GEOGRAPHIC VARIATION IN *JUNIPERUS PHOENICEA* FROM THE CANARY ISLANDS, MOROCCO AND SPAIN, BASED ON RAPDS ANALYSIS

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ABSTRACT

Populations of *J. phoenicea* var. *phoenicea* and *J. p*. var. *turbinata* from the Canary Islands, Morocco, and Spain were analyzed by Random Amplified Polymorphic DNAs (RAPDs). The Canary Islands and Moroccan populations were very similar to *J. p*. var. *turbinata* from Tarifa, Spain. The largest divergence from *J. p*. var. *turbinata* was in the high Atlas Mtns., Morocco population. Although the Canary Islands population is somewhat divergent, it is treated as *J. p*. var. *turbinata* rather than *J. p*. var. *canariensis*. There is insufficient support to merit the recognition of *J. p*. var. *canariensis*, *J. p.* var. *megalocarpa* or *J. p*. var. *mollis*.

KEY WORDS: *Juniperus phoenicea*, *Cupressaceae*, geographic variation, RAPDs.

Juniperus phoenicea L. is a small tree that is native to the northern Mediterranean from Portugal to Israel (Adams, 2004). It is also native to North Africa in Algiers and Morocco as well as the Canary Islands (Adams, 2004). Gaussen (1968) discussed several infraspecific taxa: *J. p*. var. *turbinata* (Guss.) Parl.(= *J. p.* var. *oophora* Kunze) with female cones elongated (turbinate) in littoral sites throughout the Mediterranean; *J. p*. var. *canariensis* Guyot on the Canary Islands; var. *lycia* (L.) Gaussen

(*pro specie*) (= *J. phoenicea*), France littoral zone; var. *mollis* M & W., common in Morocco; and var. *megalocarpa* Maire, dunes near Mogador (now Essaouira), Morocco. Later, LeBreton and Thivend (1981), on the basis of total proanthocyanidins and the ratio of procyanidine to prodelphinidine, recognized *J. phoenicea* subsp. *eu-mediterranea* Lebr. & Thiv. as occurring on the Mediterranean islands, North Africa and southwestern Portugal. LeBreton (1983) expanded his work to include more sample locations and showed all of the southwestern coastal populations of Portugal and Spain to have high proanthocyanidins (implying *J. p.* subsp. *eu-mediterranea*).

Adams, Barrero and Lara (1996) sampled plants from the area of LeBreton's population 70, his pure *J. phoenicea* population (66-65) and *J. p.* var. *turbinata* from Tarifa as well as a reference population of *J. phoenicea* in Greece. Based on leaf essential oils, Adams, Barrero and Lara (1996) concluded that *J. phoenicea* var. *turbinata* and *J. p.* subsp. *eu-mediterranea* were conspecific.

Recently, Rezzi et al. (2001) reported on infraspecific variation in the leaf essential oils of *J. phoenicea* var. *turbinata* from Corsica. They found two chemical types: high α-pinene, low β-phellandrene, low α-terpinyl acetate (cluster I, 35 indvs.); and low α-pinene, high β-phellandrene, high α-terpinyl acetate (Cluster II, 15 indvs.). No morphological differences were found.

Adams et al. (2002) analyzed the RAPDs of *J. phoenicea* from Portugal (*J. p.* subsp. *eu-mediterranea*), Spain, Canary Islands, Corsica, and Greece. They found that *J. phoenicea* was clearly divided into var. *phoenicea* and *var. turbinata* and affiliated populations. *Juniperus p.* subsp. *eu-mediterranea* from Portugal was confirmed to be conspecific with *J. p.* var. *turbinata*. By nomenclatural priority, *J. p.* subsp. *eu-mediterranea* is a synonym of *J. p.* var. *turbinata*. Putative *J. p.* var. *canariensis* from the Canary Islands showed a strong affinity to plants from southern Greece and to *J. p.* var. *turbinata* plants. Based on RAPDs data and morphological observations, only two infraspecific taxa of *J. phoenicea* are recognized: var. *phoenicea* and var. *turbinata.*

Random Amplified Polymorphic DNAs (RAPDs) have been used in several *Juniperus* studies and have proved useful in systematics (Adams, 1999, 2000a-d, 2001) when stringent laboratory procedures are followed (Adams, Flournoy and Pandey, 1998). In the present study, plants were analyzed from several of the aforementioned populations (Adams et al., 2002), plus collections of putative *J. p.* var. *megalocarpa*,

Essaouira, and *J. p.* var. *mollis*, high Atlas Mtns., Morocco.

