Ann. Bot. Fennici 50: 327–336 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 5 September 2013

Population structure and genetic diversity of *Prunus scoparia* in Iran

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Received 2 Aug. 2012, final version received 14 Aug. 2013, accepted 27 Mar. 2013

Mehdigholi, K., Sheidai, M., Niknam, V., Attar, F. & Noormohammadi, Z. 2013: Population structure and genetic diversity of *Prunus scoparia* in Iran. — *Ann. Bot. Fennici* 50: 327–336.

Over 30 *Prunus* species and taxa below the rank of species are known from Iran. These wild taxa provide an enlarged gene pool and may be considered a valuable germplasm source for breeding cultivated almonds. The present study is a genetic diversity analysis of six *P. scoparia* populations using six nuclear SSR markers. We also studied correlations between the population genetic differences, morphological differences and geographical distance. All six SSR primers produced amplification. The highest number of alleles occurred in the Fars and Lorestan populations, with 121 and 114 alleles, respectively. Some of the alleles were shared by all populations, while some others were specific to one population only. The observed heterozygosity ranged from 0.675 in the Tehran population to 0.900 in the Fars and Lorestan populations, while the expected heterozygosity ranged from 0.783 in the Tehran population to 0.948 in the Fars population. Bayesian model-based clustering showed a good separation of populations at $K = 6$. AMOVA indicated significant differences both among individuals and among populations. Mantel's test of SSR and morphological trees or geographical distance did not show any distinct pattern. Neighbour-joining and reticulation trees, as well as the STRUCTURE plots revealed admixture among the populations, indicating genetic exchange and presence of ancestral gene loci among them.

Introduction

According to different authors (Lee & Wen 2001, Bortiri *et al*. 2002, Potter *et al*. 2007), the genus *Prunus* (Rosaceae) contains five or six subgenera. In the six subgenera system, the subgenera are: (1) *Amygdalus*, almonds and peaches, with the type *P. dulcis* (almond); (2) *Prunus*, plums and apricots, with the type *P. domestica* (plum); (3) *Cerasus*, cherries, with the type *P. cerasus* (sour cherry); (4) *Lithocerasus*, with the type *P. pumila* (sand cherry); (5) *Padus*, bird cherries, with the type *P. padus* (European bird cherry); and (6) *Laurocerasus*, cherry laurels (mostly evergreens), with the type *P. laurocerasus* (European cherry laurel).

Prunus contains about 200 species, which are of economic importance (Reynders & Salesses 1990). Members of the genus can be deciduous or evergreen. A few species have spiny stems. The leaves are simple, alternate, usually lanceolate, unlobed, and often with nectaries on the petiole. The flowers are usually white to pink, sometimes red, with five petals and five sepals, and contain numerous stamens. The flowers are borne singly, or in umbels of two to six or sometimes more on racemes. The fruit is a fleshy drupe (a "prune") with a single relatively large, hard-coated seed. Bortiri *et al*. (2001) showed that *Prunus* is monophyletic and descended from some Eurasian ancestor.

Prunus trees are very common in Iran, Afghanistan and throughout the Turco-Iranian area. According to Zohary (1963) and Ladizinsky (1999), Iran and Anatolia are the centers of origin for several *Prunus* species. It is however believed that over 30 *Prunus* "species" described by botanists may actually be subspecies or ecotypes (Browicz & Zohary 1996, Kester *et al*. 1991, Kester & Gradziel 1996), which have morphologically diverged from their parental species and adapted to different environmental conditions. Iran too has numerous endemic *Prunus* species (Etemadi & Asadi 1999, Ghahreman & Attar 1999, Sorkheh *et al*. 2009) that may contain several infraspecific taxa.

Wild *Prunus* species can provide an extensive gene pool, which can be used in breeding of cultivated almond, for their desirable characteristics such as late blooming, self-fertility and resistance to drought, salinity and low winter temperatures and resistance to abiotic and biotic stresses (Sorkheh *et al*. 2009).

