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Genetic relationship among *Juniperus spp*. in Taif, Saudi Arabia, based on, molecular marker (RAPD), SDS-PAGE profile and seed morphological taxonomy

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Juniperus L. (*Cupressaceae*), an aromatic evergreen plant, comprises of up to 67 species spread around the world. Here, two species naturally distributed in Saudi Arabia were investigated genetically for identification using molecular markers. Also, SDS-protein profile and exomorphic seed characters by LM, SEM and AFM were examined. Three samples from each species were gathered from different areas of Taif governorate especially from the center of the western slopes of the mountains. Genetic distances were assessed based on total 527 RAPD bands to build a dendrogram tree by means of unweighted pair group method of arithmetic means and clustered in two main groups. It was found that in every group 90.55- 97.98 % similarities and between the two main groups average 62.92 % similarity. Genetic distance values ranged from average 0.93 within a group to 0.63 between main groups *Juniperus procera* and *Juniperus phoenicea* along these results on the protein level all distinguishable bands were considered as species-specific bands and could be used as biochemical marker for each species, besides exomorphic seed characters of the studied *Juniperus spp*., depending on LM, SEM and AFM results seeds weight were none significantly among the examined characters, but general shape varies in *Juniperus* spp., spear in *J.procera* and elliptic in *J. phoenicea*., number of seeds in the fruit, seed color, seed surface, seed hilum, seed shape and seed size average varies significantly among the examined taxa. It looks that this research is the first complete morphologically and molecular studied on Saudi Arabian *Juniperus* species.

Keywords: *Juniperus*; RAPD markers; molecular identification; Genetic distance; SEM.

INTRODUCTION

The genus Juniperus L is the second most diverse genus of coniferous species in the world, and it belongs to the *Cupressaceae* family. This genus contains 67 species and 34 varieties. All species are confined to the Northern Hemisphere except the J. procera Hochst. ex Endl species, which is also found along the rift mountains of eastern Africa to the Southern Hemisphere (Adams 2008). This genus is separated into three groups: *Juniperus* (10 species), *Caryocedrus Endlicher* (1 species) and *Sabina* (Miller) Spach (56 species) (Adams 2008).

This type of plant is often found in altitudes communities between 2000 and 3000 m. The importance of these woodland ecosystems is that they are an important source of biological diversity, protection from corrosion and water storage. Furthermore, it is well-known that this type of tree is considered as an important source of durable timber in some countries (Negash 1995). The western mountains area of Saudi Arabia have a high plateau (almost 3,000 meters high) and the steep slopes of these mountains deliver an environment suitable for the existence of many rich and biologically diverse plants.

However, *J. procera* trees have shown significant deterioration in this area over the last decade. On the other hand, *J. phoenicea* is characterized as an evergreen monoecious or dioecious tree. It is found native in some areas of the Mediterranean basin from Portugal to Lebanon, Jordan and western Saudi Arabia. Also it found in North Africa countries as Algeria, Morocco and Egypt, as well as the Canary Islands (Adams et al., 2002; El-Bana et al., 2010).

In Saudi Arabia, this species extends from the Mediterranean region southwards to as far south as Taif (Chaudhary, 1999)*.* There are only two in the province of Taif (*Juniperus procera* and *Juniperus phoniceae*). *Juniperus phoniceae* is dominant type on the higher altitudes of 1700 m above sea level is the in the northern part of the mountains Sarawat and *Juniperus procera* in the southern part, while at an altitude of 1700-3000 m above sea level, the two types of juniper exist together.

The purpose of this research is to examine the relationships between local *Juniperus spp*. based on morphology, Taxonomy, SDS-PAGE of seeds beside DNA markers using random amplified polymorphic DNA (RAPD) for the characterization and establishing the diversity and relationships among different Juniper species in Taif, Saudi Arabia.

MATERIALS AND METHODS

Plant material: Plants samples were collected from their natural growth area at different localities in Al-Hada and Al-Shafa at Taif province which is located in the center of the western slopes of the mountains in the Kingdom of Saudi Arabia at an altitude of almost 2,000 meters above sea level (21 ° 16 N - 40 ° 25 east). Young leaves (first completely prolonged leaves) were sampled, three plants at each location.

