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Genetic diversity, population structure, and relationships among wild and domesticated almond (*Prunus* spp.) germplasms revealed by ISSR markers

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Abstract: The use of diverse almond genetic resources to expand the genetic bases of commercial cultivars is important for almond breeders. Iran is within the center of origin for almond and enjoys a huge diversity of wild species and local cultivars of this important nut crop. Despite some reports, there is still a critical need to collect comprehensive information on the genetic diversity of almond germplasm in Iran. This study was conducted to evaluate the genetic diversity, structure, and relationships among a total of 75 individuals from 10 populations of 4 wild and cultivated almond species by using 12 inter-simple sequence repeat (ISSR) primer pairs. A total number of 353 DNA fragments were obtained of which 352 were polymorphic (99.69%). The average of polymorphism information content (PIC), marker index (MI), and resolving power (Rp) were 0.932, 27.211, and 7.882, respectively which indicated high discriminatory power of markers. Gene flow between wild and cultivated gene pools is shown to be moderate to high (Nm = 2.7607), which verifies the hypothesis of low genetic differentiation among populations. Cluster analysis based on unweighted pair-group, classified individuals into 7 major gene pools which showed the entire provenances were divided into 7 main groups. Overall high levels of genetic diversity were confirmed and useful information obtained on the differentiation and genetic structure of the studied almond germplasms. Future evaluation on morphological and physiological aspects, is necessary to identify the most promising individuals to be used directly in afforestation, landscape development as well as nut and oil production or indirectly in future almond and stone fruits breeding programs.

1. Introduction

Almond [*Prunus dulcis* (L.) Batsch] belongs to the Rosaceae family and

is one of the most important nut crops in the world which is known for its high nutritional value. Almond domestication occurred nearly 5000 years ago in the Fertile Crescent (Velasco *et al*., 2016). United States of America, Spain, Iran, Italy, Turkey, Tunisia, Morocco, Syria, Greece and Australia are the ten major producers of almond (Ardjmand *et al.*, 2014). Iran is the fifth world producer of almond (Gharaghani *et al.*, 2017) which produced approximately 2.99% of the total world production of cultivated almonds (Sorkheh *et al.*, 2016).

Due to the narrow genetic background of commercial cultivars, breeding programs of almond face many challenges. In modern plant breeding, native plants are considered as valuable gene pools for crossing programs which can be used to introduce new traits into commercial relatives. Wild almond species are found in the mountains and deserts of Central Asia from western China to Iran and Turkey (Rahemi *et al.*, 2012). Wild almond species could be valuable gene pools for breeding purposes due to late bloom, early maturity, adaption to drought and salinity, resistance to winter lower temperatures, reduced insect infestation and fungal attacks (Gharaghani *et al.*, 2017). Thus, knowledge about genetic diversity of wild genetic resources of almond is an essential prerequisite for involvement of native germplasm in almond breeding programs. On the other hand, assessment of genetic diversity and population structure is necessary to evaluate the existing levels of genetic variability and its patterns of distribution among the local populations, which is considered as a guarantee for conservation management of natural populations (Cohen *et al.*, 1991; Sreekanth *et al.*, 2012).

Iran is a center for genetic diversity of almond and nearly twenty wild species of almond have been reported from arid and semi-arid regions of this country (Sorkheh *et al.*, 2009). Different regions of Iran have variable environmental conditions including subtropical climate in the south, temperate in the north, and extended deserts in the middle which helps the distribution of wild species such as almonds. Wild almond germplasm forms the main part of distributed plant species in the mountainous and plain sub-regions of ecological zones in the Zagros of Iran where the annual precipitation rate is more than100 mm (Sabeti, 1994). Almond stands of the Irano-Turanian region have been observed in Badamak, Mohammadabad Maskun and Badameshk forests in Fars, Kerman and South Khorasan province of Iran, respectively (Talebi *et al*., 2013). Fars and Charmahal-o-Bakhtiari provinces cover parts of the central and southern Zagros where *Prunus scoparia* (Spach) C.K. Schneid*., P. elaeagnifolia* Spach., and *P. eburnean* Spach. are widely distributed (Gharaghani *et al.*, 2017).

Owing to some traits such as leaf shedding during hot seasons, and the remarkable capability of roots in water absorption, some of the wild almond species can resist draught (Madam *et al*., 2011). *P. scoparia* is a potentially multi-purpose wild almond species in Iran which has the potential to become the crop of choice for soil stabilization and landscape in arid and semi-arid areas (Mozaffarian, 2005). It has been used as a dwarfing rootstock for almond for centuries (Gharaghani and Eshghi, 2015). *P. scoparia* is a potential source of vegetable oil for human nutrition and health with relatively higher oxidative stability, higher unsaturated to saturated fatty acids ratio, calculated oxidisability value, total tocopherols and phenolics contents, and unsaponifiable matter contents, than those of olive oil (Farhoosh and Tavakoli, 2008). Zedu gum is exuded from the bark of *P. scoparia*, and its kernel oil are used in Iranian traditional medicine (Zargari, 1997). Zedu gum is also being used as emulsifier in cosmetic and textile industries (Rahimi *et al*., 2013). These species lie among the rare trees which naturally grow in barren soils (Ali *et al*., 2015). *P. elaeagnifolia* has been used as a rootstock for plum (Gholami *et al.,* 2010) in drought conditions in Iran and some other species have also been used as rootstocks for almond and peach by ancient Iranians in arid lands (Denisov, 1988). Grafting nectarine on wild almond trees as rootstock has also been reported by Alberghina in 1978. Wild almond species have also been used to afforest barren lands and to protect vegetative cover (Mardani, 2006).

