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Evaluation of genetic relationships among Iranian pistachios using microsatellite markers developed from *Pistacia khinjuk* Stocks

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ABSTRACT

To evaluate the genetic relationships among wild and cultivated *Pistacia* species grown in Iran and the analysis of genetic variation among Iranian pistachio genotypes, two DNA libraries enriched for dinucleotide $(AG)_n$ and trinucleotide $(ATG)_n$ microsatellite motifs were developed from *Pistacia khinjuk* genome. Following screening of clones by colony PCR technique, 44 clones were sequenced and 27 pairs of primers designed from flanking regions of the repeats. The examination of primer pairs, designed from *P. khinjuk* sequences, showed successful cross-species amplification within the genus *Pistacia*. A dendrogram constructed on the basis of the Minimum Evolution clustering algorithm revealed that *Pistacia vera* has closer relationships with *P. khinjuk*, than with *Pistacia integerrima*, *Pistacia palaestina*, *Pistacia atlantica* and *Pistacia mutica*. The dendrogram further distinguished the wild Sarakhs pistachio from the rest of *P. vera* genotypes suggesting that the domesticated genotypes of *P. vera* are evolved from *P. vera* var. Sarakhs and then this wild genotype likely develops to other local pistachios. Hence, it seems that the wild Sarakhs pistachio plays an important role in evolutionary trend of the edible pistachios in Iran. The results indicated that microsatellites developed in *P. khinjuk* are distributed in the genome of indigenous pistachio species including *P. vera* genotypes and therefore they will be useful in characterization of Iranian pistachio genotypes.

1. Introduction

The genus *Pistacia* is a deciduous and wind pollinated member of the family Anacardiaceae (Shuraki and Sedgley, 1994), which comprises 11 species (Zohary, 1952). Among them, *Pistacia vera* L., *Pistacia atlantica* subsp. *mutica* (Fisch. & C. A. Mey.) Rech. f. (*Pistacia mutica*), and *Pistacia khinjuk* Stocks, are the species that occur in Iran (Sheibani, 1996), of which only *P. vera* has economical importance and its cultivation, as a traditional nut crop, is extended to the dry land areas of the country. Wild *P. vera* var. Sarakhs called Sarakhs is also indigenous to the region located in north east Iran, north Afghanistan and Turkmenistan (Zohary, 1996; Sheibani, 1996). Many ecological and physiological traits, particularly tolerance to biotic and abiotic stresses, made it possible to use Sarakhs for reforestation in arid zones or representing rootstock material (Esmail-pour, 2003). However, the importance of its involvement as a source of variability for pistachio-breeding strategies, in order to develop new cultivars and rootstocks, has been neglected.

The genetic variability of *P. vera* is relatively high in Iran and information on such variability is essential for its conservation, breeding and commercial production. Several molecular markers such as Isozymes (Dollo, 1993), RFLPs (Parfitt and Badenes, 1997), RAPDs (Mirzaei et al., 2006; Hormaza et al., 1998; Kafkas and Perl-Treves, 2001) and AFLPs (Katsiotis et al., 2003; Kafkas, 2006; Salehi Shanjani et al., 2009) have been used to assess the genetic diversity of pistachio cultivars and important *Pistacia* species. However, it seems that simple sequence repeats (SSRs) or microsatellite markers, due to their high information content, codominancy nature, reproducibility (Powell et al., 1996) and potential for analysis of intracultivar variability issues (Cipriani et al., 2002; Lopes et al., 2004) should be more relevant for germplasm characterization, DNA fingerprinting and genetic mapping of fruit trees (Wunsch and Hormaza, 2002a; Sanchez-Perez et al., 2005). Also, SSRs developed for one species could be used in related plant species, considering that the success of cross-species amplification depends on the evolutionary relatedness (Dayanandan et al., 1997). The transferability of SSR loci has been reported by locus amplification success across species and genera such as, *Malus* (Pierantoni et al., 2004), *Olea* (Rallo et al., 2003), *Prunus* (Wunsch and Hormaza, 2002b; Xu et al., 2004) and *Vitis* (Arnold et al., 2002; Di Gaspero et al., 2000).