Seeds collection: Mature seeds were collected from both cultivars from the natural habitat of Taif. The external macro-morphological aspects of the seeds including seed size, general shape, seed surface, color, and hilium shape were investigated with the aid of light microscope and scanning electron microscope (SEM). Also, seeds size average were calculated by Atomic Force Microscope device.

DNA extraction: total genomic DNA was extracted from the young leaves using the modified CTAB method of Murray and Thompson (1980). The extracted DNAs were adjusted to a final concentration of 50 ng/µL and stored at -20°C until use. The quality of the DNA was investigated on 1% agarose gel.

Table 1. Detailed information of randomly amplified polymorphic DNA primers used in this assay.

No.	Oligo name	Sequences 5'-3'	Tm (°C)
1	P116	TACGATGACG	30
2	P134	AACACACGAG	30
3	P153	GAGTCACGAG	32
4	P204	TTCGGGCCGT	34
5	P212	GCTGCGTGAC	34
6	P218	CTCAGCCCAG	34
7	P239	CTGAAGCGGA	32
8	P249	GCATCTACCG	32
9	P250	CGACAGTCCC	34
10	P265	CAGCTGTTCA	30
11	P327	ATACGGCGTC	32
12	P338	CTCTGGCGGT	34
13	P346	TAGGCGAACG	32
14	P347	TTGCTTGGCG	32
15	P375	CCGGACACGA	34
16	P391	GCGAACCTCG	34

RAPD DNA fingerprinting:

Sixteen 10 mers RAPD-PCR primers belonging to the group of Oligo (P116, P134, P153, P204, P212, P218, P239, P249, P250, P265, P327, P338, P346, P347, P375, P391.) were used for determining the DNA fingerprinting of the plant samples of collected *Juniperus* plant samples. Reaction solutions and PCR program amplification of RAPD were performed using the protocol described by Jambhale *et al*. (2007). PCRs were performed in a 25 µL reaction volume in the presence of 50-100 ng/µL genomic DNA as a template. Cycling conditions was 94°C for 4 minutes (1 cycle); 34X each of denaturation at 94ºC for 45 sec, an annealing temperature of 36 ºC for 45 sec and elongation at 72ºC for 60 sec. Final elongation was 72ºC for 10 minutes; and 4ºC hold forever. A negative sample was used as a control PCR tube containing all components except genomic DNA with some primers to examine for contamination.

DNA Electrophoresis:

Amplifications of RAPD were performed in a PCR Thermal Cycler C-1000 (Bio-Rad) in 0.2 mL tubes. Products of RAPD were resolved in TAE buffer by 1.5% and 2% agarose gel electrophoresis, respectively, at 80V for 1 hour.

The gels were stained with 0.5 µg/mL ethidium bromide (100 mg/mL) and visualized under ultraviolet light as reported by Sambrook *et al.* (1989).

SDS-PAGE of total seed proteins:

Total proteins were isolated from the mature healthy seeds according to the methods of (Badr et al., 1998). SDS-PAGE gel electrophoresis was achieved in 12% acrylamide slab gels according to Laemmli (1970). After complete migration, gel was photographed and analyzed by gel Doc 2000 Bio-Rad system.

Data analysis:

DNA polymorphism of each of RAPD was analyzed. The similarity coefficient (F) and the formula of a dendrogram were determined using un-weighted pair group method and arithmetic average (UPGMA) as reported by Rohlf (1990) and (Nei and Li1979), respectively.

The presence or absence of each band was recorded as 1 or 0, respectively. The bands that were unpredictable in replicate analyzes were excluded. Also, bands that occurred only once or did not show fidelity within the replicate of each taxon were rejected. Binary matrix was used to evaluate genetic similarities and distances between samples, by employing Dice index (Nei and Li, 1979). These similarity co-effcients were used to make a dendrogram using the un-weighted pair group method of arithmetic means (UPGM).

