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ORIGINAL ARTICLE

Genetic relationships among *Pistacia* species studied by SAMPL markers

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Abstract Pistachio is economically important in Iran. Selection of suitable rootstocks, resistant to unfavorable and soil conditions and diseases, is important for increasing yield and the acreage of this crop. It is essential to identify the genetic relationships among *Pistacia* species for the breeding of pistachio rootstocks. The goal of this study was to determine the genetic relationship among *Pistacia* species (*P. vera* L., *P. khinjuk* Stocks., *P. eurycarpa* Yalt., *P. atlantica* subsp. *atlantica* Zoh., *P. atlantica* subsp. *mutica* Fish et C.A. Mey and *P. atlantica* subsp. *cabulica* Stocks.) with potential in the breeding of rootstocks using the selective amplification of microsatellite polymorphic loci (SAMPL) technique. Six primer combinations produced a total of 182 bands, with an average of 30.33 bands per primer pair, of which 128 were polymorphic. Three branches were obtained, the first containing *P. vera*, and the second containing *P. khinjuk*, *P. eurycarpa*, *P. atlantica* and subspecies *mutica* and *cabulica*, with numerous leaflets clustered in the third branch. UPGMA analysis separated *P. atlantica* subspecies from *P. eurycarpa*.

Keywords *Pistacia* · *P. khinjuk* · *P. eurycarpa* · *P. atlantica* · SAMPL · UPGMA

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Introduction

The genus *Pistacia* includes 13 or more species, and of these *Pistacia vera* L. has commercially important edible nuts. The other species grow in the wild and their seedlings are used mainly as rootstocks for pistachio (Kafkas and Perl-Treves 2002). There are two main centers of diversity for *Pistacia*: one comprises the Mediterranean region of Europe, Northern Africa and Middle East countries. Other is the Eastern part of Zagros Mountains (Iran) and Caucasus regions from Crimea to the Caspian Sea (Zohary 1952). Iran is a center of origin for four important *Pistacia* species, *P. vera* L., *P. khinjuk* Stocks. *P. eurycarpa* Yalt. (*P. atlantica* subsp. *kurdica* Zoh.) and *P. atlantica* Dsef. (Karimi et al. 2009b).

Pistachio is usually propagated by budding or grafting selected scions onto seedling rootstocks of the same species or other *Pistacia* species. Different rootstocks are used in the different growing areas; thus, *P. atlantica*, *P. integerrima* (Stewart) Zoh. and their hybrids are the main rootstocks in California. *Pistacia vera* seedlings are main rootstocks used in Iran and Turkey. Resistance to both aerial (salinity and drought) and soil fungal diseases (*Phytophthora*, *Verticillium* and nematodes) are interesting traits in the breeding of pistachio rootstocks. *Pistacia mutica* and *P. khinjuk* are the rootstocks most resistant to root-knot nematode and drought, respectively. *Pistacia* species have a high genetic diversity due to their dioecious character, pollination mechanism and high heterozygosity. Because of these factors high selectivity in rootstocks breeding is required, and therefore knowledge of the genetic relationships among *Pistacia* species would be very useful in pistachio rootstock breeding. Different markers have been used for studying *Pistacia* species and pistachio cultivars (Karimi et al. 2009a; Kafkas 2006; Ahmad et al. 2003;

Katsiotis et al. 2003; Parfitt and Badenes 1997), but no study has been reported in which the SAMPL (selective amplification of microsatellite polymorphic loci) marker technique was used. SAMPL is a modified amplified fragment length polymorphism (AFLP) technique in which a compound microsatellite sequence is used as one of the two AFLP primers in selective amplification (Witssenboer et al. 1997), generally in place of *EcoRI* primers.

The objective of this study was to assess the genetic relationships among *Pistacia* species using the SAMPL marker technique.