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It is believed that, *P. vera* and *P. khinjuk* are the most primitive species and also postulated that *P. khinjuk* was directly descended from *P. vera* (Zohary, 1952) as a bridge to other *Pistacia* species. Morphological characteristics (Zohary, 1952) with genetic relationship analysis of the species within genus *Pistacia* (Parfitt and Badenes, 1997; Golan-Goldhirsh et al., 2004) provide sufficient information to support the relatedness of *P. vera* and *P. khinjuk*. Although these two species share many similar characteristics, based on RAPD analysis of Iranian pistachios, it was suggested that *P. vera* var. Sarakhs placed in between *P. khinjuk* and commercial genotypes of *P. vera*. The notion that encourages concluding that wild *P. vera* var. Sarakhs has an important role in the evolving of pistachio cultivars (Mirzaei et al., 2006; Salehi Shanjani et al., 2009; Pazouki et al., 2010). However, no other study has been addressed to evolutionary significance of *P. vera* var. Sarakhs in the diversification of *Pistacia* sp.

To design high transferable SSR markers for *Pistacia* genus, it is necessary to select a focal species for generating SSR markers to test them as potential markers through cross-amplification in related species. Recently SSRs were developed from *Pistacia lentiscus* (Albaladejo et al., 2008) and *P. vera* (Ahmad et al., 2003) which are used to differentiate pistachio cultivars collected from different regions (Ahmad et al., 2003; Pazouki et al., 2010). However the poor transferability (Albaladejo et al., 2008) and low level of polymorphism of these microsatellites across Iranian pistachio accessions (Ahmad et al., 2003) made them relatively less efficient for precise discrimination of species and cultivars of pistachio grown in Iran. Therefore, due to genetic relatedness of *P. khinjuk* to *P. vera*, its long-term existence and wide spread in different regions of Iran, *P. khinjuk* was selected as a focal species to develop SSR markers for germplasm analysis of pistachio accessions in Iran.

The objectives of this study were the development of microsatellite markers from genomic library of *P. khinjuk* enriched for AG and ATG repeats using enrichment method, evaluation of their transferability to related species of *Pistacia* sp. grown in Iran, and use them to examine the inter-relations among Iranian wild and cultivated *Pistacia* accessions.

2. Materials and methods

Total genomic DNA was extracted from young leaves of *P. khinjuk* (Hormaza et al., 1994). Two microsatellite enriched libraries of $(AG)_n$ and $(ATG)_n$ repeats were constructed according to Hamilton et al. (1999), with some modifications. DNA was digested with a combination of restriction enzymes *Rsa*I, *Alu*I, *Hae*III and *Nhe*I (Promega, USA), and the fragments generated in range between 200 and 1000 bp (estimating based on their migration on agarose gel) were dephosphorylated and ligated to double-stranded universal SNX linkers. The resultant DNA fragments were separately hybridized to 5′-biotinilated microsatellite repeat oligos $(AG)_{15}$ or $(ATG)_{10}$ (synthesized by MWG Biotech AG, Germany). After extensive washing to remove non-specific binding DNA fragments, repeat-containing DNA fragments were recovered using streptavidin coated magnesphere paramagnetic particles (Promega, USA). The eluted fragments were amplified by PCR employing primers complementary to the linker region and the amplicons were then ligated to dephosphorylated pBlueScript plasmid and transformed into competent cells of *Escherichia coli* JM109.

Following blue/white screening, colony PCR technique with M13 forward and reverse primers plus one of the related synthesized repeat primer was used to direct selection of insert-containing transformants. To do so, a loop of each colony was transferred to a tube containing 50 μl of sterile deionized water, and then boiled for 5 min. After cooling on ice, each sample was diluted 20-fold and used in PCR reaction in a final volume of 15 μl with 4 μl of lysate, 1× PCR-buffer, 0.15 mM dNTP, 2 mM $MgCl_2$, 1 U *Taq* DNA Polymerase (Fermentas, USA), 0.3 μM M13 primers and 0.6 μM of repetitive primers (according to enrichment step). The conditions of the PCR were as the following: an initial step of 95 °C for 2 min, followed by 15 cycles of 94 °C for 20 s, touchdown from 65 °C to 50 °C for 30 s, 72 °C for 1.5 min, and 20 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 1.5 min with a final extension step at 72 °C for 10 min. Amplified products were separated by electrophoresis on a 1.2% agarose gel in TBE, stained with ethidium bromide and visualized under a UV transilluminator. All samples yielding two bands PCR products with appropriate sizes were selected as the desired motif containing colonies and subjected for further analysis. Plasmids of selected clones with an approximately 300–1000 bp insert were extracted by plasmid mini kit (SEQLAB, Germany) and sent out for sequencing with T3 or T7 universal primers (SEQLAB Sequence laboratories, Germany).