DNA-based molecular markers are important tools to study genetic variation in population genetics. Various molecular markers including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), Inter-simple sequence repeat (ISSR), and single nucleotide polymorphism (SNP) have been previously used to describe genetic diversity and structure in the genus *Prunus* (Martins *et al.*, 2003; Shiran *et al.*, 2007; Sorkheh *et al.*, 2007; Wu *et al.*, 2008; Bouhadida *et al.*, 2009; Rahemi *et al.*, 2012). Among different DNA markers, ISSRs have greater reliability and reproducibility in comparison with RAPD system, as well as the lower cost of the analyses than AFLP

and SSR (Rodrigues *et al.*, 2013). Moreover, ISSR markers seem to be especially useful to study closely related individuals which show low levels of polymorphism (Zietkiewicz *et al.*, 1994).

Local wild species make up an excellent source of genetic diversity which can be used for crop improvement and breeding programs (Khadivi-Khub and Anjam, 2014). Despite some reports, there is still a critical need to collect more information on the genetic diversity of almond germplasm in Iran. Because of high value of *P. scoparia*, it is important that the necessary steps be taken to comprehensively evaluate, utilize and ensure the conservation of this unique wild species. The purpose of our study was to study the genetic diversity and population structure of a collection of wild and cultivated almond populations in Iran using ISSR markers. The emphasis of this study is on the populations of *P. scoparia* collected from different sites in central and southern Zagros regions, experiencing less natural precipitation and higher temperature comparing to other natural habitats of this species in Iran. These special climatic condition made these wild populations a promising source of genes evolved for drought and high temperature tolerance, which will be very valuable in facing harsh effects of climate change. We also sought to compare the diversity and illustrate the relationships of these populations with some populations of three other almond species including *P. elaeagnifolia*, *P. eburnea* and *P. dulcis* (common almond) from the same geographical region, to put more shed on the possible gene flow among them as well as to detect the footprint of these species in the genetic background of cultivated almond. The results of this study are useful for conservation of these wild stands as well as for decision making on direct or indirect utilization of them for afforestation, nut and oil production, landscape purpose and through breeding programs.

2. Materials and Methods

Field sampling

To detect higher genetic diversity (resulting from cross-pollination in natural habitat of the plant materials used herein) as well as the feasibility of plant materials collection (seeds instead of leaf samples) we chose to use raised seedling populations instead of natural populations in this study. In total, seeds of 72 wild almond trees were sampled from southern and central regions of Zagros Mountain in Iran during late spring to early summer of 2014. These regions are placed in Fars and Chaharmahal-and-Bakhtiari provinces. The studied genotypes belong to *P. scoparia* and *P. eburnean* in section Spartioides Spach. as well as *P. elaeagnifolia* and *P. dulcis* in section Euamygdalus Spach (Kester and Gradziel, 1996). In addition, seeds of 5 almond cultivars were sampled. Characteristics of the populations (collection sites, latitude, longitude, altitude, etc.) are listed in Table 1.

Plant materials and DNA extraction

The seeds of all species were mechanically scarified and then soaked in water for 24 h. They were mixed with perlite and stratified at 4±1°C for 45 days. After stratification, nuts were directly sown in 5 kg pots filled with a mixture of fine sand, soil and leaf mold. The pots were then transferred to the greenhouse with an average temperature of 26±3°C under daylight illumination conditions i.e. 800 $\mu mol\, m^{-2}\, s^{-1}$ about 10 hours. In December 2017 a total of 75 seedlings (each seedling represents an individual tree in natural habitat) comprised 10 populations (3 to 11 individuals per population) were selected. Stem pieces (200 mg) for each individual were collected into aluminum foil, immediately snap-frozen in liquid nitrogen and stored at -80°C until the DNA was extracted. Total genomic DNA was isolated following the cetyltrimethylammonium bromide (CTAB) protocol with minor modifications (Doyle and Doyle, 1987). DNA quantity and quality were determined by spectrophotometry and visual comparison of DNA electrophoresed on 1% agarose gel.

ISSR genotyping

In total, 12 ISSR primer pairs were selected based on literature review (Carvalho *et al.*, 2002; Martins *et al.*, 2003; Dje *et al.*, 2006; Zhao *et al.*, 2007; Oliveira *et al.*, 2010; Moulin *et al.*, 2012; Ahmed *et al.*, 2013; Muraseva *et al.*, 2018) and synthesized (by Metabion, Germany). Polymorphism of markers was first tested in a subset of samples and then polymerase chain reaction (PCR) conditions were optimized. The list of primers and their information are presented in Table 2.