Materials and methods

We used 28 wild and cultivated pistachios accessions in this study. The accessions belonged to *P. vera*, *P. khinjuk*, *P. eurycarpa*, *P. atlantica* subsp. *atlantica*, *P. atlantica* subsp. *mutica* and *P. atlantica* subsp. *cabulica*. Samples were originally from Iran, Turkey, US and Syria (Table 1). Leaf samples were used for DNA extraction, and genomic DNA was extracted using the CTAB method of Doyle and Doyle (1987) with minor modifications (Kafkas and Perl-Treves 2002). Leaf samples (1 g) were ground in liquid nitrogen and mixed with 6 ml of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 2% polyvinylpyrrolidone, 0.2% β-mercaptoethanol, 0.1% $Na_2S_2O_5$). The samples were then incubated at 65°C for 1 h, followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was recovered and mixed with an equal volume of cold isopropanol and left at −20°C for 24 h. The precipitated nucleic acids were recovered by centrifugation at 1,000 rpm for 2 min, washed with ammonium acetate (10 mM) in 76% ethanol, dried and resuspended in double-distilled water. The DNA concentration was estimated by comparison with known DNA concentrations, after 0.8% agarose gel electrophoresis and ethidium bromide staining, and the concentration was adjusted to 50 ng/μl for the SAMPL procedure.

For the SAMPL reaction, 50 ng of genomic DNA was used. Restriction and ligation were performed using 5 U of *EcoRI* and 1 U of *MseI* enzymes, 5 pmol of each *EcoRI* and *MseI* adaptor, 1 U of T4 DNA ligase, 1× ligase buffer, 1.1 μl of 0.5 M NaCl and 0.55 μl of bovine serum albumin (1 ng/μl), in a final volume of 11 μl. The preselective amplification reaction mixture contained 4 μl restricted-ligated DNA as PCR template, 5 pmol of each preselective amplification primer ($EcoRI_{+}$A and $MseI_{+}$C), 3 mM dNTPs, 2 μl PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.4 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase. The thermocycle conditions were as described by Vos et al. (1995) with minor modifications (Kafkas 2006). The adaptor sequences, preselective amplification primers and selective primers are listed in Table 2.

Table 1 *Pistacia* species used in the phylogenetic analysis by the SAMPL marker method, and their countries of origin

No	Species	Accession	Country of origin
1	*P. vera* 'Uzun'	PV-1	Turkey
2	*P. vera* 'Kirmizi'	PV-2	Turkey
3	*P. vera* 'Siirt'	PV-3	Turkey
4	*P. vera* 'Ghazvini'	PV-4	Iran
5	*P. vera* 'Ghazvini'	PV-5	Iran
6	*P. vera* 'Badami'	PV-6	Iran
7	*P. vera* 'Badami'	PV-7	Iran
8	*P. vera* 'Badami'	PV-8	Iran
9	*P. vera* 'Sarakhs'	PV-9	Iran
10	*P. vera* 'Sarakhs'	PV-10	Turkey
11	*P. vera* 'Sarakhs'	PV-11	Turkey
12	*P. khinjuk*	PK-1	Iran
13	*P. khinjuk*	PK-2	Iran
14	*P. khinjuk*	PK-3	US
15	*P. khinjuk*	PK-4	US
16	*P. eurycarpa*	PE-1	Turkey
17	*P. eurycarpa*	PE-2	Turkey
18	*P. atlantica* subsp. *atlantica*	PAA-1	Syria
19	*P. atlantica* subsp. *atlantica*	PAA-2	Syria
20	*P. atlantica* subsp. *atlantica*	PAA-3	Turkey
21	*P. atlantica* subsp. *atlantica*	PAA-4	Turkey
22	*P. atlantica* subsp. *mutica*	PAM-1	Iran
23	*P. atlantica* subsp. *mutica*	PAM-2	Iran
24	*P. atlantica* subsp. *mutica*	PAM-3	Iran
25	*P. atlantica* subsp. *mutica*	PAM-4	Turkey
26	*P. atlantica* subsp. *mutica*	PAM-5	Turkey
27	*P. atlantica* subsp. *cabulica*	PAC-1	Iran
28	*P. atlantica* subsp. *cabulica*	PAC-2	Iran