Table 1
List of pistachio accessions and their code assessed in this study.

Accession	Code	Accession	Code
Momtaz	PvMO	Sarakhs	PvSR
Badami-ravar	PvBR	Ohadi	PvOH
Akbari	PvAK	fandoghi-riz	PvFR
Ahmad-aghaii	PvAH	Kale-ghoochi	PvKQ
Gholam-rezai	PvGR	*Pistacia khinjuk*	PkRI
Ghazvini	PvQZ	*Pistacia mutica*	PmRI
Italiai-zoodras	PvIZ	*Pistacia atlantica*	PaRI
Khanjari-damghan,	PvKD	*Pistacia integerrima*	PiRI
Nish kalaghi	PvNK	*Pistacia palaestina*	PpRI

The DNA sequences from each SSR-containing *P. khinjuk* insert were checked against the GenBank, using NCBI BLASTN tools. The clones that possess SSRs with sufficient flanking region length were considered for primer design using the Primer3 software (Rozen and Skaletsky, 2000). The primers were synthesized commercially by Isogen Life Science (The Netherlands).

SSR primer sets were initially checked for interspecies amplification of *Pistacia* sp. For this purpose the total genomic DNA of young leaves of each plant samples was extracted according to previous method (Hormaza et al., 1994). PCR was performed in a 20 μl reaction mixture containing 1× PCR buffer, 25 ng template DNA, 0.15 μM of each primer, 0.15 mM of dNTPs, 2 mM $MgCl_2$ and 1.0 unit of *Taq* DNA polymerase. PCR conditions were optimized for each primer pair using gradient PCR program with base annealing temperature ranges of 65–50 °C to determine optimum annealing temperatures for each SSR primer pair. The reactions were done on an Eppendorf thermal cycler (Germany), programmed for the initial denaturation step at 95 °C for 3 min followed by 35 cycles of 94 °C for 30 s, at gradient annealing temperatures for 30 s and 72 °C for 60 s (extension), followed by a final elongation step at 72 °C for 10 min.

The SSR primer pairs yielded scorable amplicons in primer screening tests were used to assess genetic diversity among pistachio accessions collected from National Pistachio Collection Centre, Rafsanjan, Iran (Table 1). Following extraction of their total genomic DNA (Hormaza et al., 1994), PCR reactions were performed under the same conditions with optimized cycling program consisting of 3 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 60 °C or 65 °C (depending on the primer pair), 90 s at 72 °C and a final extension step of 72 °C for 10 min. Microsatellite fragments were detected on silver stained 8% non-denaturing polyacrylamide gel.

The amplified bands were scored manually by considering those in the range of best resolution in the polyacrylamide gel. After determining the allelic profile at each SSR locus, the program PowerMarker Ver. 3.25 (Liu and Muse, 2005) was used to estimate the number of alleles per locus, observed heterozygosity (H_o), gene

diversity (expected heterozygosity, H_e), and the polymorphism information content (PIC). Power of discrimination was calculated with the formula $PD = 1 - \sum g_i^2$, where g_i is the frequency of the *i*th genotype (Kloosterman et al., 1993).

The evolutionary distances among all the pair-wise combinations of genotypes were computed using the Kimura 2-parameter method (Kimura, 1980), which suitable to clarify the genetic relationships among accessions by means of the Minimum Evolution method (Rzhetsky and Nei, 1992). The dendrogram was resulted and the robustness of its nodes was assessed with bootstrap analysis using 1000 replacements by MEGA software version 4 (Kumar et al., 2004).

3. Results

3.1. SSR isolation and characterization

Constructing two genomic libraries of *P. khinjuk* enriched for AG and ATG repeats led to generating a total of 44 sequences, 36 of which contained microsatellites that only one of them was redundant. Among these, a higher proportion of microsatellites (71%) were simple perfect and the remaining SSRs identified as interrupted perfect (17%) and complex imperfect (11%) repeats. Nine of the sequences contained too short flanking DNA sequence to design primer pairs, thus only 27 primers, having repeats in the range of three to 32, were designed for 35 unique SSR sequences.