The PCR mix contained 10 ng template DNA, 10 pmol of primer in a final 20 µl reaction volume. Conditions of the PCR amplification were as follows: 94°C (3 min), then 35 cycles at 94°C (45 s) / 38-61°C (varied for each primer according to Table 2) (45 s) / 72°C (1 min) and final extension at 72°C for 7 min. The amplified products were separated by 1% (w/v) agarose gel electrophoresis in 1× TBE buffer at constant voltage (100) for 45 min, stained with

Table 1 - List of the studied genotypes with indication of their regions and geographical coordinates of the collection sites

Species	No.	Population	E	N	Altitude (m)
Prunus scoparia	1	Shiraz 1	52 34.558	29 37.082	1535
	2	Shiraz 2	52 35.822	29 44.275	1820
	3	Shiraz 3	52 35.784	29 44.287	1816
	4	Shiraz 4	52 35.811	29 44.269	1819
	5	Shiraz 5	52 34.601	29 37.103	1569
	6	Shiraz 6	52 34.449	29 37.465	1565
	7	Shiraz 7	52 33.704	29 37.946	1549
	8	Shiraz 8	52 34.586	29 39.490	1670
	9	Shiraz 9	52 32.642	29 40.263	1728
	10	Shiraz 10	52 35.789	29 44.353	1821
	11	Shiraz 11	52 35.785	29 44.351	1818
P. scoparia	12	Nourabad 3	51 20.875	30 4.695	1179
	13	Nourabad 4	51 39.730	29 48.513	934
	14	Nourabad 5	51 32.377	30 1.167	1086
	15	Nourabad 6	51 23.931	30 0.745	1285
	16	Nourabad 7	51 39.823	29 48.605	946
	17	Nourabad 8	51 21.011	30 6.513	1183
	18	Nourabad 9	51 31.694	30 1.252	1067
	19	Nourabad 10	51 58.427	30 01 08.4	1592
P. scoparia	20	Marvdasht 1	52 54.983	30 3.162	1728
	21	Marvdasht 2	52 54.800	30 6.249	1730
	22	Marvdasht 3	53 00.807	30 6.800	1812
	23	Marvdasht 4	53 12.117	30 5.742	1837
	24	Marvdasht 5	53 10.524	30 1.617	1828
	25	Marvdasht 6	53 12.643	29 59.116	1803
	26	Marvdasht 7	53 14.080	29 57.712	1765
	27	Marvdasht 8	53 6.493	29 48.754	1667
	28	Marvdasht 9	53 6.535	29 48.816	1663
	29	Marvdasht 10	53 8.662	29 47.443	1640
P. scoparia	30	Firuzabad 2	52 32.509	29 8.712	1725
	31	Firuzabad 3	52 34.486	28 58.157	1503
	32	Firuzabad 4	52 33.874	28 57.406	1530
	33	Firuzabad 5	52 32.400	28 55.816	1445
	34	Firuzabad 6	52 38.719	29 5.885	1917
	35	Firuzabad 7	52 23.202	28 53.222	1524
	36	Firuzabad 8	52 38.310	29 3.808	1732
	37	Firuzabad 9	52 32.801	29 9.124	1763
	38	Firuzabad 10	52 41.274	28 13.319	1275
	39	Firuzabad 11	-----	-----	1578
P. scoparia	40	Mian Jangal Fasa 1	52 46.117	29 26.327	1481
	41	Mian Jangal Fasa 2	52 50.130	29 19.653	1526
	42	Mian Jangal Fasa 3	--	--	2187
	43	Mian JangalFasa 4	53 24.351	29 9.542	1729
	44	Mian Jangal Fasa 5	53 23.894	29 9.939	1754
	45	Mian Janga lFasa 6	53 26.033	29 7.617	1720
	46	Mian Jangal Fasa 7	53 26.054	29 7.632	1716
	47	Mian Jangal Fasa 8	53 24.138	29 9.082	1756
	48	Mian Jangal Fasa 9	53 22.759	29 10.821	1815
	49	Mian Jangal Fasa 10	53 19.277	29 12.351	1825

to be continued...

Table 1 - List of the studied genotypes with indication of their regions and geographical coordinates of the collection sites

Species	No.	Population	E	N	Altitude (m)
P. scoparia	50	Eqlid 3	52 40.003	30 15.126	1701
	51	Eqlid 4	52 38.310	30 16.346	1742
	52	Eqlid 5	52 36.405	30 18.062	1800
	53	Eqlid 6	52 35.080	30 22.196	2321
	54	Eqlid 7	52 23.051	30 19.324	1843
	55	Eqlid 8	52 23.764	30 19.060	1750
	56	Eqlid 9	52 24.085	30 18.382	1715
P. scoparia	57	Lordegan 1	51 11.609	31 33.619	1752
	58	Lordegan 2	51 13.020	31 34.354	1962
	59	Lordegan 3	--	--	1948
	60	Lordegan 4	--	--	1963
P. elaeagnifolia	61	*P. elaeagnifolia* 1	52 35.806	29 44.098	1801
	62	*P. elaeagnifolia* 2	52 35.746	29 44.158	1804
	63	*P. elaeagnifolia* 3	--	--	2128
	64	*P. elaeagnifolia* 4	--	--	2570
	65	*P. elaeagnifolia* 5	52 34.880	30 22.732	2458
	66	*P. elaeagnifolia* 6	52 34.898	30 22.748	2455
P. eburnea	67	*P. eburnea*2	52 22.173	30 19.865	1912
	68	*P. eburnea*3	53 24.368	29 09.592	1732
	69	*P. eburnea*4	--	--	2280
	70	*P. eburnea*5	52 24.585	30 18.376	1711
	71	*P. eburnea*7	52 22.130	30 19.854	1902
	72	*P. eburnea*8	53 24.101	29 9.071	1763
P. dulcis	73	Mamaei	---	---	1910
	74	Ferragnes	---	---	1910
	75	Badam talk	---	---	1910

SimplySafe (EURx, Poland) and photographed with UV light (Nade Gel Documentation and Analysis System JS-6800, China). The size of produced fragments was defined according to size marker (Fermentas, Germany).