RESULTS AND DISCUSSION

The genomic DNA is concentrated and became suitable for a variety of applications, including amplification by PCR, Yields are typically ofthe order of 100-200 ug/g fresh weight of tissue, (Fig. 1).

RAPD analysis.

Out of 16 random RAPD primers, only ten were showed clear and reproducible loci. These 10 RAPD primers gave 527 total scorable loci (number of samples analyzed × number of scorable loci with all primers) of the whole tested plants, with an average of 5-9 loci per primer. The number of produced bands for each RAPD primer was varied from 4 bands (P212) to 8 bands (P338). The amplification products ranging size of all primers were from 208 bp (P212) to 1689 bp (P327).

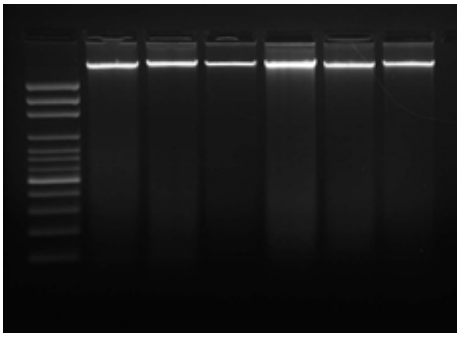

Figure. 1: Total extracted genomic DNA from all *Juniper* young leaves.

The maximum number of 9 loci was present within the size range of 208 to 1689 bp, followed by 5 loci of less than 300 bp in size to highest 1689 bp at the same primer (P327). However, some bands were absent in some other samples. A pairwise Jaccard's similarity values ranged from 0.91 to 0.97 (average 0.93) for inside each group separately. Genetic similarities among six Juniper individuals using RAPD primers were in all the three samples showed in every group 90.55-97.98 % similarity and between the two main groups average 62.92 % similarity.

Genetic distance values between samples the dendrogram constructed is presented in Figure 3. This analysis shows two major groups. The first group contains *Juniperus procera* and the second group includes *Juniperus phoenicea* L. (section Sabina) in Saudi Arabia morphological character differences were found to possess considerable DNA differences.

SDS-protein profile

The data obtained by SDS-PAGE technique in Figure (3) shows the profile of the two samples belonging to *J. procera* and *J. phoenecea* data analysis revealed a total number of 9 detectable bands (subunits) with molecular mass (Mr) ranging from 114.4 to 26.0 kDa. High polymorphism percentage (100%) was recorded in the protein pattern. Sample 1 was characterized by 5 unique bands at 78.6, 52.7, 35.0, 29.3 and 26.0 kDa, while 4 bands with a molecular mass of 114.4, 81.7, 36.4 and 28.3 kDa distinguished Sample 2. All distinguishable bands were considered as species-specific bands and could be used as biochemical marker for each species. (Fig. 4)

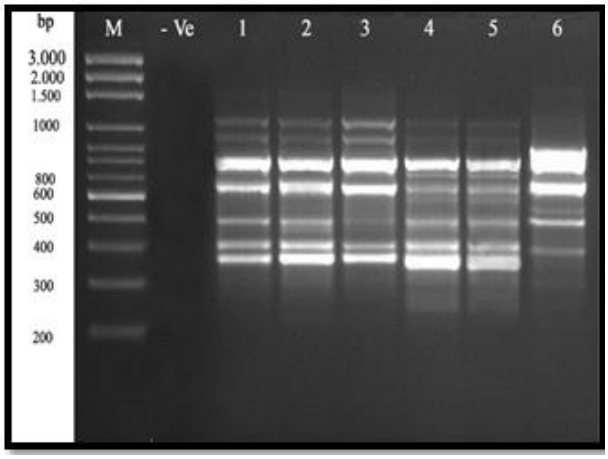

Figure. 2: RAPD fingerprints using of primer P204. Two groups (*Lanes 1-3* and lanes 4-6 and every group of *Juniper plant samples from different locations with the same phenotype*).