There have been several large-scale studies on *Prunus*. These investigations were mainly concerned with the taxonomy and molecular systematics. Different molecular markers have been used, including isozymes (Vezvaei 1994), AFLPs (Shiran *et al*. 2009), ISSRs (Shahi-Gharahlara 2011), EST and genomic SSRs (Tahana *et al*. 2009, Liu *et al*. 2012), combination of nuclear and chloroplast SSRs (Zeinalabedini *et al*. 2008), RAPD and SSR markers (Shiran *et al*. 2007), and RAPD and ISSR markers (Martins *et al*. 2003). However, there has been no attempt to show intraspecific (interpopulation) genetic differentiation in the wild *Prunus* species of Iran. We carried out a genetic diversity analysis of six *P. scoparia* populations by using nuclear SSR markers. We aimed to establish if the populations are genetically isolated by geographic distance or if there is a gene flow among them, and if the populations' genetic distance is correlated with their morphological and geographical distance.

Material and methods

Plant material

Molecular and morphological studies were carried out on the following six populations of *P. scoparia* (synonyms used regionally: *P. dulcis*, *P. amygdalus*, *Amygdalus communis*, *A. dulcis*): Fars (locality Firoozabad, alt. 1470 m a.s.l.), Lorestan (locality Poledokhtar alt. 885 m a.s.l.), Tehran (locality Tehran, alt. 1660 m a.s.l.), Semnan (locality Reshm, alt. 1300 m a.s.l.), Khorasan (locality Deihouk, alt. 1300 m a.s.l.), Qom (locality Salafchegan, alt. 1400 m a.s.l.). Forty trees were randomly collected from each population and used for further studies. Forty leaves were randomly selected from each plant and used for the DNA extraction.

DNA extraction and visualization of amplified SSR (microsatellite) fragments

A piece of 1 cm^2 obtained from each leaf was ground to powder in liquid nitrogen with a Mixer Mill (MM 300, Retsch). Total DNA was extracted using the protocol of Dumolin *et al*. (1995) based on CTAB/dichlormethane. DNA concentrations were then measured and dilutions containing 10 ng μl^{-1} DNA were prepared and stored at –20 °C.

Six microsatellites were analysed for *P. scoparia* (Table 1). A high throughput microsatellite genotyping method was applied using two sets of primer pairs. Set 1 comprised of four loci (UDP96-005, UDP98-411, UDP98-412 and BPPCT040), and Set 2 of two microsatellite loci (UDP98-410 and BPPCT034) (Testolin *et al*. 2000, Schüler *et al*. 2003). The microsatellite fragments were amplified by multiplexing all primers of each set. The cycling consisted of a

Table 1. SSR primers and the number of alleles in the *Prunus* populations studied.

Loci	Fars	Lorestan	Tehran	Semnan	Khorasan	Qom
UDP96-005	27	27	19	15	19	19
UDP98-411	18	21	16	11	8	12
UDP98-412	15	1	13	13	12	11
BPPCT040	14	14	9	10	10	5
UDP98-410	25	20	13	7	13	12
BPPCT034	22	15	15	10	14	14

denaturation step of 4 min at 94 °C, followed by 35 cycles of 25 s at 94 °C, 25 s at 60 °C (Set 1) and 56 °C (Set 2), respectively, and an extension of 45 min at 65 °C; the final extension took 45 min at 60 °C. We used a 25 μl PCR reaction mix for each sample as described in Jolivet *et al*. (2011). Amplified SSR fragments were analysed with an Amersham MegaBace 1000 capillary sequencer (GE Healthcare, Freiburg) and individual genotypes were determined with the Fragment Profiler software ver. 1.2.

Statistical analyses

To assess the morphological diversity among the studied populations, MANOVA was performed using nine morphological characters (*see* Table 2). Normality of the data was verified by normal probability plot.

To assess the genetic diversity, genetic parameters including the number of common alleles and their frequency, the number of specific alleles, Shannon's information index, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity and fixation index (Weising *et al*. 2005, Freeland *et al*. 2011) were determined. Mantel's test was used to determine correlations between genetic diversity parameters and location (altitude and longitude) of the populations studied.