Comparing of two enriched libraries in specificity of their resultant microsatellites showed a relatively high proportion of nonspecific repeats (eight out of 18) for ATG library consisting of AG (7) and AGC (1) motifs,

3.2. Microsatellite amplification and cluster analysis

Of 27 primer pairs tested, 25 pairs successfully amplified SSR loci in *P. khinjuk* with expected size (based on the sequence data). Five primer pairs were subsequently discarded in phylogenic study because of low rate of amplification across species of *Pistacia* sp. The remaining SSRs amplified unambiguous and scorable PCR products in 18 genotypes related to six *Pistacia* species (Fig. 1), including two primer pairs (PKATG012 and PKAG021) amplified what appear to be duplicated loci (Table 2).

Across 18 polymorphic primer pairs (20 loci), a total of 114 alleles were distinguished in pistachio accessions tested, of which 56 alleles belonged to *P. vera* genotypes. In *P. vera* genotypes, the average number of presumed alleles per locus was 2.8, ranging from one to six. The highest number of polymorphic bands was obtained with Primer pairs PKATG021, PKAG001 and PKAG012 with 18, 15 and 11 alleles respectively in all accessions and 11, five and six alleles in *P. vera* genotypes. In all *Pistacia* accessions, the average values of H_e and PIC were 0.61 and 0.56, respectively, while values of these diversity parameters calculated 0.45 and 0.38 when only *P. vera* genotypes were considered. The variability parameters for 20 polymorphic loci on all accessions and *P. vera* genotypes are presented in Table 3.

Minimum Evolution dendrogram including all accessions was constructed based on their genetic distance (Fig. 2). The resultant dendrogram clearly separated 18 genotypes into two major clusters. The first cluster contained the Iranian cultivated pistachios, wild *P. vera* var. Sarakhs and *P. khinjuk*. In this cluster a close relationship was found between wild Sarakhs and *P. vera* cultivars, while *P. khinjuck* was rather distant from the remainder genotypes. The second cluster included the rest of *Pistacia* species: *Pistacia palaestina*, *Pistacia integerrima*, *P. atlantica* and *P. mutica*. Node values from the Bootstrap analysis showed the consistency of the results.

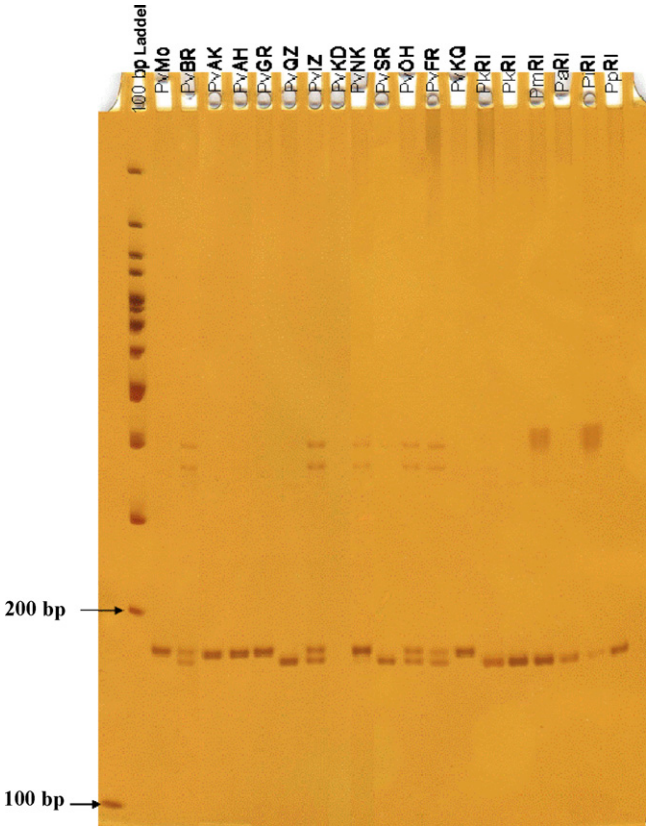

Fig. 1. The determination of the SSR alleles present at the PKATG011 locus for pistachio accessions. Lane codes correspond to the names of accessions in Table 1.

