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Molecular characterization and RAPD analysis of *Juniperus* species from Iran

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ABSTRACT. The genus *Juniperus* L. (Cupressaceae), an aromatic evergreen plant, consists of up to 68 species around the world. We classified five species of *Juniperus* found in Iran using molecular markers to provide a means for molecular identification of Iranian species. Plants were collected (three samples of each species) from two different provinces of Iran (Golestan and East Azarbayejan). The DNA was extracted from the leaves using a Qiagen Dneasy Plant Mini Kit. Amplification was performed using 18 ten-mer RAPD primers. Genetic distances were estimated based on 187 RAPD bands to construct a dendrogram by means of unweighted pair group method of arithmetic means. It was found that *J. communis* and *J. oblonga* were differentiated from the other species. Genetic distance values ranged from 0.19 (*J. communis* and *J. oblonga*) to 0.68 (*J. communis* and *J. excelsa*). *Juniperus foetidissima* was found to be most similar to *J. sabina*. *Juniperus excelsa* subspecies *excelsa* and *J. excelsa* subspecies *polycarpos* formed a distinct group.

Key words: *Juniperus*; RAPD markers; DNA amplification; Genetic distance; Iran

INTRODUCTION

The genus *Juniperus* L. (Cupressaceae) consists of up to 68 species around the world. It is an aromatic, drought-resistant plant that is found in mountainous parts of the northern hemisphere (altitude: 500-3000 m) except for some species in Africa (Adams and Pandey, 2003).

The *Juniperus* species of Iran mainly consist of five species with two vegetation forms (tree or shrub): *J. communis* L., *J. oblonga* M.B., *J. sabina* L., *J. foetidissima* Willd., and *J. excelsa* M.B. with two subspecies namely *J. excelsa* M.B. subspecies *excelsa* and *J. excelsa* M.B. subspecies *polycarpos* (K. Koch) Takhtajan. They are widely spread evergreen plants that grow in several provinces of Iran (Khorasan, Golestan, Semnan, Mazandaran, Gilan, Azarbayejan, Ardabil, Fars, Yazd, Kerman, and Hormozgan). In Iran the key character separating *J. communis* from other species is the morphological characterization. It is a shrub, 2.2 m tall, multistemmed, decumbent, crown generally depressed, altitude 2063 m. *J. oblonga* is a shrub to small tree, 1-4 m, decumbent-upright, growing in East Azarbayejan Province, altitude 1500 m. This seems to imply that these species possess similarities, although they grow in distinct regions. *J. sabina* is a shrub, 2-3 m, prostrate, growing in Golestan Province, altitude 2050 m. *J. foetidissima* is a tree, up to 16 m, growing in East Azarbayejan Province, altitude 1400 m. *J. excelsa* subspecies *excelsa* is a monoecious plant with taxonomic problems in Iran. *J. excelsa* subspecies *polycarpos* is a tree, up to 25 m, growing in different parts of Iran, altitude 500-3000 m (Emami et al., 2007). DNA fingerprinting studies were used alone or together with the leaf volatile oils for *Juniperus* classification (Adams, 2000a,b,c; Adams et al., 2006). An isoenzyme analysis was performed on 109 individuals from 11 populations of *J. excelsa* complex collected from Iran (Hojjati et al., 2009). Random amplified polymorphic DNA (RAPD) markers have also been used to assess differences among Juniper and Cedar cultivars (Hsiang and Huang, 2000).

The objective of the present study was to investigate the ability of RAPD markers to cluster 5 species of *Juniperus* from Iran.

MATERIAL AND METHODS

Plant material

Plant materials were obtained from their main local growth area of Golestan and East Azarbayejan Provinces (Table 1). Fresh leaves were preserved at -80°C using an ultra-low freezer until DNA extraction. Voucher specimens were deposited in the Ferdowsi University of Mashhad Herbarium (FUMH).

Table 1. List of location, altitude, collection date, and FUMH voucher number of *Juniperus* samples used in this study.