Data analysis

Marker results (reproducible distinct bands with high resolution) were dominantly scored in a data matrix. The matrix was used for calculation of population genetic variation indices.

The informativeness of primer pairs in genotyping and subsequent evaluation of genetic diversity and population structure was compared using the polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolving power (Rp). For each primer, the polymorphic information content (PIC) was estimated by PowerMarker v3.25 (Liu and Muse, 2005). Marker index for each primer was calculated as a product of polymorphic information content and effective multiplex ratio: MI = EMR * PIC (Varshney *et al*., 2007). The resolving power (Rp) of each primer was calculated as $Rp = \sum Ib$, where Ib shows the informative fragments. The Ib may be shown on a scale of 0/1 by the following formula; $Ib = 1 - (2 \times |0.5 - pi|)$ where pi is the proportion of populations containing the *ith* band (Prevost and Wilkinson, 1999).

Based on ISSR bands identified in the individuals, some basic parameters for genetic diversity including the total number of bands (TNB), the number of polymorphic bands (NPB), the percentage of polymorphic bands (PPB), mean Nei's gene diversity index (H), Shannon's information index (I), the observed number of alleles per locus (Na), the effective number of alleles per locus (Ne), the level of gene flow (Nm), population diversity (Hs), the total gene diversity (Ht), inter-population differentiation (Gst), genetic identity and genetic distance were calculated for each population using software POPGENE 1.32 (Yeh *et al*., 1999). Private bands (referring to the bands found only within one population) and major allele frequency were estimated by power marker software.

To illustrate the relationship among populations,

an unweighted pair group method with arithmetic mean (UPGMA) dendrogram was constructed based on Nei's genetic distance using POPGENE 1.32 (Yeh *et al.*, 1999). The dendrogram was generated using TreeView program.

To further understand the relationships among populations, a Bayesian clustering-based structure analysis was performed on the entire data set using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) to reveal the number of genetic pools. Two runs of analysis using the admixture model were performed. Initial runs were performed with a burn-in length of 50000 and 750000 MCMC (Markov Chain Monte Carlo) replicates for 10 times at each K from 1 to 10. The probable number of groups was estimated. The second run was 100000 for burn-in length and 300000 for MCMC replicates, 10 times for each K. To estimate the best K value. The Evanno test was performed on STRUCTURE results using ''Structure Harvester'' (Evanno *et al*., 2005). The results were summarized in a bar plot using DISTRUCT (Rosenberg, 2004).

3. Results

Informativeness of markers

The mean of PIC values was analyzed for all loci to evaluate markers efficiency. PIC value ranged from 0.845 (primer 4) to 0.973 (primer 1). The mean PIC value for all loci was 0.932. The highest EMR value of 38 (primer 1) and the lowest of 20 (primer 12), with an average EMR value of 29.25 per primer were obtained. The highest (36.97) and the lowest (19) MI values were observed with primers 1 and 12, respectively. The mean MI value was 27.211 per primer. The highest Rp value was observed with primer 1 (13.183) and the lowest with primer 4 (3.518) with an average Rp of 7.882 per primer (Table 2).

Genetic diversity

The 75 individuals of wild and domesticated almond assigned to 10 populations and were amplified with 12 selected primers (Table 2). A total of 353 bands were scored with an average band number of 29.33 per primer across 75 individuals. Among the 353 bands, 352 bands (99.69%) were polymorphic. The percentage of polymorphic bands (PPB) varied from 96.29% for primer 3 to 100% for the other primers (Table 2). At the population level, PPB ranged from 18.70% in *P. dulcis* to 58.07% in *P. scoparia* (Shiraz and Firuzabad populations) with a mean value of 49.66% (Table 3).

The N_a ranged from 19.63 for ISSR 3 to 20.00 for other ISSRs. Across the populations, N_a ranged from 11.870 for *P. dulcis* to 15.807 for *P. scoparia* (Shiraz and Firuzabad populations). The N_e ranged from 11.453 for ISSR 4 to 14.254 for ISSR 5 with an average of 13.003 alleles per locus. Across the populations, N_e

Table 2 - ISSR primers used in this study and their results

Primers	Primer sequences (5'-3')	Tm	Total number of alleles (a)	Number of polymorphic alleles (b)	% Polymorphism (b/a)*100	PIC	MI	EMR	MAF	Rp
1	GAC AGA CAG ACA GAC A	48	38	38	100	0.973	36.97	38.00	0.120	13.183
2	GTG CGT GCG TGC GTG C	58	30	30	100	0.952	28.56	30.00	0.173	4.932
3	GTG GTGGTGGTGGTG-	61	27	26	96.29	0.948	23.72	25.03	0.186	8.132
4	CTC TCT CTC TCT CTC TTG	54	27	27	100	0.845	22.68	27.00	0.373	3.518
5	GAG AGA GAG AGA GAG	50	23	23	100	0.953	21.85	23.00	0.173	9.160
6	CAC CACCAC GC	38	33	33	100	0.947	31.02	33.00	0.200	9.946
7	ACA CAC ACA CAC ACA	54	23	23	100	0.855	19.55	23.00	0.360	4.889
8	GAA GAAGAAGAAGAA-	50	28	28	100	0.958	26.60	28.00	0.173	8.172
9	GTC GTCGTCGTCGTCGTC	61	32	32	100	0.873	27.84	32.00	0.333	4.692
10	GAG AGA GAG AGA CC	44	35	35	100	0.961	33.60	35.00	0.160	12.177
11	BDB ACA ACAACAACAA-	49	37	37	100	0.956	35.15	37.00	0.160	9.531
12	YHY GTG TGT GTG TG	42	20	20	100	0.959	19.00	20.00	0.133	6.252
Min.	---	---	20	20	96.29	0.845	19.00	20.00	0.120	3.518
Max.	---	---	38	38	100	0.973	36.97	38.00	0.373	13.183
Means	---	---	29.41	29.33	99.69	0.932	27.21	29.25	0.212	7.882
Total	---	---	353	352	---	---	326.54	351.03	2.544	---