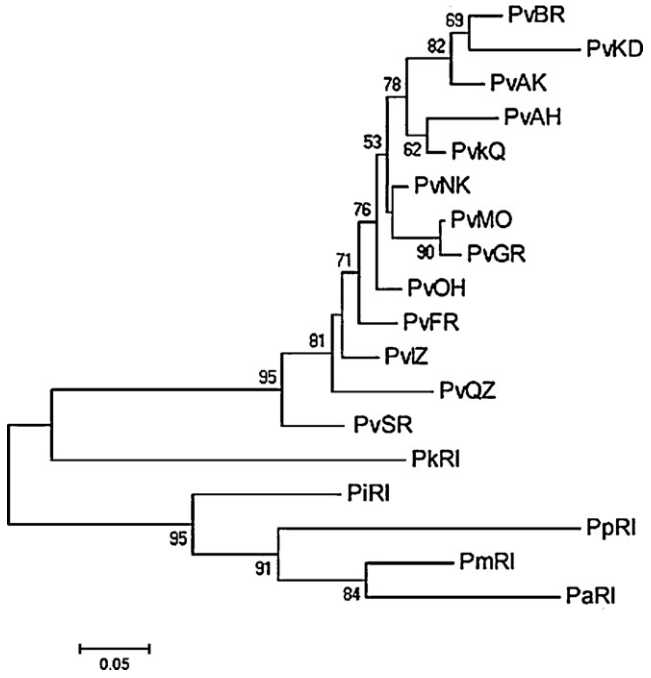

Fig. 2. Minimum Evolution dendrogram for 18 pistachio accessions based on Kimura-2 parameter. The numbers shown on each branch represent the bootstrap percentages obtained from 1000 pseudo-replications. Codes correspond to the names of accessions in Table 1.

Table 2
Microsatellite names, primer sequences, repeat motif, PCR product size, and primer annealing temperatures of 27 microsatellites developed from *Pistacia khinjuk*.

NCBI acc. No.	SSR name	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)	Repeat motif	Length[a] (bp)	T_a (°C)
FJ984494	PKAG001	TGAAAAGTGTATTCATCTGTG	AAACAATGTCACCTGTCC	$(AG)_{26}$	229	60
FJ984495	PKAG002	GCAACAGCGGACTGACTTAC	ATGATTACTTGCACTCACCTCAC	$(AG)_6$	149	65
FJ984496	PKAG003	CAAAGACAATCTCCACCCTTG	TTGTGTGTGTGTGTGTGTGTGT	$(TC)_{25}$	115	60
FJ984497	PKAG004	AACTTGAGGAGGCGGAAAG	CTAGCAGAAGCCCAATATCCA	$(TG)_{17}$	191	50
FJ984502	PKAG005	GTCGGACTGCAGGCTTATAG	GTCGCAGATAGCGAGTTGAG	$(AC)_8$–$(TC)_{13}$	190	60
FJ984518	PKAG006	GCCACTTGGTAGTTCCGTTTC	GCCTTTACAATACCAGGGGAAG	$(AG)_{17}$	237	65
FJ984503	PKAG007	TCCCATGGGTATTCCTTATTG	GCTAGAACAATTGCCGACTTC	$(AG)_{27}$	250	60
FJ984501	PKAG008	CAAACCATATATAAGCGCATGTTC	TTAAACCCCCTAATCACTCGAC	$(AG)_{11}$	169	65
FJ984519	PKAG009	GGATGCTCAGGTAGCAAATC	CTGGGATAAGAAGGAGGAAGAC	$(AG)_{31}$	238	60
FJ984498	PKAG010	CCGGCTTCAATAATCAAGAA	GACGTTGGTGCCCAATA	$(AG)_{17}$	222	55