Species	Location	Altitude (m)	Collection date	Voucher No.
J. communis	Between Damab and Cephali, Golestan Province	2063	Oct. 4, 2002	FUMH37069
J. excelsa subspecies *excelsa*	Kelisa Kharabeh, East Azarbayejan Province	1400-1600	Nov. 30, 2002	FUMH37074
J. excelsa subspecies *polycarpos*	Chopoughlou Darahsi, East Azerbayejan Province	1593	Sept. 21, 2002	FUMH37065
J. oblonga	Between Makidi and Vainagh, East Azerbayejan Province	1500	July 6, 2002	FUMH37071
J. sabina	Sourkesh, Golestan Province	2050	Oct. 3, 2002	FUMH37072
J. foetidissima	Between Makidi and Vainagh, East Azerbayejan Province	1400	Sept. 23, 2002	FUMH37067

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from the leaves using the Qiagen Dneasy Plant Mini Kit (Qiagen, Germany). Extracted DNA was quantified by spectrophotometer (Nanodrop ND-1000, USA), at an absorbance level of 260/280 nm. The quality was further checked on 0.8% agarose gel.

Ten-mer primers were purchased from Microsynth (Switzerland). Sequence information for the primers is presented in Table 2.

Table 2. Detailed information of randomly amplified polymorphic DNA primers used in this assay.

No.	Oligo name	Sequences 5'-3'	Size (nt)	MW (g/mol)	Tm (°C)
1	P116	TACGATGACG	10	3052.2	30
2	P134	AACACACGAG	10	3030.0	30
3	P153	GAGTCACGAG	10	3077.1	32
4	P204	TTCGGGCCGT	10	3035.3	34
5	P212	GCTGCGTGAC	10	3044.2	34
6	P218	CTCAGCCCAG	10	2973.1	34
7	P239	CTGAAGCGGA	10	3077.1	32
8	P249	GCATCTACCG	10	2988.2	32
9	P250	CGACAGTCCC	10	2973.1	34
10	P265	CAGCTGTTCA	10	3003.3	30
11	P327	ATACGGCGTC	10	3028.2	32
12	P338	CTCTGGCGGT	10	3035.3	34
13	P346	TAGGCGAACG	10	3077.1	32
14	P347	TTGCTTGGCG	10	3050.4	32
15	P375	CCGGACACGA	10	3022.0	34
16	P391	GCGAACCTCG	10	3013.1	34
17	P413	GAGGCGGCGA	10	3118.0	36
18	P431	CTGCGGGTCA	10	3044.2	34

Size (nt) = number of nucleotides in a primer; MW = molecular weight in gram per mol; Tm = melting temperature.

PCR was performed in a volume of 15 µL containing 50 mM Tris-HCl, pH 9, 2.0 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTP, 0.36 µM of each primer, 0.3 ng genomic DNA, 15 ng BSA and 0.6 U Taq DNA polymerase (Fermentas, Germany). A control PCR tube containing all components, but no genomic DNA, was performed with each primer to check for contamination. DNA amplification was performed in a Programmable Thermal Cycler (Techne Research Inc., UK). The thermal cycle included: 94°C (1.5 min) for initial strand separation, then 40 cycles of 38°C (2 min), 72°C (2 min), 91°C (1 min). Two additional steps were used: 38°C (2 min) and 72°C (5 min) for final extension (Asili et al., 2010). Amplification products were loaded onto 1% agarose gels (Sigma, Germany). DNA ladder (100 bp; Fermentas, Germany) was loaded on the last lane of each gel.

The presence or absence of each band was scored as 1 or 0, respectively. Bands that were inconsistent in replicate analyses were not scored. Bands that occurred once or did not show fidelity within the two samples of each taxon were eliminated. Binary matrix was used to estimate genetic similarities and distances between pairs, by employing Dice index (Nei and Li, 1979). These similarity coefficients were used to generate a dendrogram by means of the unweighted pair group method of arithmetic means (UPGMA).

RESULTS AND DISCUSSION

One hundred and eighty-seven RAPD bands were detected (Figure 1).

