), the Mantel test was used to examine the correlation between pairwise Fst and the corresponding matrix of geographical distances. Estimating the significance of correlation coefficients between geographical and genetic distances amongst the populations was performed using Mantel's test (Mantel 1967) with PopTools software Ver. 2.5.5. We examined the genetic structure amongst populations using the Bayesian approach and the Markov chain Monte Carlo (MCMC) algorithm implemented in STRUCTURE version 2.2 (Pritchard et al. 2000). The optimum number of subpopulations (K) was confirmed after 10 independent runs for each value of K between 1 and 10. The length of the burn-in period and number of MCMC reps after burn-in were set to 5000 and 50 000, respectively.

3 Results

The average lengths of the three cpDNA regions were approximately 1800 bp for *DT*; 3800 bp for *CD* and 2700 bp for *K1K2*. All seven analyzed primer-enzyme combinations (*CD-Hinf*I, *DT-Hinf*I, *DT-Taq*I, *DT-Eco*RI, *K1K2-Hinf*I, *K1K2-Taq*I and *K1K2-Eco*RI) revealed genetic variations. Amongst these variable regions, The *CD-Hinf*I fragments were the most varied, displaying seven polymorphic fragments (Table 2). In total, 18 of the cpDNA haplotypes were identified in the four populations (Table 3). The main characteristics of the haplotype distribution are a high number of unique haplotypes and the absence of a clear geographic structuring of the haplotypes, with each of the four populations possessing one or more private haplotypes. Haplotype C had the highest frequency (60%) and was found in Marmishoo. Haplotypes K and N were the next most frequent (33%) and appeared in Shahrestanak and Siahmarzkoh, respectively (Fig. 1). The remaining 15 haplotypes were the least frequent haplotypes with frequencies ranging from 16% to 20%.

The parameters obtained from the analysis of genetic diversity and population differentiation of the four populations are shown in Table 4. The total genetic diversity in all populations (H_T) was high (0.932, Table 4). Average intra-population genetic diversity was estimated as $H_S = 0.729$, and average differentiation of populations (G_{ST}) was 0.218 (Table 4). The highest H_T was observed in Sangedeh (0.946). The IBD test revealed a significant relationship between Fst and geographical distances for the four populations ($r = 0.77$, $p < 0.05$). The results of the hierarchical AMOVA of cpDNA indicated that 66% variation was partitioned within populations, whilst the variance amongst the four populations was only 34% (all partitions were significant at $p < 0.01$) (Table 5). The population genetic structure amongst the four populations is shown in Fig. 2 and Table 6. Two major groups of *B. pendula* were supported by the STRUCTURE analysis. The Sharestanak and Sangedeh populations (1 and 2) formed one group, whereas the Siahmarzkoh and Marmisho (3 and 4), formed the other group.

Table 2. The PCR-RFLP chloroplast DNA haplotypes in four isolated populations of *Betula pendula* in Iran.

Haplotypes	CD-*Hinf*I (NPB=7)	K1K2-*Hinf*I (NPB=3)	K1K2-*Taq*I (NPB=4)	K1K2-*EcoR*I (NPB=3)	DT-*Hinf*I (NPB=2)	DT-*Taq*I (NPB=2)	DT-*EcoR*I (NPB=3)
A	0000000	001	1001	111	11	11	011
B	0010000	001	1001	100	00	11	011
C	0011111	101	1001	111	01	01	011
D	0111111	001	1001	101	01	01	011
E	0111111	001	1111	101	01	11	011
F	0111111	001	1111	111	00	11	011
G	0111111	101	1001	111	11	11	011
H	1010000	001	1001	111	00	11	011
I	1010000	001	1001	111	00	11	111
J	1010000	011	1011	111	00	11	011
K	1110000	001	1001	111	00	11	011
L	1110000	101	1001	111	00	11	011
M	1111111	001	1001	100	11	01	111
N	1111111	001	1001	101	01	11	011
O	1111111	001	1001	111	01	11	011
P	1111111	001	1111	101	01	11	011
Q	1111111	011	1101	100	00	01	011
R	1111111	101	1001	101	11	01	011

NPB = Number of the polymorphic bands were shown by digestion of the PCR fragments; Only fragment polymorphism associated with each haplotype is represented.