FJ984505	PKATG011	CCCTCCTCTTTTCTCCTTCTCT	GAGGTCGTTCAAGTCCTGATG	$(ATC)_{13}$	156	60
FJ984506	PKATG012	TCCCATCAAGCATCATCATC	GCTAGTTGTCCCCTTGCATT	$(ATC)_8$	241	60
FJ984509	PKATG013	GACATTCACACCAACCCAAG	CGCTAAAGCGGACTGACTTAC	$(TC)_{11}$	188	65
FJ984508	PKATG014	AGCATCTGCCTTAGCACTAGAC	CGTCAACACCGCAAGTATC	$(ATC)_5$–NN–$(ATC)_3$	189	60
FJ984510	PKATG015	GATACCAAAACTGCAGAGAAAG	CTGCCCACAGATATTCTTGA	$(GTA)_5$	172	60
FJ984507	PKATG016	CCTTGTTGAGGACATGATGAT	GAGTTCCTCCAAACCAGACG	$(AGC)_6$	133	60
FJ984511	PKATG017	CCCTACTCACAGCCACCATC	CTTATGACCGGCCAACTCAG	$(ATC)_9$	92	60
FJ984512	PKATG018	CACCACCCACAATATGCAAC	CAGAGAAATGAGAAGGTGAAGACA	$(AG)_{15}$–GG–$(AG)_5$	154	55
FJ984499	PKAG019	ACCCTCGTCACGATTCTACC	AGTGCCCTAGAGCCAATCAA	$(AAT)_3$	157	55–60
FJ984496	PKAG020	CGACTCCACCTCCTCTCTCT	GTGGTCATCTAGGTTGGTGGT	$(TC)_{25}$–$(AC)_{15}$	119	55–60
FJ984513	PKAG021	GGTATGATATCCATAATAGACCTACAG	GAAATCTTGCTGACGACAAA	$(AC)_{17}$	145	55–60
FJ984515	PKATG022	GGCAACAATAAATGCAATCAAC	CCTAAATGGAATAGGGTTTTAATTGT	$(AG)_{15}$	198	?
FJ984516	PKAG023	CGTTGTTCGATGGCTCTGA	CAGGCATTTTGTGTTGGAGAG	$(AG)_{12}$	183	55
FJ984517	PKAG024	GCCGGATTTAGTCCTGACAT	GAGTAAGGTTAGGTCGCGTTC	$(TC)_{22}$	223	55
FJ984500	PKATG025	GCGATAGCGGACTGACTTACA	GGTCCTGACAATCACACCAAC	$(AG)_{31}$	226	?
FJ984502	PKAG026	GGGGTTGTGGAGTGCTTGTA	CAGAAGAGCCGTCGCATAA	$(TC)_7$ & $(AC)_7$	193	55–60
FJ984514	PKATG027	CACTCACCAACGTTACTCACTAAGA	TGATGATGATGATGAGGACACTC	$(TCA)_{11}$–NN–$(TCA)_5$	106	60