The selective amplification reaction was conducted in a final volume of 20 μl containing 4 μl of diluted preselective amplification products as template, 5 pmol each of labeled *EcoRI* site primer (SAMPL-6 or SAMPL-7) and *MseI* primer, 3 mM dNTPs, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$ and 1 U *Taq* DNA polymerase. The SAMPL program included one cycle of 2 min at 94°C, followed by ten cycles of denaturation at 94°C for 45 s, gradient annealing at 65°C (−1°C per cycle) for 45 s, and extension at 72°C for 2 min. An additional 20 cycles of denaturation, annealing and extension at 94°C for 45 s, 56°C for 45 s and 72°C for 2 min were applied, followed by a final incubation for 30 s at 60°C. The PCR products were separated on an ABI Prism 3130 Genetic Analyzer. The polymorphic bands were scored as present (1) or absent (0). A Jaccard's similarity

Table 2 Sequences of the oligonucleotide adaptors and primers used in phylogenetic analysis of 28 *Pistacia* accessions by the SAMPL marker method

Adaptor/primer	Code	Sequence
Adaptors		
EcoRI adaptor		5′-CTC GTA GAC TGC GTA CC-3′
		3′-CAT CTG ACG CAT GGT TAA-5′
MseI adaptor		5′-GAC GAT GAG TCC TGA G-3′
		3′-TAC TGA GGA CTC AT-5′
Pre selective amplification primers		
EcoRI primer + A		5′-GAC TGC GTA CCA ATT C + A-3′
MseI primer + C		5′-GAT GAG TCC TGA GTA A + C-3′
Selective amplification primers		
MseI + 3-CAG	M_{CAC}	5′-GAT GAG TCC TGA GTA A + CAG-3′
MseI + 3-CAC	M_{CAT}	5′-GAT GAG TCC TGA GTA A + CAC-3′
MseI + 3-CAA	M_{CCA}	5′-GAT GAG TCC TGA GTA A + CAA-3′
MseI + 3-CCC	M_{CCC}	5′-GAT GAG TCC TGA GTA A + CCC-3′
SAMPL-6		5′-ACACACACACACACATATAA-3′
SAMPL-7		5′-TGTGTGTGTGTGTGTATAT-3′

Table 3 Banding pattern information in DNA fingerprinting of 28 *Pistacia* accessions with SAMPL markers

Primer combination	No. of bands	No. of polymorphic bands	Percent polymorphism
SAMPL-6/M_{CCA}	32	22	62.50
SAMPL-6/M_{CAG}	23	13	56.52
SAMPL-6/M_{CAC}	20	15	75.00
SAMPL-7/M_{CCA}	48	37	75.00
SAMPL-7/M_{CCC}	37	24	64.86
SAMPL-7/M_{CAG}	22	17	77.27
Total	182	128	–
Mean	30.33	21.33	68.52

matrix was calculated using numerical taxonomy and the multivariate analysis system NTSYSpc ver 2.11 (Rohlf 2004) and the dendrogram produced using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm.

Results

SAMPL polymorphism and discrimination capacity

Six SAMPL primer combinations were used in the molecular characterization of 28 pistachio accessions originating in different countries. These combination primers generated in total 182 scorable bands, with an average of 30.33 bands per primer pair, of which 128 were polymorphic. The number of total bands varied from 20 (SAMPL-6/M_{CAC}) to 48 (SAMPL7/M_{CCA}). The maximum number of polymorphic bands was 37 for primer combination SAMPL-7/M_{CCA} and the lowest number was 13 for SAMPL-6/M_{CAG} (Table 3).

Genetic relationships among *Pistacia* accessions

According to Jacquard's similarity matrix (Table 4) and UPGMA clustering, accessions at a similarity distance of 0.55 were separated into three main branches (Fig. 1). The first branch included *P. vera* that comprised two groups: the first group comprised pistachio accessions originating in Turkey, and the second group comprised accessions originating in Iran. In the Turkish group, the 'Siirt' cultivar was separated by the greatest distance from the others. In the Iranian group, the 'Badami' cultivar was more similar to the 'Ghazvini' cultivar, whereas the 'Sarakhs' variety was separated by the greatest distance from the others.The second branch included *P. khinjuk* accessions from Iran and the US. In this cluster Iranian samples were separated from US samples. *Pistacia eurycarpa* and all of the subspecies of *P. atlantica* (*atlantica*, *mutica* and *cabulica*) were located in the third branch. This branch included three groups: The first group comprised *P. eurycarpa* accessions from Turkey; the second group comprised *P. atlantica* subsp. *atlantica* from Turkey and Syria plus *P. atlantica*