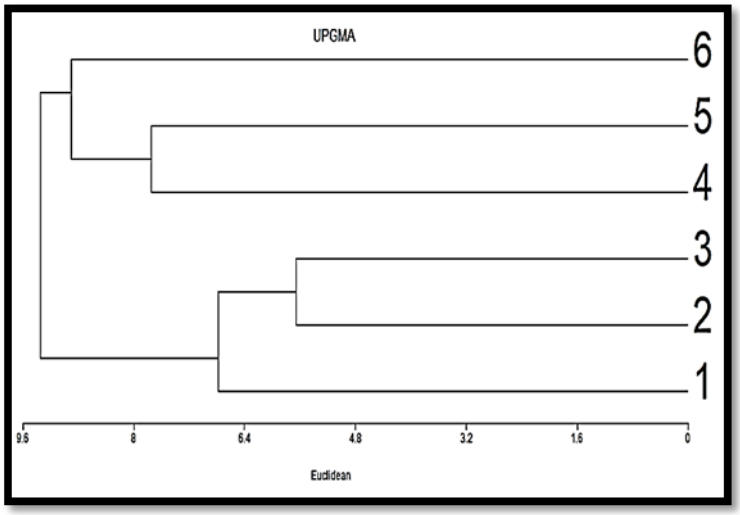

Figure. 3: UPGMA dendrogram generated based on the cluster analysis of 527 RAPD bands of six samples of *Juniper.*

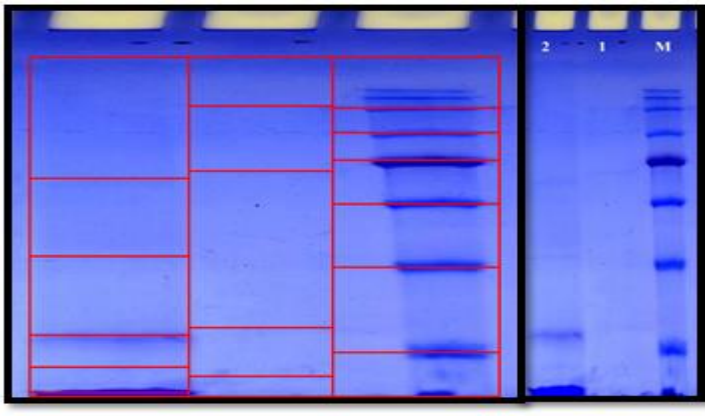

Figure. 4: SDS-protein profile for the tow samples belonging to *J. procera* and *J. phoenecea*.