[a] The length was determined from the sequencing results for the isolated *P. khinjuk* clones.

4. Discussion

The library enrichment procedure adopted here was successful in both developed libraries, since the final proportion of microsatellite-containing clones appears to be high (82%) which corresponds to the results obtained by others in developing SSR markers in plants such as tall fescue (Saha et al., 2006), potato (Ashkenazi et al., 2001), apple (Gianfranceschi et al., 1998), pistachio (Ahmad et al., 2003), peach (Dirlewanger et al., 2002) and pine (Echt et al., 1996). The results indicated that PCR-colony screening method has high efficiency to identify SSR-containing clones before sequencing (Wang et al., 2007). It seems that combination of enrichment method with subsequent colony-PCR screening is a better choice to obtain reasonable amount of SSR markers.

Nonspecific repeats from ATG library were more frequent than AG, the event which was observed in trinucleotide SSR libraries of other plants such as hazelnut (Bassil et al., 2005) and avocado (Ashworth et al., 2004). Probably short length of microsatellites in trinucleotide motifs leads to weaker target hybridization of probes

Table 3
Summary statistics of genetic diversity across all *Pistacia* accessions and *P. vera* genotypes only, based on 20 microsatellite loci developed from *P. Khinjuk*.

Marker	Indices of all accessions					Indices of *P. vera* genotypes only				
	Allele No.	H_e[a]	H_o[b]	PIC[c]	PD[d]	Allele No.	H_e	H_o	PIC	PD
PKAG001	15	0.80	1.00	0.78	0.66	5	0.63	1.00	0.56	0.38
PKAG002	4	0.62	0.83	0.54	0.38	2	0.49	0.92	0.37	0.14
PKAG003	8	0.66	1.00	0.61	0.33	2	0.50	1.00	0.37	0.00
PKAG004	3	0.53	1.00	0.42	0.13	2	0.50	1.00	0.37	0.00
PKAG005	7	0.74	1.00	0.70	0.67	3	0.62	1.00	0.55	0.47
PKAG006	7	0.66	1.00	0.60	0.46	4	0.57	1.00	0.48	0.14
PKAG007	4	0.42	0.28	0.39	0.45	1	0.00	0.00	0.00	0.00
PKAG008	4	0.61	1.00	0.53	0.43	2	0.50	1.00	0.37	0.00
PKAG009	5	0.72	0.89	0.67	0.68	3	0.62	1.00	0.55	0.50
PKATG011	8	0.46	0.28	0.45	0.54	2	0.07	0.08	0.07	0.14
PKATG012a	5	0.71	0.65	0.67	0.84	3	0.60	0.50	0.52	0.74
PKATG012b	6	0.64	0.89	0.59	0.60	3	0.62	0.92	0.54	0.38
PKATG013	3	0.55	1.00	0.45	0.20	3	0.54	1.00	0.43	0.14
PKATG014	2	0.28	0.20	0.24	0.42	1	0	0.00	0.00	0.00
PKATG015	2	0.50	0.24	0.37	0.65	2	0.44	0.33	0.35	0.61
PKATG016	3	0.42	0.06	0.35	0.43	1	0	0.00	0.00	0.00
PKATG018	4	0.68	0.78	0.61	0.43	2	0.5	1.00	0.37	0.00
PKAG021a	10	0.83	0.54	0.82	0.84	6	0.75	0.50	0.72	0.76
PKAG021b	8	0.83	0.29	0.81	0.88	5	0.77	0.27	0.73	0.83
PKATG027	6	0.55	0.44	0.52	0.71	4	0.28	0.31	0.26	0.49
Mean	5.7	0.61	0.67	0.56	0.54	2.8	0.45	0.64	0.38	0.29

[a] Expected heterozygosity.
[b] Observed heterozygosity.
[c] Polymorphism information content (PIC).
[d] Power of discrimination (PD).

at enrichment steps comparing to dinucleotide repeats (Katti et al., 2001).

The results of current study demonstrated a relatively high transferability of microsatellite markers from *P. khinjuk* to five different species belonging to *Pistacia* sp. Comparing to other investigations (Peakall et al., 1998), The high level cross-transferability of these SSR markers represents that SSRs derived from *P. khinjuk* and their flanking regions are conserved in studied species due to its low rate of mutation and recent emerging of species in the genus (Parfitt and Badenes, 1997). Hence, these microsatellites may efficiently and reliably be used as genetic markers to determine genetic relatedness and taxonomic proximity of the genotypes of three pistachio species (*P. khinjuk*, *P. vera* and *P. atlantica* subsp. *mutica*) commonly found throughout Iran.

Amplification of more than two bands in *Pistacia* sp. by Markers PKATG012 and PKAG021 was in accordance with a nearly similar banding patterns as it was previously observed in pistachio (Ahmad et al., 2003), apricot (Hormaza, 2002) and peach (Aranzana et al., 2002). Since pistachio is a diploid plant (Ila et al., 2003) and there is no possibility of tetraploidy, hence amplification of more than two loci probably implies the duplication of some loci in the whole genome (Sanchez-Perez et al., 2005).

The high percentage of polymorphic loci (90%), average number of alleles per locus ($A = 5.7$), expected heterozygosity ($H_e = 0.61$) and polymorphism information content (PIC = 0.56) confirm that SSR markers developed from *P. khinjuk* are useful tools for genetic studies in five pistachio species. The PIC values of 20 loci among the genus ranged between 0.24 and 0.82, of which 13 loci with PIC > 0.5 could be classified as very informative markers. The mean PIC value for dinucleotide microsatellites (0.61) was higher than for trinucleotides (0.46) in genotype analysis of pistachios, supporting the hypothesis for higher levels of polymorphism for di-nucleotide repeats than trinucleotide repeats in pistachios (Ahmad et al., 2003). Although increasing the level of polymorphism with raising SSR array length was reported (Chambers and MacAvoy, 2000), in agreement with Ahmad et al. (2003), in the present study no correlation was found between the number of microsatellite repeats and their polymorphism or informativeness.