Table 4 Genetic similarity among studied *Pistacia* accessions based on Jaccard's coefficient

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	PV-1	–																											
2	PV-2	0.83	–																										
3	PV-3	0.77	0.79	–																									
4	PV-4	0.62	0.57	0.56	–																								
5	PV-5	0.60	0.56	0.59	0.88	–																							
6	PV-6	0.60	0.59	0.65	0.61	0.65	–																						
7	PV-7	0.62	0.62	0.65	0.59	0.59	0.70	–																					
8	PV-8	0.57	0.57	0.60	0.63	0.61	0.71	0.82	–																				
9	PV-9	0.58	0.56	0.53	0.52	0.54	0.57	0.59	0.54	–																			
10	PV-10	0.53	0.50	0.54	0.51	0.52	0.53	0.57	0.52	0.61	–																		
11	PV-11	0.59	0.51	0.54	0.51	0.52	0.56	0.56	0.56	0.68	0.65	–																	
12	PK-1	0.50	0.48	0.44	0.48	0.44	0.41	0.48	0.47	0.48	0.44	0.49	–																
13	PK-2	0.52	0.48	0.49	0.48	0.43	0.42	0.48	0.46	0.48	0.43	0.47	0.79	–															
14	PK-3	0.49	0.47	0.48	0.54	0.48	0.47	0.46	0.48	0.46	0.46	0.47	0.63	0.75	–														
15	PK-4	0.45	0.45	0.44	0.52	0.48	0.46	0.46	0.48	0.47	0.45	0.46	0.66	0.66	0.75	–													
16	PE-1	0.48	0.50	0.49	0.44	0.41	0.44	0.50	0.49	0.49	0.47	0.46	0.54	0.54	0.52	0.51	–												
17	PE-2	0.54	0.49	0.49	0.47	0.45	0.36	0.49	0.45	0.47	0.50	0.48	0.55	0.54	0.50	0.50	0.71	–											
18	PAA-1	0.55	0.52	0.51	0.43	0.43	0.44	0.52	0.48	0.50	0.47	0.51	0.51	0.53	0.49	0.50	0.65	0.66	–										
19	PAA-2	0.54	0.53	0.49	0.40	0.41	0.45	0.47	0.45	0.48	0.47	0.52	0.48	0.50	0.46	0.48	0.63	0.64	0.81	–									
20	PAA-3	0.51	0.54	0.51	0.39	0.38	0.41	0.47	0.43	0.48	0.47	0.44	0.50	0.51	0.48	0.46	0.65	0.64	0.75	0.79	–								
21	PAA-4	0.54	0.54	0.54	0.41	0.39	0.42	0.47	0.43	0.47	0.46	0.45	0.51	0.53	0.50	0.45	0.67	0.66	0.73	0.75	0.83	–							
22	PAM-1	0.52	0.50	0.47	0.44	0.42	0.36	0.43	0.39	0.47	0.48	0.43	0.47	0.50	0.48	0.45	0.63	0.68	0.66	0.62	0.68	0.73	–						
23	PAM-2	0.49	0.51	0.50	0.44	0.42	0.34	0.43	0.40	0.44	0.41	0.42	0.47	0.48	0.49	0.46	0.66	0.68	0.65	0.68	0.76	0.74	0.79	–					
24	PAM-3	0.50	0.49	0.47	0.42	0.39	0.38	0.46	0.46	0.49	0.50	0.48	0.48	0.48	0.47	0.46	0.67	0.73	0.63	0.66	0.69	0.71	0.76	0.78	–				
25	PAM-4	0.56	0.55	0.50	0.45	0.44	0.40	0.47	0.42	0.47	0.46	0.47	0.55	0.54	0.50	0.50	0.62	0.65	0.73	0.69	0.75	0.73	0.69	0.72	0.63	–			
26	PAM-5	0.57	0.57	0.55	0.45	0.43	0.42	0.50	0.45	0.50	0.45	0.45	0.52	0.56	0.50	0.50	0.66	0.64	0.75	0.69	0.80	0.80	0.70	0.74	0.69	0.77	–		
27	PAC-1	0.51	0.51	0.49	0.46	0.43	0.40	0.44	0.45	0.45	0.46	0.44	0.46	0.50	0.49	0.50	0.70	0.65	0.64	0.69	0.72	0.74	0.73	0.78	0.76	0.62	0.73	–	
28	PAC-2	0.50	0.50	0.46	0.44	0.41	0.36	0.43	0.42	0.49	0.47	0.46	0.49	0.52	0.48	0.47	0.69	0.66	0.63	0.62	0.68	0.70	0.72	0.72	0.75	0.66	0.70	0.78	–