Table 3. The location and distribution of PCR-RFLP chloroplast haplotypes over populations. The Haplotype frequency (Hf) and the total number of haplotypes (tN) found in each of the populations are also given.

Populations	Haplotypes																	tN	Hf
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R		
Siahmarzkoh	0	0	0	1	1	0	0	0	0	0	0	0	1	2	0	1	0	0	6	0.942
Sangdeh	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	5	0.946
Shahrestanak	0	1	0	0	0	1	0	0	0	0	2	1	0	0	0	0	0	1	6	0.942
Marmishoo	0	0	0	0	0	0	0	3	1	1	0	0	0	0	0	0	0	0	5	0.901
(H_f)%	20	16	20	16	16	16	20	60	20	20	33	16	16	33	20	16	20	16	-	-

Table 4. Estimates of average gene diversity within four isolated populations of *Betula pendula* in Iran (H_S), total gene diversity (H_T), inter-population differentiation (G_{ST}), and the number of substitution types (N_{ST}), with permut, using a permutation test with 1000 permutations.

Populations	H_S	H_T	G_{ST}	N_{ST}
Syahmarzkouh	0.776	0.942	0.197	-
Sangedeh	0.800	0.946	0.188	-
Shahrestanak	0.776	0.942	0.197	-
Marmisho	0.560	0.901	0.305	-
Total	0.729	0.932	0.218	0.281

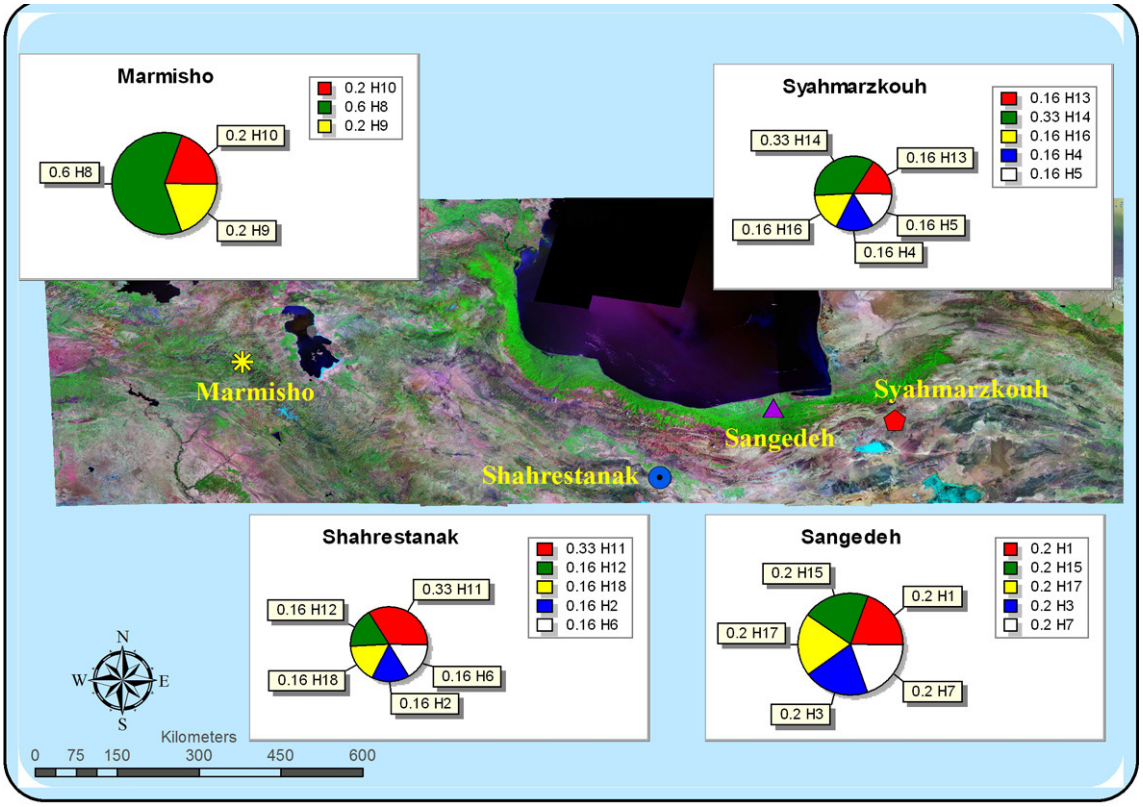

Fig. 1. The geographical distribution of cpDNA haplotypes of four isolated populations of *Betula pendula* in Iran.