In order to study the genetic differences among populations, Analysis of Molecular Variance (AMOVA) was performed. The genetic variance was partitioned into three levels: (1) among populations, (2) within populations, and (3) among individuals. Significance of fixation indices was tested using a nonparametric permutation approach with 1000 permutations with Genealex ver 6. Neighbor joining (NJ) tree was constructed for the specimens based on Nei's genetic distance.

The Bayesian model-based clustering (Pritchard *et al*. 2000) was used to elucidate the genetic structure of the populations by using STRUCTURE ver. 2.3. This program implements a model-based clustering method for inferring population structure using genotype data consisting of unlinked markers. The model applied in the analysis assumes the existence of K clusters. Applications of this model include demonstrating the presence of population structure, assigning individuals to a single population or to two or more populations if their genotypes

Table 2. Measurements of morphological characters. All sizes cm; $n = 5$ in all cases.

Character	Fars	Lorestan	Tehran	Semnan	Khorasan	Qom
Leaf length	11.2 ± 1.1	9 ± 1.22	11.4 ± 1.78	10.8 ± 2.95	12.2 ± 1.3	9.1 ± 0.55
Leaf width	9.8 ± 0.27	9.7 ± 1.44	9.1 ± 1.19	9.8 ± 3.49	11.3 ± 2.41	5.4 ± 0.55
Calyx length	3.5 ± 0.01	3.8 ± 0.27	4.6 ± 0.42	3.3 ± 1.35	4 ± 0.61	3.2 ± 0.27
Calyx width	3 ± 0.01	3.4 ± 0.22	2.4 ± 0.22	3.3 ± 0.97	3.7 ± 0.76	2.48 ± 0.04
Stamens number	33.8 ± 1.64	28.8 ± 0.45	25.6 ± 1.95	24.6 ± 5.73	31.8 ± 0.45	31.8 ± 0.45
Size of outer stamen	5.3 ± 0.27	4.6 ± 0.55	5.3 ± 0.45	4.8 ± 1.35	5.8 ± 0.27	3.96 ± 0.09
Size of inner stamen	3.98 ± 0.04	3.58 ± 0.53	4.3 ± 0.45	4.3 ± 1.6	4.8 ± 0.27	2.98 ± 0.04
Hyphantium length	5.48 ± 0.04	3.4 ± 0.22	4.8 ± 1.1	3.6 ± 0.96	4.6 ± 0.42	5 ± 0
Hyphantium width	3.48 ± 0.04	5.6 ± 0.22	3.9 ± 0.89	3.8 ± 0.91	3.18 ± 0.29	2.98 ± 0.04

indicate that they are admixed. It is assumed that within populations, the loci are at the Hardy-Weinberg and linkage equilibria.

We used admixture ancestry model under the correlated allele frequency model. The Markov chain Monte Carlo simulation was run 20 times for each value of K (2–6) for 10^6 iterations after a burn-in period of 10^5. All other parameters were set at their default values. The proportional membership of each cluster was estimated for each individual and each population. Reticulation (network) tree was constructed by Darwin ver. 5 to show the presence of common alleles or genetic exchange among populations.

The populations' genetic relationships were determined by Nei's genetic distance followed by NJ (Neighbor Joining) tree construction. We used UPGMA (Unweighted Paired Group using Average Method) clustering method to study populations' morphological similarity. Mantel's test (Podani 2000) was performed to study association between molecular distance, morphological distance and geographical distance of the populations by NTSYS ver. 2 (1998).

Results

Allele frequency and genetic diversity

All six SSR primers produced polymorphic bands, or distinct alleles (Table 1). The highest number of alleles occurred in the Fars and Lorestan populations with 121 and 114 alleles, respectively. Some of the alleles were shared by all the populations studied, while some others were specific to one population. For example, in the first locus, alleles 156 and 162 were shared by all the populations. The same was true for alleles 183, 185 and 187 of the locus 2. However, alleles 133, 155 and 156 of the locus 4 occurred only in the Semnan, Lorestan and Tehran populations, respectively. Some of the alleles were absent from one population only. For example, alleles 142 and 144 (locus 5) were absent from the Qom population, while allele 146 of the same locus was absent from the Semnan population.