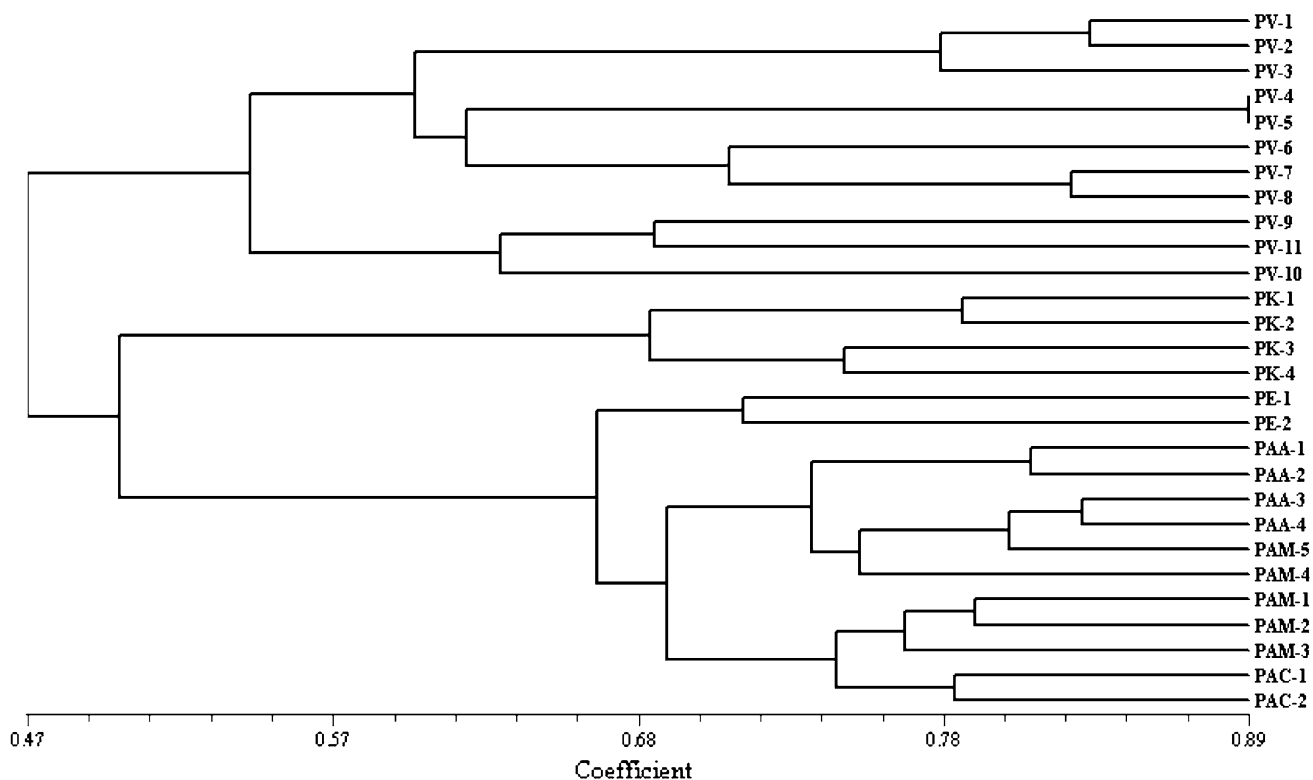

Fig. 1 UPGMA dendrograms of 28 accessions by SAMPL analysis using Jaccard's similarity matrices