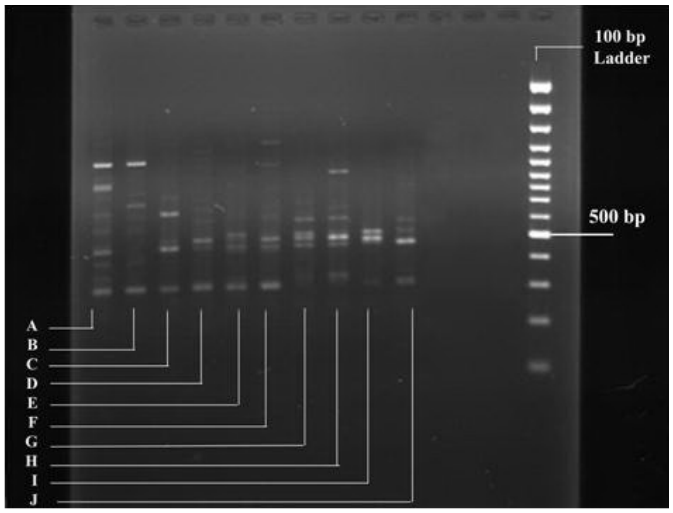

Figure 1. RAPD fingerprints with the use of primer P204. *Lane A = Juniperus communis* (female); *lane B = J. communis* (male); *lane C = J. excelsa* subspecies *polycarpos* (female); *lane D = J. excelsa* subspecies *polycarpos* (male); *lane E = J. sabina* (female); *lane F = J. sabina* (male); *lane G = J. foetidissima* (female); *lane H = J. foetidissima* (male); *lane I = J. oblonga*; *lane J = J. excelsa* subspecies *excelsa*.

The analysis of the RAPD bands were generated by 18 random primers and yielded an average of 10.3 bands/primer. Genetic similarities among five *Juniperus* species using RAPD primers are presented in Table 3.

Table 3. Genetic distance values obtained for 6 Iranian *Juniperus*.

Species	1	2	3	4	5	6
1	*					
2	0.35	*				
3	0.54	0.46	*			
4	0.51	0.57	0.44	*		
5	0.19	0.62	0.57	0.52	*	
6	0.68	0.13	0.38	0.33	0.62	*

1 = *J. communis*; 2 = *J. excelsa* subspecies *polycarpos*; 3 = *J. sabina*; 4 = *J. foetidissima*; 5 = *J. oblonga*; 6 = *J. excelsa* subspecies *excelsa*.

Genetic distance values between five *Juniperus* species ranged from 0.214 (*J. excelsa* subspecies *polycarpos* and *J. excelsa* subspecies *excelsa*) to 0.733 (*J. communis* and *J. excelsa* subspecies *excelsa*). The dendrogram constructed is presented in Figure 2.

This analysis shows two major groups. The first group contains *J. communis* and *J. oblonga*. The second large group includes *J. excelsa* subspecies *excelsa*, *J. excelsa* subspecies *polycarpos*, *J. sabina*, and *J. foetidissima*, that separate into two subgroups.

This study has compared all species of *Juniperus* in Iran. Species that are separated by even minute morphological character differences (*J. excelsa* subspecies *excelsa*, *J. foetidissima* and *J. excelsa* subspecies *polycarpos*) were found to possess considerable DNA differences.

J. sabina is native to the mountains of central and southern Europe and western and central Asia, from eastern Spain to eastern Siberia, typically growing at altitudes of 1000-3300 m. It is very

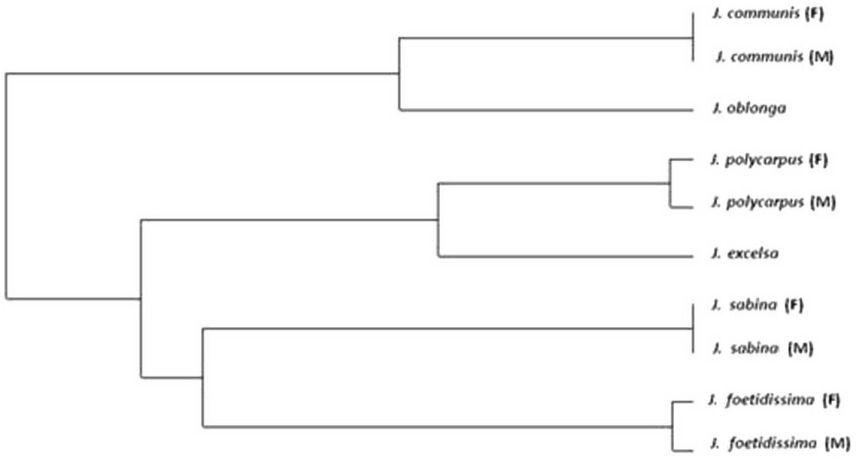

Figure 2. UPGMA dendrogram generated based on the cluster analysis of 187 RAPD bands of five species of *Juniperus*.