Table 3. AMOVA results.

Source	df	SS	MS	Est. Var.	Percentage of variation
Among pop.	5	100.706	20.141	0.218	8
Among indiv.	234	637.300	2.724	0.133	5
Within indiv.	240	590.000	2.458	2.458	87
Total	479	1328.006		2.809	

F-statistics	Value	*p*
F_{st}	0.078	0.010
F_{is}	0.051	0.010
F_{it}	0.125	0.010

The observed heterozygosity (H_o) ranged from 0.62 in locus 5 to 0.97 in locus 4, while the expected heterozygosity (H_e) ranged from 0.55 in locus 5 to 0.94 in locus 1. A high level of average heterozygosity was found for all six loci studied (> 0.81). AMOVA showed significant among-population differences for the six studied loci (*see* Table 3).

The H_o value ranged from 0.729 in the Lorestan population to 0.871 in the Fars population, while H_e varied from 0.743 in the Semnan population to 0.922 in the Fars population (*see* Table 4).

The highest numbers of alleles and effective alleles were found in the Fars population (13.97 and 2.747, respectively), while the lowest values of the same parameters were found in the Semnan population (4.296 and 1.739, respectively). No significant relationship between genetic diversity parameters and altitude or location of the populations studied was found.

A data matrix of 12×240 was formed for NJ clustering (Fig. 1). The two hundred and forty trees studied were distributed among six major clusters. Specimens collected from the Semnan population along with some trees from Fars, Tehran and Khorasan formed the first major cluster. Specimens collected from the Lorestan and Tehran populations, were intermixed and formed the second major cluster. The third major cluster is a mixture of trees from almost all the populations, while the forth cluster was formed mainly by trees collected from the Qom and Lorestan populations. The trees from the Fars, Khorasan and Qom populations formed the fifth and sixth clusters.

In the STRUCTURE plots, grouping of K = 6 showed a better separation of trees, although

Table 4. Genetic diversity parameters in the *Prunus* populations. N = number of individuals, N_a = number of alleles, N_e = number of effective alleles = $1/\sum p_i^2$, I = Shannon's Information Index = $-\sum(p_i \ln p_i)$; H_o = observed heterozygosity = H_e/N; H_e = expected heterozygosity = $1 - \sum p_i^2$; UH_e = unbiased expected heterozygosity = $[2N/(2N-1)]H_e$; F = Fixation Index = $1 - (H_o/H_e)$; p_i is the frequency of the ith allele in the population.

Population		N	N_a	N_e	I	H_o	H_e	UHe	F
Fars	Mean	40	20.167	13.975	2.747	0.871	0.922	0.934	0.055
	SE	–	2.182	1.775	0.121	0.027	0.010	0.010	0.032
Lorestan	Mean	40	19.000	10.825	2.614	0.842	0.906	0.917	0.070
	SE	–	1.949	0.698	0.073	0.031	0.006	0.006	0.035
Tehran	Mean	40	14.167	7.829	2.254	0.800	0.864	0.875	0.074
	SE	–	1.376	0.850	0.111	0.047	0.015	0.015	0.054
Semnan	Mean	40	11.000	4.296	1.739	0.729	0.743	0.752	0.012
	SE	–	1.125	0.567	0.140	0.053	0.040	0.041	0.062
Khorasan	Mean	40	12.667	7.363	2.158	0.850	0.854	0.865	0.004
	SE	–	1.542	0.699	0.122	0.041	0.021	0.021	0.045
Qom	Mean	40	12.167	6.193	2.021	0.825	0.824	0.835	–0.004
	SE	–	1.851	0.682	0.152	0.032	0.026	0.027	0.041

containing some degree of admixture (*see* Fig. 2).

The highest value of Nei's genetic distance was found between the Semnan and Lorestan populations (1.143) followed by that between the Semnan and Tehran populations (1.081) (Table 5). In the NJ tree (Fig. 3), the Tehran and Qom populations had the highest genetic similarity and were placed close to each other, while the Lorestan population joined them at some distance. Two populations from Semnan and Khorasan also showed genetic affinity and were placed close to each other. The Fars population showed the least genetic similarity to the other populations studied.