Exomorphic seed characters study

In this study exomorphic seed characters of the studied *Juniperus* spp. as shown by LM and SEM are reviewed in the following (Table 1; Fig. 1–2).We can notice the general shape varies in *Juniperus* spp., spear in *J.procera* and Elliptic in *J. phoenicea.* Also about the number of seeds in the fruit we find a difference in *J.procera* only four but in *J. phoenicea* we count six. But in seed weight none significantly among the examined. Whereas the weight of the seed *J. procera* 0.01gm, while the weight of the seed *J. phoenicea* 0.09gm. Another difference appears in the seed size, *J.procera* 4 – 6.5 **(µm),** *J. phoenicea* 3.8 –7.7 **(µm)** and seed color *J.procera* has yellowish and it is red-brown in *J. phoenicea*. On the other hand the seed hilum shape varies significantly among the examined taxa. It varies from ovate in *J. procera* and *J. phoenicea* to linear. In this study, DNA fingerprinting of Saudi Arabian Junipers revealed that *Juniperus procera* and the second group includes *Juniperus phoenicea* L (Sabina group) clustered weakly together (Figure 4). At the molecular weight of the fragments, it ranged from 114.4 to 26.0 KDa. Percent of similarities between DNA fingerprinting were reached to 81.8-100.0%. As a conclusion, the presences of three types of fragments, i.e., monomorphic, polymorphic and unique fragments showed that RAPD analyses of DNA polymorphisms of six locations collected genotypes confirmed the genetic variability between them. It is suggested that no significant level of differences between individuals inside groups of populations can result from the earlier divergence and considerably different mutation rate within a population or low level of gene flow between them, or between groups a different origin of different environment conditions, Juniper trees of Saudi Arabia are very similar to subspecies in terms of vegetation characters, such as shape, type of leaves, seeds and height. The RAPD analysis in other studies revealed that UPGMA dendrogram generated based on the cluster analysis of RAPD bands of tested species of *Juniperus*, although they were different, their varieties have a high similarity morphologically (Adams, 1999). *J. communis* and its diversities especially var. Oblonga have been investigated previously. Using 191 RAPD bands, (Adams and Pandey 2003) stated that there was little evidence to support the recognition of *J. communisvar*. oblonga (Adams and Pandey, 2003). Also, Adams (1999) elucidated that the divergences of morphologically near-identical taxa in their terpenoids and DNA fingerprints. He elucidate that even species separated by minute morphological character differences have substantial terpenoid and DNA differences, which propose that evolution profits at different rates for different character sets. The use of several character sets seems practical in *Juniperus* taxonomy and evolutionary studies (Adams, 1999). Along those experiments the exomorphic seed characters of the studied *Juniperus* spp. as shown by LM, SEM and AFM are reviewed previously general shape varies in *Juniperus* spp., spear in *J.procera* and Elliptic in *J. phoenicea.* Also about the number of seeds in the fruit, seed color but seed sizes none significantly among the examined. (Fig. 6). On the other hand the seed surface coarse-flaklyin *juniperus procera* and reticulate in *juniperus phoenicea*, seed hilum shape varies significantly among the examined taxa. It varies from ovate in *J. procera* and *J. phoenicea* to linear. (Fig. 8). Also there is a different between the seeds size average of *juniperus* spp. (Fig. 7).

Figure. 5: General view of seeds in light microscope. A; *Juniperus procera* fruit shape and color , A_1; seed shape, A_2; number of seed in fruit (1-4), B;J*uniperus phoenicea* fruit shap and color B_1; seed shape B_2; number of seed in fruit (>5).

Table (2) Morphological seed characters of the studied as *Juniperus* spp. revealed by LM, SEM and AFM.

N.	Taxon	Fruit color	Seed surface	Seed shape	Seed color	Seed size (µm)	Seed Weight (gm)	Coat	Hilum shape	N. of seeds in the fruit
1	*J. procera*	Blue	coarse-flakly	Spear	Yellowish	4 – 6.5	0.01	thick	Ovate	4
2	*J. phoenicea*	Red	reticulate	Elliptic	Red-brown	3.8 –7.7	0.09	thick	Linear	6