widely distributed from Spain through Europe to Siberia. It is generally a small shrub, less than 1 m in height and ranging from 1-2 m in width. However, in the Sierra Nevada of Spain, it forms a prostrate shrub on rocky areas and in Mongolia it occurs as a prostrate plant on sand dunes (Adams and Schwarzbach, 2006). Iranian *J. sabina* is a shrub, up to 2-3 m tall, prostrate, and occurs at an altitude of 2050 m. DNA fingerprinting revealed that *J. sabina* from the Eastern hemisphere is a multiseeded *Juniperus*, clusters weakly with *J. semiglobosa-talassica* and *J. jarkendensis*, and is distinct from *J. excelsa*, *J. excelsa* var. *polycarpos* and *J. foetidissima* (Adams, 1999). There was support (67%) that *J. sabina* from the Mongolian sand dunes is a distinct taxon (Adams and Schwarzbach, 2006). In this study, DNA fingerprinting of Iranian Junipers revealed that *J. sabina* clusters weakly with *J. foetidissima* (Figure 2). Researchers have suggested that *J. sabina* acts as a nurse plant for *J. communis* (Verdu et al., 2004). *J. foetidissima* is a medium-sized tree reaching 6-25 m in height, with a trunk up to 2.5 m in diameter and two forms of leaves. *J. excelsa* subspecies *excelsa* is a large tree reaching 6-20 m in height. It is largely monoecious, often occurs together with *J. foetidissima*. *J. excelsa* subspecies *excelsa* (Greek Juniper) is a Juniper found throughout the Eastern Mediterranean, from Greece across Turkey to Syria and the Caucasus Mountains. *J. excelsa* subsp *polycarpos*, known as the Persian Juniper, occurs in the Alborz and other mountains of Iran and to the east to northwestern Pakistan. Some botanists treat *J. polycarpos* syn. *J. macropoda* as a distinct species (Verdu et al., 2004). Iranian *J. excelsa* subspecies *excelsa* and *J. foetidissima* have the same characters as these species in other countries, but the Iranian *J. excelsa* subspecies *excelsa* is a monoecious plant. *J. excelsa* subspecies *polycarpos* of Iran is very similar to the *J. excelsa* subspecies *excelsa* in terms of vegetation characters, such as shape, type of leaves, seeds and height, but it is largely some older dioecious data sets that support the recognition of the *J. excelsa* subspecies *polycarpos* as a subspecies of *J. excelsa*. In the Balochistan Province, the Pakistan Juniper initially was called *J. excelsa*, but later it was suggested that it should be referred to as *J. turcomanica* var. *seravschanica* not *J. excelsa* or *J. macropoda*. Adams showed that clustering based on 126 RAPDs revealed three groups: *J. excelsa*, *J. procera* and the *J. polycarpos-seravschanica-turcomanica* complex. These three groups were clustered at about the same level. This supports the concept of three species: *J. excelsa*, *J. polycarpos* and *J. procera*. This was in contrast to his previous article. The RAPD analysis revealed that *J. foetidissima* and *J. excelsa* are distinct. *J. foetidissima* is clus-

tered with *J. sabina*. *J. excelsa* closely clustered with *J. polycarpos* (Adams, 1999).

J. communis and its varieties have been studied. One of them was *J. communis* var. *oblonga* collected from Lake Sevan, Armenia. Based on 191 RAPD bands, they found that there was little evidence to support the recognition of *J. communis* var. *oblonga* (Adams and Pandey, 2003).

Adams (1999) explained that the most surprising aspect of his research has been the divergences of morphologically near-identical taxa in their terpenoids and DNA fingerprints. He showed that even species separated by minute morphological character differences possess considerable terpenoid and DNA differences, which suggest that evolution proceeds at different rates for different character sets. The use of multiple character sets seems prudent in *Juniperus* taxonomy and evolutionary studies (Adams, 1999).

It seems that this study is the first complete research on Iranian *Juniperus* species DNA fingerprinting. The authors believe that further studies need to be based on geographic variation of *Juniperus* in Iran.

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