Morphometry

The populations studied differed significantly in their morphological characters (MANOVA: $F_{45,74.76} = 10.72$, $p << 0.001$, Wilk's $\lambda = 0.0001$). The Tehran and Lorestan populations showed the highest level of morphological similarities followed by the Semnan and Qom populations, while the Fars and Khorasan populations differed the most from the other populations (Fig. 4). Mantel's test did not show correlation between molecular and morphological distance ($r = 0.27985$, $t = 0.95$, $p = 0.83$), or between molecular and geographical distance ($r = 0.02$, $t = 0.12$, $p = 0.54$).

Combined analysis of SSR and morphological data

A combined data matrix of molecular and morphological characters was used for grouping the populations. The UPGMA tree and PCoA plot produced similar results, therefore only the PCoA plot is given here (Fig. 5). The Fars, Lorestan, Tehran and Semnan populations are fairly well separated. The specimens collected from Khorasan and Qom populations were intermixed.

Table 5. Nei's genetic distance among the *Prunus* populations.

	Fars	Lorestan	Tehran	Semnan	Khorasan
Lorestan	0.654	–			
Tehran	0.606	0.860	–		
Semnan	0.753	1.143	1.081	–	
Khorasan	0.583	0.711	0.883	0.544	–
Qom	0.711	1.033	0.685	0.840	0.849