Detecting low level of polymorphism among Iranian cultivated pistachios may suggest the existence of a limited genetic diversity among Iranian genotypes of *P. vera* as described previously (Ahmad et al., 2003). These results are inconsistent with recent conclusions addressing broad genetic background of Iranian pistachio cultivars (Salehi Shanjani et al., 2009; Pazouki et al., 2010). However, high rate of allelic diversity of SSRs developed from *P. khinjuk* in current study in comparison to those used by Pazouki et al. (2010) together with the findings reported previously (Ahmad et al., 2003; Mirzaei et al., 2006) supports low variation in genetic background of Iranian commercial pistachio germplasm. This narrow genetic base could be indicative of probable co-ancestory of Iranian cultivars (Ahmad et al., 2003; Mirzaei et al., 2006), the clonally propagation of genotypes, the long juvenility and long life-span of pistachio trees (Maggs, 1973), high selection pressure for commercially important traits such as nut size or productivity (Ahmad et al., 2003).

Adjacent grouping of *P. vera* to *P. khinjuk* was consistent with the results obtained from morphological traits analysis (Zohary, 1952) and molecular clustering of *Pistacia* sp. (Parfitt and Badenes, 1997; Kafkas and Perl-Treves, 2001; Katsiotis et al., 2003; Kafkas, 2006; Salehi Shanjani et al., 2009; Pazouki et al., 2010). The closer intimacy of *P. khinjuk* to *P. vera* rather than to other species in this study supports Zohary's hypothesis that these most primitive species belong to Eu-Butmella and *P. khinjuk* to be directly descended from *P. vera* as an intermediate species between *P. vera* and other *Pistacia* species (Zohary, 1952).

In resulted dendrogram, *P. atlantica* and *P. mutica* were the closest species with bootstrap value of 98%, and therefore *P. mutica* should be considered as a subspecies of *P. atlantica*. Previous morphological and molecular studies also retained *P. mutica* as the subspecies of *P. atlantica* (Zohary, 1952; Kafkas, 2006) within the group of Eu-Butmella (Zohary, 1952). Although the findings based on RFLP, RAPD and AFLP analysis, placed *P. vera*, *P. khinjuk*, and *P. atlantica* in the same group (Parfitt and Badenes, 1997; Kafkas and Perl-Treves, 2001, 2002; Kafkas, 2006), the estimation of genetic distances between species in current study provided another molecular proof that there is a modest genetic affinity between *P. atlantica* and Eu-Terebinthus group. Within the latter major cluster, *P. palaestina* and *P. integerrima* were clustered together and split up from *P. atlantica* and *P. mutica* which were organized as a separate subgroup. Recently Salehi Shanjani et al. (2009) noted a close relationship between foreign pistachio cultivars and *P. atlantica* var. *kurdica*, a relationship not acknowledged by others. Although an unexpected variation among the genotypes could be of interest to future research programs, this assumption is unlikely to be justified, since it is obvious from previous reports that all commercial pistachios belong to *P. vera* (Zohary, 1952; Parfitt and Badenes, 1997; Golan-Goldhirsh et al., 2004; Kafkas, 2006) which is adjacent to *P. khinjuk*, and there is no indication to support a closer relationship between non-Iranian pistachios and *P. atlantica* than *P. vera*.

The *P. vera* genotypes, with low intraspecific genetic variability among the accessions, formed a distinct cluster from the rest of species. Clustering of wild *P. vera* var. Sarakhs along with cultivated pistachios in this group indicated that they are sharing certain common alleles. This finding together with low divergence among wild and cultivated *P. vera* lead to presume that the cultivated *P. vera* accessions share a common ancestor and probably the wild pistachios such as Sarakhs and Ghazvini were the progenitor of primitive Iranian pistachio cultivars. This conclusion is similar to those suggested that pistachio cultivars are recently evolved and diverged from their wild relatives (Maggs, 1973; Sheibani, 1996; Salehi Shanjani et al., 2009; Pazouki et al., 2010). Later, it was shown that *P. vera var.* Sarakhs is a highly diverse genotype (Salimi et al., unpublished data) and therefore, such genotype might be a useful source of genetic material for a breeding program of pistachios in the future. Better understanding of this relationship in natural habitats of wild pistachios is a prerequisite to their use as a very rich gene pool for introduction of new important genes to cultivated pistachios. This can help in the choice of genetic sources to enlarge the diversity of pistachio cultivars and should decrease the risk of pistachio narrow genetic base.

In conclusion, the 18 polymorphic SSR markers developed from *P. khinjuk* in this study were transferable to local *Pistacia* species and can be used for inter-specific and intra-specific genetic studies across related species. Their reproducibility will allow the exchange of information across laboratories and eventually result in a framework SSR-based linkage map for *Pistacia* species.

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