Y = (C, T); D = (A, G, T); V = (A, C, G); B = (C, G, T); R = (A, G) Rp= resolving power; PIC= polymorphism information content; MI= marker index; EMR effective multiplex ratio; MAF= major allele frequency.

Table 3 - Genetic diversity within the populations of almond in Iran exhibited by inter simple sequence repeat (ISSR)

Population	No.	Observed no. of alleles (Na)	Effective no. of alleles (Ne)	Shannon's in formation index (I)	Nei's genetic diversity (H)	Percentage of polymorphic loci (PPB)	No. bands	No. private Bands
Shiraz	11	15.807	12.853	0.2749	0.1774	58.07	205	7
Nourabad	8	15.212	12.657	0.2570	0.1664	52.12	184	3
Marvdasht	10	15.581	12.723	0.2653	0.1708	55.81	197	6
Firuzabad	10	15.807	12.812	0.2737	0.1759	58.07	205	11
Mian Jangal Fasa	10	15.666	12.879	0.2738	0.1774	56.66	200	8
Eqlid	7	15.524	12.968	0.2816	0.1838	55.24	195	12
Lordegan	4	14.419	13.161	0.2652	0.1817	44.19	157	1
P. elaeagnifolia	6	14.703	12.577	0.2453	0.1607	47.03	167	6
P. eburnea	6	15.071	13.099	0.2778	0.1854	50.71	179	8
P. dulcis	4	11.870	11.496	0.1190	0.0831	18.70	66	0
Mean	7.5	14.966	12.722	0.2533	0.1662	49.66		

ranged from 11.496 for *P. dulcis* to 13.161 for *P. scoparia* (Lordegan population) (Tables 3 and 4). Across the populations, the highest values of I (0.2816) and H (0.1838) indexes were observed for *P. scoparia* (Eqlid populations). However, *P. dulcis* showed the lowest I (0.1190) and H (0.0831) values (Table 3). However, for studied accessions, the average values of Na and Ne were 14.966 and 12.722, respectively.

Genetic similarity and cluster analysis among populations

The dendrogram derived from UPGMA cluster analysis was generated for all populations (Fig. 1). Among seven distinct groups obtained by dendrogram, four groups represent populations of *P. scoparia*. The group I consisted of two populations of *P. scoparia* (Shiraz and Mian Jangal-e-Fasa) collected from Fars province. The group II was composed of the other three populations of *P. scoparia*(Nourabad, Marvdasht, and Firuzabad) sampled from Fars province. The Eqlid population of *P. scoparia*

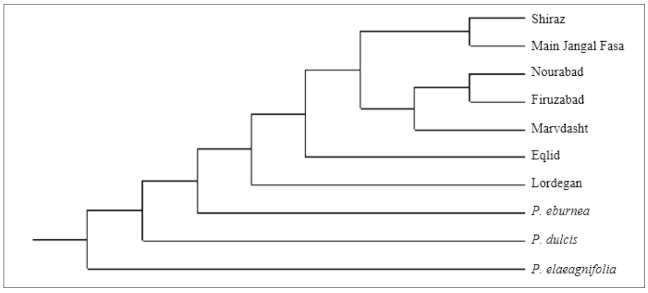

Fig. 1 - UPGMA dendrogram on the basis of Nei's (1978) evaluation of genetic distance among 10 populations of almond.

Table 4 - A summary of genetic parameters across inter-simple sequence repeat loci

Locus	Na	Ne	I	h	Ht	Hs	Gst	Nm
1	20.00	13.759	0.3872	0.2418	0.2412	0.2034	0.1568	2.6884
2	20.00	11.847	0.2547	0.1408	0.1427	0.1197	0.1615	2.5953
3	19.630	13.564	0.3529	0.2244	0.2273	0.1959	0.1385	3.1111
4	20.00	11.453	0.2178	0.1165	0.1183	0.0974	0.1771	2.3231
5	20.00	14.245	0.4103	0.2623	0.2538	0.2088	0.1774	2.3181
6	20.00	13.447	0.3652	0.2247	0.2234	0.1939	0.1319	3.2900
7	20.00	12.437	0.3061	0.1780	0.1775	0.1522	0.1428	3.0022
8	20.00	13.268	0.3392	0.2095	0.2092	0.1726	0.1751	2.3559
9	20.00	11.649	0.2255	0.1237	0.1234	0.1063	0.1387	3.1053
10	20.00	14.034	0.3857	0.2474	0.2426	0.2125	0.1244	3.5201
11	20.00	12.712	0.2869	0.1739	0.1761	0.1426	0.1903	2.1274
12	20.00	13.630	0.3593	0.2261	0.2216	0.1904	0.1408	3.0504
Mean	19.90	13.003	0.3242	0.1974	0.1964	0.1663	0.1546	2.7906

Na= Observed number of alleles; Ne= Effective number of alleles; I= Shannon's Information index; Nei's genetic diversity; Ht= Total gene diversity; Hs= Population diversity; Gst= Inter-population differentiation; Nm= Estimate of gene flow.

sampled in north of Fars province was separated in the group III. The group IV comprised Lordegan population of *P. scoparia* sampled from Charmahal and Bakhtiari province in central Zagros region. Groups V, VI and VII included populations of *P. eburnea*, *P. dulcis*, and *P. elaeagnifolia*, respectively.