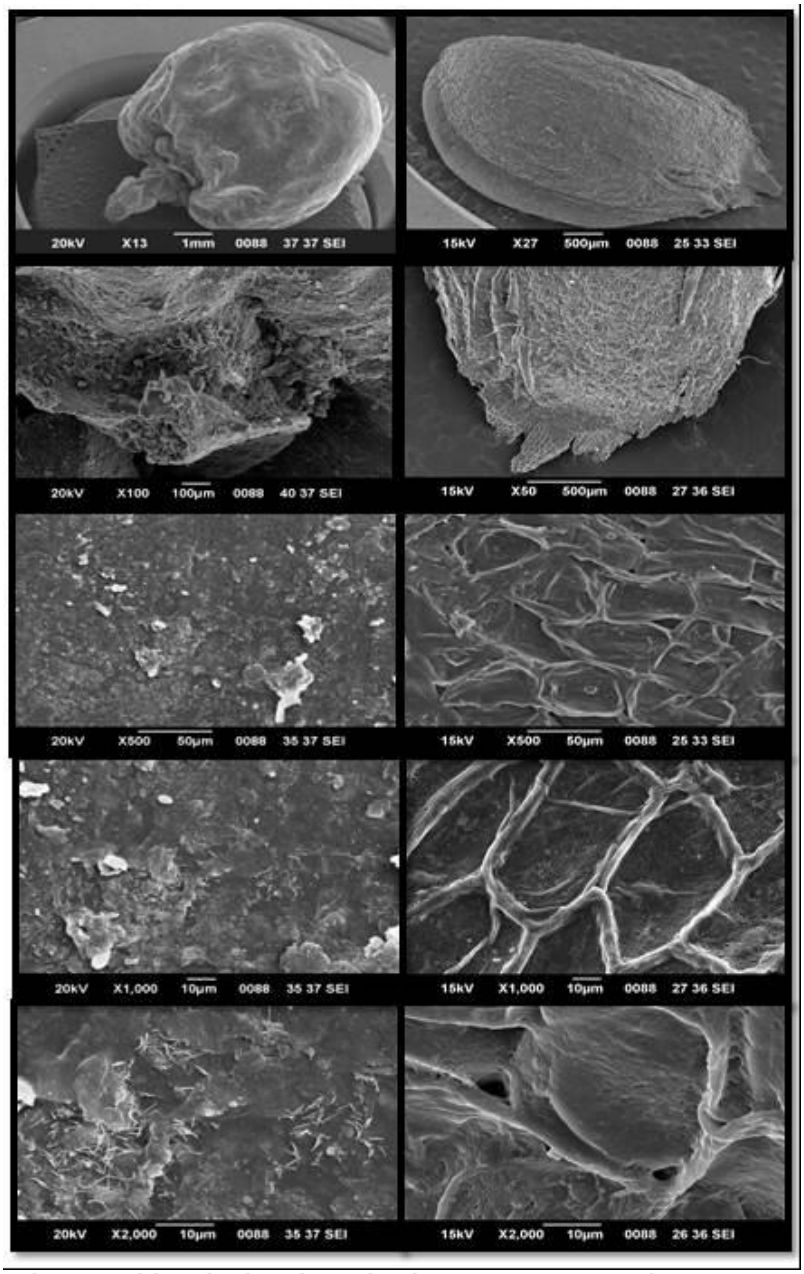

Figure. 6: 3D-AFM image with calculated seeds size average A; *Juniperus procera*, B; *Juniperus phoenicea*.

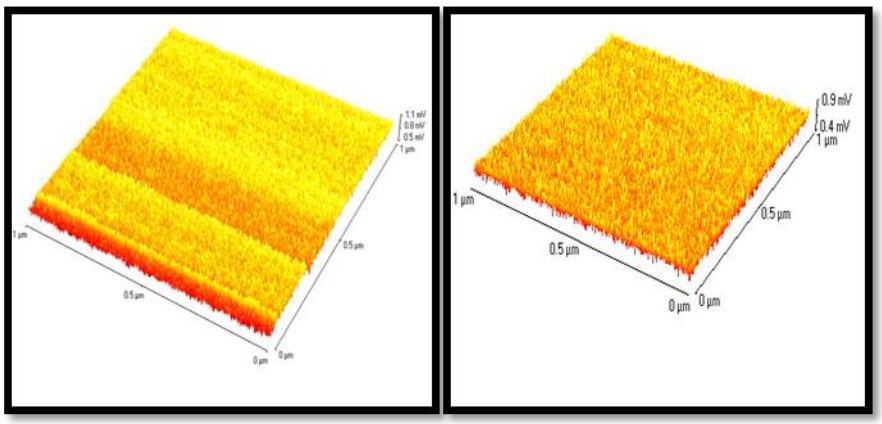

Figure. 7. A: *Juniperus procera*, B: *Juniperus phoenicea*; (A-B) SEM photographs of seed shape, (A_1-B_1); Hillum shape; (A_2-B_2); seed surface (x500-5µm); (A_3-B_3); seed surface (x1000-10µm); seed surface (x2000-10µm) (A_4-B_4).

CONCLUSION

It seems that this research is the first complete molecular and morphological research on Saudi Arabian *Juniperus* species. The genetic diversity was observed at species and population levels. The high level of differentiation in the high-mountains Sarawat populations and the lower populations of two clustered groups reflects a high possibly a different origin and the other variation between same group individuals may be for long period of isolation. It is believed that further studies needed to be based on the geographic variation of *Juniperus* sp.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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