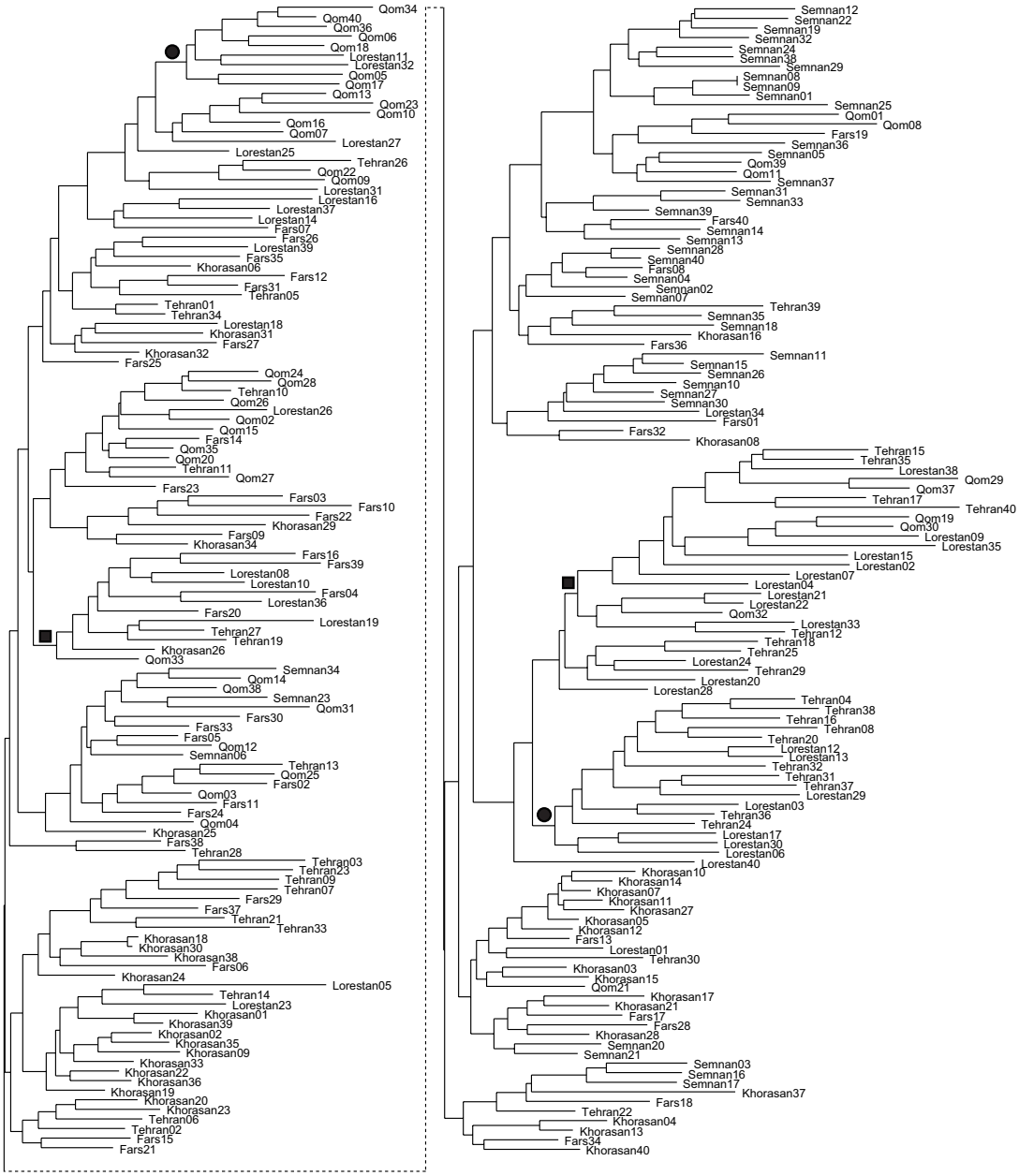

Fig. 1. Neighbor joining (NJ) tree of the sampled specimens. The symbols ● and ■ indicate clusters between which genetic exchange has taken place.

Discussion

Genetic polymorphism

The presence of different SSR alleles indicates genetic polymorphism in the studied *Prunus* populations. Moreover, the presence of specific alleles in some of the plants/genotypes illustrates the occurrence of unique insertions/deletions in the DNA. This SSR allelic variation is in agreement with the report of Fathi *et al*. (2008), who showed that irrespective of the cross-amplification ability of microsatellite markers across the *Prunus* species, a high level of heterozygosity occurs for all loci.