The Nei's genetic distance ranged from 0.0077 to 0.0452 and genetic identity ranged from 0.9558 to 0.9923 (Table 5). The genetic identity between Nourabad and Firuzabad populations of *P. scoparia* was 0.9923 having the closest genetic relationship; however, the farthest genetic identity was 0.9558 between Lordegan population of *P. scoparia* and *P. elaeagnifolia* populations.

Population structure

The amount of gene flow (Nm) among populations was 2.7607, showing the moderate to high gene flow among populations studied herein. The genetic diversity within populations (*H*s) and the total genetic diversity (*H*t) of the species were 0.1663 and 0.1964, respectively (Table 6). The genetic differentiation among the populations (Gst) was 0.15 which shows that 15% of the total genetic variability was among populations and 85% was within populations.

The results of the structure analysis with ISSR markers are presented in figure 2. The structure plot suggested a lack of definite structure although

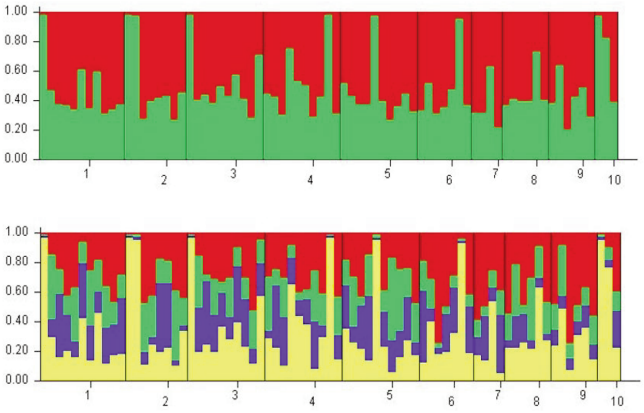

Fig. 2 - Population structure of almond populations for K = 2 and K = 4, showing a high degree of genotypic admixture among individuals. On the horizontal axis, the following population are illustrated: (1): *Prunus scoparia* (Shiraz); (2): *Prunus scoparia* (Nourabad); (3): *Prunus scoparia* (Marvdasht); (4): *Prunus scoparia* (Firuzabad); (5): *Prunus scoparia* (MianJangalFasa); (6): *Prunus scoparia* (Eqlid); (7): *Prunus scoparia* (Lordegan); (8): *Prunus elaeagnifolia*; (9): *Prunus eburnea*; (10): *Prunus dulcis*.

Evanno's test indicated that the most informative number of populations was K = 2 and K = 4.

Using the defined strategies of DNA purification with the selected primers, good patterns could be attained for the different accessions under study. Instance of patterns of amplification attained by ISSR

Table 5 - Genetic identity (above diagonal) and genetic distance (below diagonal) estimates between populations across all loci based on Nei (1978)

Population	Shiraz	Nourabad	Marvdasht	Firuzabad	Mian Jangal	Eqlid	Lordegan	*Prunus elaeagnifolia*	*Prunus eburnea*	*Prunus dulcis*
Shiraz	****	0.9862	0.9898	0.9899	0.9896	0.9773	0.9774	0.9693	0.9715	0.9722
Nourabad	0.0139	****	0.9910	0.9923	0.9882	0.9806	0.9806	0.9789	0.9792	0.9756
Marvdasht	0.0102	0.0091	****	0.9900	0.9841	0.9868	0.9756	0.9677	0.9709	0.9721
Firuzabad	0.0102	0.0077	0.0101	****	0.9894	0.9835	0.9783	0.9714	0.9777	0.9789
MianJangalFasa	0.0104	0.0119	0.0161	0.0107	****	0.9749	0.9779	0.9675	0.9725	0.9737
Eqlid	0.0230	0.0196	0.0132	0.0167	0.0254	****	0.9730	0.9600	0.9625	0.9728
Lordegan	0.0229	0.0196	0.0247	0.0219	0.0224	0.0273	****	0.9558	0.9718	0.9567
P. elaeagnifolia	0.0312	0.0214	0.0328	0.0290	0.0330	0.0409	0.0452	****	0.9713	0.9609
P. eburnea	0.0289	0.0210	0.0296	0.0225	0.0279	0.0382	0.0286	0.0291	****	0.9560
P. dulcis	0.0282	0.0247	0.0283	0.0213	0.0267	0.0275	0.0442	0.0399	0.0450	****

Table 6 - Assessment of the genetic variability among ten populations designated based on the ISSR analysis

	*H*t Total gene diversity	*H*s population diversity	Gst Inter-population differentiation	Nm Estimate of gene flow
Average	0.1964	0.1663	0.1533	2.7607
Standard deviation	0.0236	0.0168	---	-----

in various accessions of almond are shown in figure 3.

4. Discussion and Conclusions

Informativeness of markers

Due to highly variable nature and less investment in time and money than other marker systems, ISSR markers are widely used in population genetic studies (Harris, 1999). Moreover, Matesanz *et al.* (2011) reported that because of high polymorphism, only a few ISSR loci (as few as five to seven primer pairs) are enough to obtain reliable information on genetic diversity of populations.