Table 5. Analyses of molecular variance (AMOVA) for four isolated populations of *Betula pendula* in Iran by PCR-RFLP: Statistics include sums of squared deviations (SSD); mean squared deviations (MSD), variance component estimates, the percentage of the total variance contributed by each component and the probability of obtaining a more extreme component estimate by chance alone.

Source	df	SSD	MSD	Est. Var.	Pvar	*p*-value
Among population	3	31.997	10.666	1.429	34%	0.010
Within population	18	50.867	2.826	2.826	66%	0.010
Total	21	82.864		4.255	100%	

df = degree of freedom; Est. Var. = Estimates variance; Pvar = Percentage of variance.

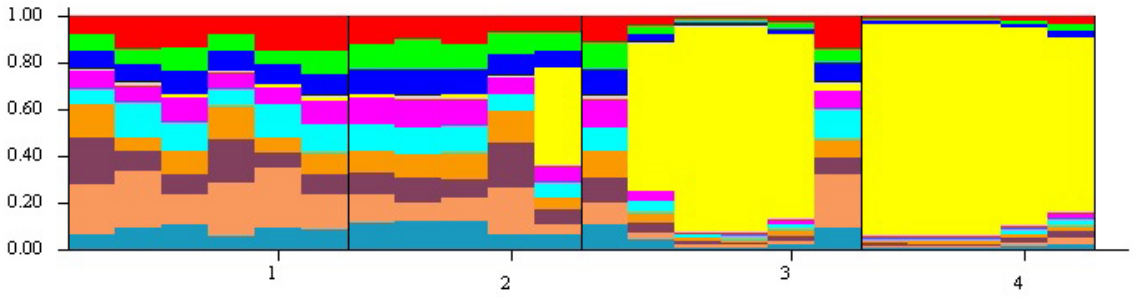

Fig. 2. The population structure of four isolated populations of *Betula pendula* in Iran estimated by STRUCTURE. In the figure, the individuals were categorized by population (1- Syahmarzkouh; 2- Sangedeh; 3- Shahrestanak; and 4- Marmisho).

Table 6. The proportion of membership of each pre-defined population (Four isolated populations of *Betula pendula* in Iran) in each of the four clusters.

Cluster	1	2	3	4
1	0.013	0.269	0.384	0.334
2	0.113	0.294	0.296	0.296
3	0.597	0.104	0.161	0.138
4	0.940	0.019	0.020	0.021
AD	0.0242	0.1741	0.1510	0.1618

AD = Average distances between individuals in same cluster.

4 Discussion

Understanding the genetic structure and diversity of endangered plant species is the first step to developing an appropriate management policy (Holsinger and Gottlieb 1991).

It is expected that species with limited geographical ranges should possess relatively lower genetic diversity than species with wide distributions (Luan et al. 2006; Wang et al. 2012; Zhao et al. 2012). As expected from birch and woody species, the Iranian birch populations are genetically diverse (H_T=0.932) because the species in general has a wide distribution. Other hand, we did identify significant differences amongst the four birch populations from Iran. Thus, even the small populations in Iran carry some of the general diversity of the species. This result is in accordance with the studies of other *Betula* species (Maliouchenko et al. 2007; Palme et al. 2003). The level of genetic diversity was reported high in the *B. pendula subsp. fontqueri* populations from the Iberian Peninsula (Martin et al. 2008). Zeng et al. (2003), in their study of *Betula alnoides* populations, found a high genetic diversity and reported that 64.1% of the RAPD markers were polymorphic. Life history characteristics, including seed and pollen dispersal mechanisms, and geographical distribution are important in influencing the pattern of genetic diversity (Hamrick et al. 1992).