The AMOVA results proved usefulness of

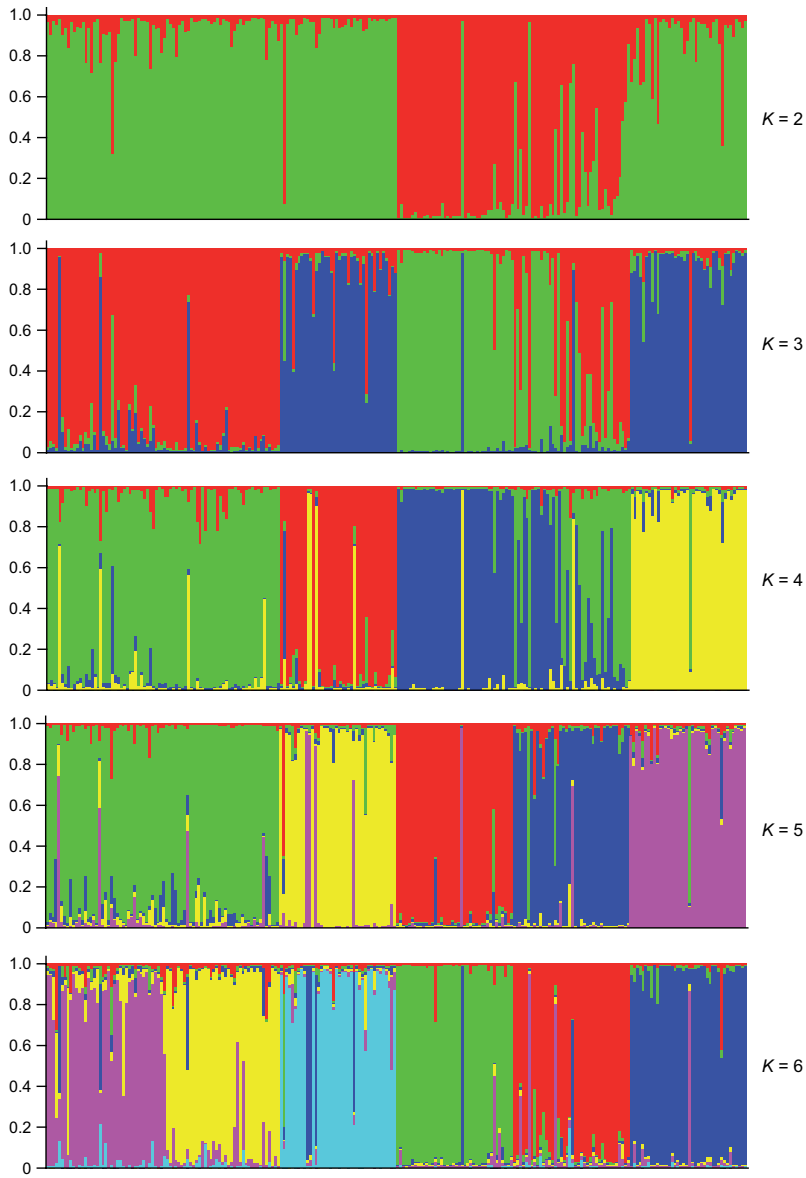

Fig. 2. Groupings based on Bayesian model-based clustering. *K* is the presumed number of populations in the analysis.

SSR loci in genetic studies of wild *Prunus* species and populations. This holds true also for cultivated *Prunus*; for example, the SSR primers CPPCT3, UDP98-412, UDP96-409, XAM05, XAM08, XAM09, XAM15 and XAM19 are known to discriminate those taxa (Fathi *et al*. 2008).

The high H_o and H_e values reported here for the studied *P. scoparia* populations are in agreement with other studies. For example, Fathi *et al*. (2008) reported a high level of heterozygosity for SSR loci (0.697) in *P. dulcis*, which was attributed to cross pollination and self-incompatibility of the plant. This may hold true for *P. scoparia* too, as the STRUCTURE analysis and the reticulation tree revealed some degree of population admixture and gene exchange.

The geographical distance among the studied populations varies from 100 to 600 km. Exactly how the genetic exchange has occurred over such long distances is an interesting question. In a similar study of four wild *Prunus* spe-

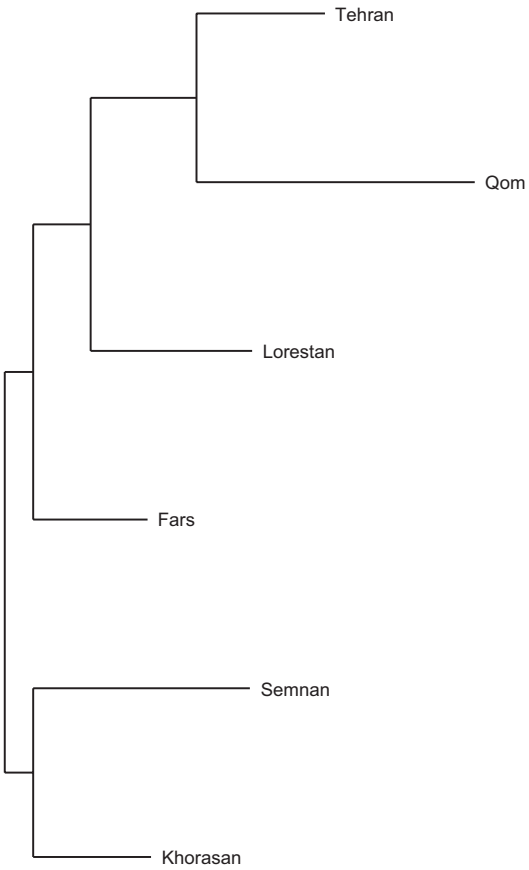

Fig. 3. NJ tree of the *Prunus* populations based on SSR data.