The efficiency of a molecular marker system in distinguishing genotypes depends largely upon the polymorphism it can discover (Guo *et al*., 2014). On the basis of high PIC values, MI, and Rp we conclude that ISSR markers used in this study were informative in the assessment of genetic diversity of almond accessions. The high PIC values with a mean of 0.932 show that all primers are informative, and this can be related to high genetic variation among accessions

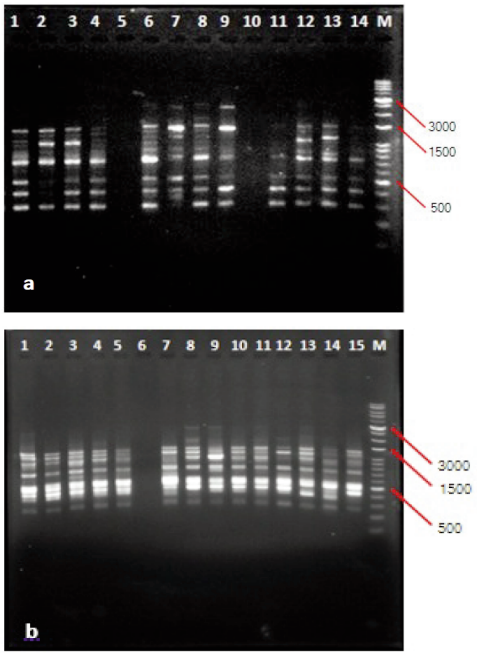

Fig. 3 - a) ISSR banding pattern generated using primer 11. Lane 1-14, Eqlid 7, Mian Jangal Fasa 6, Marvdasht 6, Eqlid 4, Nourabad 4, Firuzabad 2, *P. elaeagnifolia* 4, Nourabad 5, *P. elaeagnifolia* 2, Nourabad 10, Shiraz 8, Nourabad 9, Shiraz 2, Shiraz 6. b) ISSR banding pattern generated using primer 10. Lane 1-15, Shiraz 3, Shiraz 4, Marvdasht 8, Nourabad 7, Lordegan 1, Mamaei, Marvdasht 4, Shiraz 9, Badam talk, Shiraz 7, Mian Jangal Fasa 3, Mian Jangal Fasa 1, Eqlid 9, Marvdasht 3, Mian Jangal Fasa 4. M. 10 kb DNA ladder.

used in this research. Similar results were reported for sour cherry and *Prunus mira* (Najafzadeh *et al.,* 2014; Tian *et al.,* 2015). The variation may have been contributed by gene flow, natural hybridization, propagation by seed and human selection (Sefc *et al.,* 2000).

The Rp and MI measurements show distribution and number of alleles (bands) within the studied genotypes. Bands that are scored in the half of genotypes would possess optimal discriminatory power and with an increase in the number of bands, the Rp of a particular primer pair will be increased (Kayis *et al.*, 2010). Therefore, primers with the highest PIC, EMR, MI, and Rp values (ISSR1, ISSR10, and ISSR11) were generally the most effective in distinguishing between accessions and could be further used in almond genetic diversity studies. The similar results are reported in, *Prunu*s genus, sweet cherry, and sour cherry (Yılmaz *et al.*, 2009; Ganopoulos *et al.*, 2011; Najafzadeh *et al.*, 2014).

Genetic diversity

Information on genetic diversity and structure of wild almond populations is essential for their conservational programs. Moreover, narrow genetic background of the commercial cultivars of the genus *Prunus* restricts their cultivation in new regions with different environmental conditions. Therefore, genetic diversity among populations of this genus can be used to broaden the genetic background of commercial scion and rootstock cultivars and to overcome their distribution across different regions (Gradziel *et al.*, 2001).

Genetic variation depends on many factors such as mating system, genetic drift, gene flow, human activities, long-term evolutionary history, natural selection, and breeding systems (Schaal *et al.*, 1998; Hamrick and Godt, 1996).Populations of domesticated almonds used in this study possess restricted number of individuals and often are reproduced vegetatively. Thus, cultivated almonds shows lower levels of genetic diversity than the other species. However, higher genetic diversity was observed in certain individuals and populations of the wild almonds. This phenomenoncould be expounded by the fact that are propagated sexually whereas individuals of cultivated almonds aremainly reproduced asexually.

The PPB is a major genetic diversity index that showed high levels of genetic diversity among almond genotypes. Similar great genetic diversity

was reported by Sorkheh *et al.* (2017) in wild almond. The obtained PPB in this study was higher than values reported by Kumar *et al.* (2009) and Shuxia (2011) with *Prunus armeniaca* (96.5%) and *Prunus persica* (93%), respectively. Also, high genetic variation was shown by Rahemi *et al.* (2012) in wild almond, being similar to our results. One of the main reasons of the existing genetic variation is the process of self-incompatibility which is controlled by genes (Gouta *et al.,* 2010; Szikriszt *et al.,* 2011).The high number of generated alleles in our study may be due to use of several different genotypes that possessed high levels of genetic diversity.

The bands generated by each primer rests on the primer, sequence and the diversity size in special genotype (Shiran *et al.*, 2007). So, the number of bands differed in various genotypes. The private bands show the existence of special genes or sequences in native populations. The common bands show alleles which are shared among the cultivars studied. Thus, the private bands can be used in almond genetic fingerprinting and cultivar recognition.