Despite the destruction of birch habitats in Iran, we found that the genetic diversity of *B. pendula* populations was surprisingly high. Similar results have been reported for *Betula humilis* (Jadwiszczak et al. 2012), *Prunus spinosa* and *Nouelia insignis* (Luan et al. 2006; Zhao et al. 2012). It has been suggested that such high levels of genetic diversity may reflect only recent (post-establishment) population size reductions, especially in the last two generations. In such cases, the genetic diversity of the remaining trees is attributed to the large size and allelic richness of the original population (Luan et al. 2006; Qiao et al. 2010; Zhao et al. 2012). Also different environmental conditions can affect the amount of genetic differentiation. Xie et al. (2009) expressed the close relationship that exists between genetic diversity and geographical conditions. They stated that bottleneck is the main cause of the genetic diversity of *Betula luminifera* H.J.P.Winkl in China. Esmaeilpour et al. (2014) have demonstrated that the two studied populations (Sharestanak and Marmishoo) are currently undergoing a population bottleneck.

Gene flow is a vital element in local adaptation and bears a particularly strong influence against speciation in evolutionary processes (Slatkin 1985; Kronforst 2008; Shen et al. 2014). The high genetic differentiation amongst Iranian *B. pendula* populations (G_{ST}=0.206) may contribute to the local geographical structure and poor gene flow amongst individuals. A geographical barrier increases the probability of extinction or local adaptation of Iranian birch populations and it may cause these populations to evolve into a different types of populations with a unique genetic structure (Shen et al. 2014).

On the other hand, the amount of gene flow, being less than one, indicates the occurrence of genetic drift in Iranian birch populations. In fact, the long-term isolation has led to genetic differentiation due to drift. Lee et al. (2013) expressed that high levels of variation are expected in areas where migration waves from different refugia meet. In these areas, however, the levels of unique alleles should be low.

Iranian populations exhibited high levels of genetic diversity, population differentiation, and the presence of unique haplotypes. According to the basic expansion and contraction model (Hewitt 1996), populations of *B. pendula* in Iran can be considered as refugial because they possess the main characteristics of refugia, including high levels of genetic diversity, genetic differentiation, and a high number of unique haplotypes. Elevation gradients in Iran should have facilitated the persistence of *B. pendula* in Iran during the Pleistocene seeing as only short altitudinal shifts across the steep topography would have been required to track suitable niches during the LGM (Feliner 2011). We believe that the Iranian refugia could have possessed sufficient moisture to remain as a temperate forest during drier climatic phases of the Pleistocene. These regions are located within mountainous areas, elevations of around 2000 meters, and contain old forests recognized as important floristic areas with high species diversity and endemism for many plant taxa (Zare et al. 2010). For example, Sangedeh population is particularly one of the alpine habitats in Hyrcanian forest where 33 species out of 181 are endemic (18%), which alone makes up 9% of the endemic flora of Iran (Zare et al. 2010).

5 Conclusion

Due to more susceptible and greater risk of extinction of small isolated populations, conservation biologists advocate the comprehensive researches on isolated populations of a species. Despite the destruction of birch habitats in Iran, our results showed the *unexpectedly high genetic diversity* within small population of Iranian birch. As one of the main characteristics of glacial refugia is a high genetic diversity within populations and high genetic differentiation between populations (Hampe and Petit 2005; Hewitt 1996), it seems plausible that the Iranian populations of birch are remnants of formerly large populations that have recolonized northwards to higher latitudes during the post-glacial era. Based on our findings, we suggest that habitat fragmentation has led to significant genetic differentiation that may be attributed to low levels of gene flow due to geographic isolation. On the other hand, high genetic diversity and high population genetic differentiation within Iranian's *Betula* populations is somewhat surprising. We proposed that conservation efforts should focus on preserving the genetic material from a large number of individuals from all remaining populations in order to facilitate future restoration efforts.

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