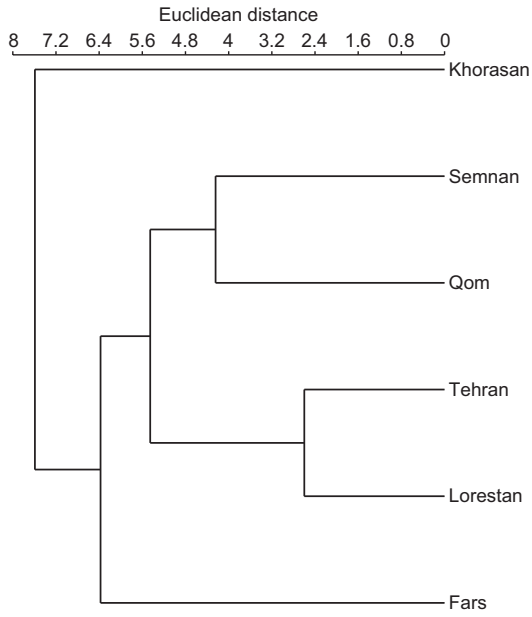

Fig. 4. UPGMA tree of morphological data.

cies in Iran (*P. eleagnifolia*, *P. hausknechtii*, *P. scoparia* and *P. lycioides*; Zeinalabedini *et al*. 2008), genetic exchange among the species was found. Therefore, gene exchange between *P. scoparia* and other wild *Prunus* taxa growing in the nearby area is possible but this was not taken into consideration in the present study.

Tahana *et al*. (2009) used STRUCTURE to study the genetic structure of populations in *P. nana* by using EST and genomic SSRs. They reported little gene exchange between *P. nana*

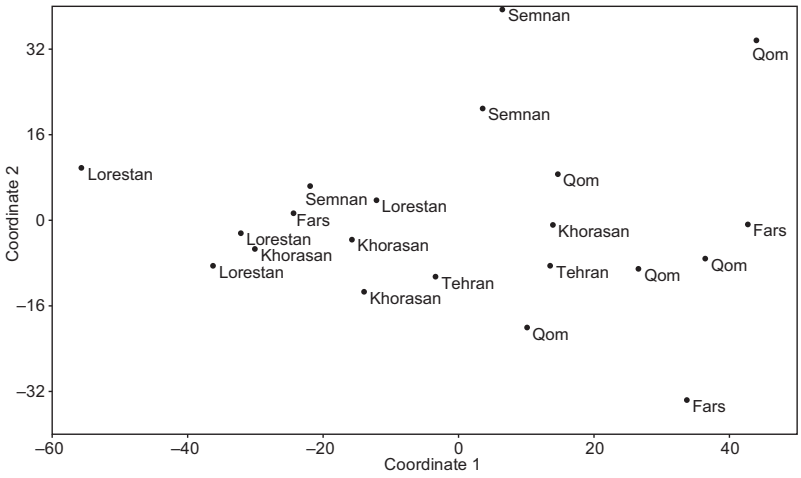

Fig. 5. PCoA plot of populations based on combined SSR and morphological characters.

(commonly known as wild almond) and *P. communis*.

In the present study, in addition to the genetic differences among the *P. scoparia* populations, we also found a high degree of genetic variation within the populations (AMOVA). Thus, the populations consist of genetically heterogeneous individuals.

Inter-population genetic diversity may indicate genetic adaptation to local conditions in geographically distant populations (Liu *et al*. 2012). Tahana *et al*. (2009) investigated genetic diversity in the wild *P. nana* and cultivated forms. They reported a higher degree of genetic diversity in the cultivated trees than in the wild genotypes, and also noticed that most of the genetic variation (82.16%) was partitioned within populations.

Morphology

Similar morphological differences as found in this study have been reported for other wild *Prunus* taxa. For example, Sorkheh *et al*. (2009) studied the leaf and fruit characteristics along with the flowering and ripening date, self-incompatibility and kernel bitterness in 137 accessions from 18 wild *Prunus* species growing in Iran. They found extensive phenotypic diversity both within and among the species. We did not found any correlation between morphological characters and altitude at which the plants grow, supporting the results of Sorkheh *et al*. (2009). They found that the differences in the average leaf dimensions within as well as among the studied species were correlated with the average rainfall, but not with the altitude of the collection site.

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