Genetic similarity and cluster analysis among populations

Cluster analysis is widely used to study the genetic relationships among germplasms (Li *et al.*, 2010). The UPGMA dendrogram obtained in this study clearly distinguished species from each other and the clades were in accordance with morphological traits. Moreover, all populations were divided into their related taxa.

Prunus scoparia (Shiraz population)which seems to be mainly an artificial (cultivated) population and *P. scoparia* (MianJangal-e-Fasa population) both were separated into the same group. Therefore, we assume that some of the *P.scoparia* stands in Shiraz region were developed artificially through seed that may have originated from MianJangal-e-Fasa. Lordegan population lay in group IV, being closely related to Eqlid populations. It may be due to the geographic proximity and climatic resemblance between these two geographical locations. *Prunus eburnean* was grouped in cluster V, close to *P. scoparia* (Lordegan) populations. This close relationship is logical because both of them belong to Spartioides section within the genus *Prunus* (Kester and Gradziel, 1996). Moreover, close relationship between domesticated population of almond (*P. dulcis*) and *P. elaeagnifolia* could be explained in the same way since they both belong to Eu amygdalus section within the genus *Prunus* (Kester and Gradziel, 1996).

Genetic proximity between the genotypes or populations from different regions, for example Nourabad and Firuzabad (Table 5), could be explained by the geographical proximity of the regions, the exchange of plant material between sites and by the probable existence of common ancestors (El Hamzaoui *et al.*, 2014). Also, Noormohammadial *et al.* (2013) reported the gene exchange among *Prunus scoparia* populations which is similar to our results.

Molecular phylogeny results obtained in this study were similar to our findings using nut and kernel morphological characteristics to cluster the same subset of plant materials with some exceptions (Rahimi Dvin *et al.*, 2017). In that work, we found that *P. eburnea* and *P. scoparia* were placed close to each other and *P. elaeagnifolia* and *P. dulcis* formed the same clade.

The genetic distance among the studied almonds in this experiment is short, indicating that a high capacity for hybridization exists between genotypes and populations. The mating system can greatly affect genetic diversity both within and among populations. Generally, most of the genetic diversity in self-pollinated plants is distributed among populations, while in out crossed plants such as almond species, most of the genetic diversity is distributed within populations (Hamrick, 1989).

Population structure

Gene flow is defined as the gene movement within and between populations (Lowe *et al*., 2009). The estimate of gene flow (Nm) has been categorized as low (Nm<1), moderate (Nm>1) and extensive (Nm>4) (Kumar *et al*., 2014). The estimate of Nm (2.7607) was higher than 1, which indicates that the number of migrants per generation can prevent population differentiation caused by genetic drift. Moreover, we conclude that high genetic diversity and lack of differentiation is due to high amount of gene flow. Almond is an important food source for both human and animals and its seeds can be easily transported by birds and nomads (which is very common in the region) increasing the amount of Nm.

Genetic differentiation coefficient is an indicator of genetic diversity and structure of species (Zia *et al.*, 2014). It should be noted that Rosaceae species usually show low levels of genetic differentiation (Fineschi *et al.*, 2005). Based on Slatkin (1985), *Nm* >1 shows no significant genetic differentiation among populations. In this study, genetic differentiation

between populations had an average value of 0.15. Similar results were reported by Li *et al.* (2013) who obtained Gst of 0.18 with apricot. Many factors can influence on genetic differentiation which may occur independently in a population. For example, high dispersal rate of seeds must be involved in low genetic differentiation (Fanciulli *et al.*, 2000). Moreover, gametophytic incompatibility, prevents self-fertilization and encourages cross-pollination (Weinbaum, 1985) which retains high levels of genetic variability within seedling populations (Arulsekar *et al*., 1986). As a consequence, populations of almonds which possess gametophytic incompatibilityshow low levels of genetic differentiation.

The genetic structure shows the history of populations with respect to their long-term evolution, mutation, recombination, genetic drift, gene flow, and natural selection (Slatkin, 1987; Schaal *et al.*, 1998). Therefore, providing information on the genetic diversity and structure of a crop is a prerequisite for the conservation and effective use of germplasms available for breeding (Laidò *et al.*, 2013).

Structure results demonstrated a high degree of admixture among individuals across 10 populations, consistent with moderate to high levels of gene flow across populations. Our results are similar to those of Mendigholi *et al.* (2013), who showed that the plots of structure exhibited the admixture of population and gene exchange which showed the existence of ancestral gene among *Prunus scoparia.*

The lack of population structure and moderate to high gene flow among the species in this study suggests the potential interbreeding among the populations. Nevertheless, a high individual genetic diversity purveys an optimistic prospect for the survival of the declining population with proper management interposition.

Results signified that ISSR primers which had been used herein had a significant distinctive power for the evaluation of the polymorphism in various almond populations. The obtained results present Iranian native almond species as a precious source of genetic diversity and recommends that they are an auspicious source of new genes for rootstock and cultivar breeding programs. Results also offer a contribution to the management and conservation of this valuable almond germplasm. Since the Iranian almond species and genotypes have not been selected for breeding programs, they are more probable to have a further diverse genetic background and may be employed in the selection of various genotypes so as to create new cultivars. It is expected that with extra experiments and analyses on morphological and physiological aspects, the most promising individuals could be identified and introducedfor direct utilization in afforestation, landscape development as well as nut and oil production or to be used by almond and stone fruits breeders in future breeding programs.

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