subsp. *mutica* from Turkey; and the third group comprised *P. atlantica* subsp. *mutica* and *cabulica* from Iran. *P. khinjuk* was more similar to *P. eurycarpa* than *P. vera*, and *P. eurycarpa* was most similar to *P. atlantica*.

Discussion

Phylogenetic analysis of related plant species can be performed using morphological descriptors and molecular markers. The use of DNA markers is one of the most powerful techniques, because they are not influenced by environmental factors and the development stage of the plant. The SAMPL marker technique is a powerful technique for the analysis of intraspecific diversity in different fruit and nut tree species. One of the main advantages of this marker is a high multiplex ratio as in AFLP, and it has some degree of locus specificity as in simple sequence repeats (SSRs), thus making it a very efficient and effective molecular markers system. In present study the SAMPL marker technique was used to study the genetic relationships among *Pistacia* species. Six combination primers generated a total 182 bands with an average of 30.33 bands per primer pair, of which 128 were polymorphic. In a similar study, Kafkas et al. (2005) found 50.9% polymorphism in the walnut using the SAMPL marker technique, Therefore the SAMPL marker can separate closely related accessions.

In this study *P. khinjuk* was more similar to *P. eurycarpa* than to *P. vera*, also there were similar genetic relationships between *P. vera* and *P. khinjuk* and *P. eurycarpa*. Similar studies (Karimi et al. 2009b, Kafkas et al. 2002) have shown that the closest species to *P. vera* is *P. eurycarpa*, followed by *P. atlantica*. Zohary (1972) found that *P. eurycarpa*, which has larger fruits and ovate and few paired leaflets, closely resembles to *P. vera*. This species is centered mainly in Iran and Afghanistan, overlapping in some places with *P. vera* and *P. khinjuk*. Yaltirik (1967) described and introduced *P. eurycarpa* as a new species.

In contrast to the findings of Karimi et al. (2009b), this study showed that *P. atlantica* and *P. atlantica* subsp. *mutica* were very similar and should be considered the same species. The findings of the present study strongly support those of Al-Yafi (1978) and Kafkas (2006) who postulated that the *mutica* subspecies should be located in *P. atlantica*.

There is a hypothesis that *P. eurycarpa* is a hybrid between *P. vera* and *P. khinjuk*, and *P. atlantica* and *mutica* are derivatives of it (Zohary 1972). According to previous studies and the present study, *P. khinjuk* and *P. eurycarpa* have similar genetic distances to *P. vera*, while *P. eurycarpa* is more similar to *P. atlantica* than to *P. khinjuk*, and it is possible that *P. eurycarpa* is a hybrid between *P. vera* and *P. khinjuk*, and *P. atlantica* and the *mutica* subspecies are derivatives of it.

In conclusion, the SAMPL marker technique was able to easily divide accessions with regard to the species *P. vera*, *P. khinjuk*, *P. eurycarpa* and *P. atlantica*, and the SAMPL marker technique may also be used to reveal the genetic relationships among *Pistacia* species for better crossing of tolerant species for rootstock production. This study showed that *P. atlantica* and *P. atlantica* subsp. *mutica* should be considered same species. According to previous studies (Parfitt and Badenes 1997), *P. vera* and *P. khinjuk* are the most similar, while in this study *P. khinjuk* appeared to be more similar to *P. eurycarpa*. Following this study we can postulate that *P. eurycarpa* may be a hybrid between *P. vera* and *P. khinjuk*, and that *P. atlantica*, *P. atlantica* subsp. *mutica* and *P. atlantica* subsp. *cabulica* are descendents of *P. eurycarpa*. Therefore, the use of more efficient and powerful markers such as SSR and RFLP is recommended for future research.

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