MATERIALS AND METHODS

Specimens collected: *J. phoenicea*: 25 km e. Guadahortuna, 720 m, El Penon, Spain, *R. P. Adams, 7077-7079*; putative *J. p*. var. *phoenicea*: 20 km sse Marrakech, 940 m, 30º 21.033'N, 07º 45.893'W, Morocco; *J. phoenicea* var. *turbinata*: Tarifa sand dunes, 30 m, 36º 04.996'N, 5º 42.104' W, 15 km w. of Tarifa, Spain, *R. P. Adams, 7202-7204*; putative *J. phoenicea* var. *turbinata*, ca. 150 m, volcanic rock, Tenerife, Canary Islands, *R. P. Adams 8147-8149;* sand dunes; ca. 180 m, 31º 30'N, 9º 47' W, 6 km e. Essaouira, Morocco, *N. Achak & R. P. Adams 10407-10411*. Voucher specimens are deposited at BAYLU.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20^{o} C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). The RAPD analyses follow that of Adams and Demeke (1993). Ten-mer primers were purchased from the University of British Colombia (5'-3'): 134, AAC ACA CGA G; 153, GAG TCA CGA G; 184, CAA ACG GAC C; 212, GCT GCG TGA C; 218, CTC AGC CCA G; 239, CTG AAG CGG A; 249, GCA TCT ACC G; 250, CGA CAG TCC C; 268, AGG CCG CTT A; 338, CTG TGG CGG T; 346, TAG GCG AAC G; 347, TTG CTT GGC G; 375, CCG GAC ACG A; 431, CTG CGG GTC A; 478, CGA GCT GGT C.

PCR stock solutions (Taq, primer, buffer) were made in bulk so that all the PCR reaction tubes for a primer were prepared using the same bulk stock. This is a critical factor for minimizing variation in band intensities from sample to sample (see Adams, Flournoy and Pandey, 1998, for protocols to minimize PCR band variation). PCR was performed in a volume of 15 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM $MgCl_2$, and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 µM primers, 0.3 ng genomic DNA, 15 ng BSA and 0.6 unit of Taq DNA polymerase (Promega). A negative control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). Samples were run in duplicate to insure reproducibility (Adams, Flournoy and Pandey, 1998). A temperature profile was obtained for each well of the thermocycler to be sure that no variation existed among wells in the heating/ cooling block. The thermal cycle used was: 94^{o}C (1.5 min) for initial strand separation, then 40 cycles of 40^{o}C (2 min), 72^{o}C (2 min), 91^{o}C (1 min). Two

additional steps were used: 40°C (2 min) and 72°C (5 min) for final extension. The temperature inside a PCR tube containing 15 µl buffer was monitored with a temperature probe, quantitated and printed for each step for each of the 40 cycles for every PCR run (Adams, Flournoy and Pandey, 1998) to insure that each cycle met temperature specifications and that each PCR run was exactly the same. Amplification products were analyzed by electrophoresis on 1.5% agarose gels, 75V, 55 min, and detected by staining with ethidium bromide. The gels were photographed over UV light using Polaroid film 667 and scanned to digital images. The digital images were size normalized in reference to pGem® DNA size markers before band scoring. Bands were scored as present (1) and absent (0). Bands that were inconsistent in replicate analyses were not scored.

Associational measures were computed using absolute character state differences (Manhattan metric), divided by the maximum observed value for that character over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis (PCO) was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967). It should be noted that problems of homology of RAPD DNA bands on agarose gels can be significant (Rieseberg, 1996), but these errors can be accounted for using multivariate statistical methods (PCO) (see Adams and Rieseberg, 1998). A minimum spanning diagram was constructed by selecting the nearest neighbor for each taxon from the pair-wise similarity matrix, then connecting those nearest neighbors as nodes in a network that was superimposed on a geographic map (Adams et al. 2003).

RESULTS AND DISCUSSION

The major trend in the minimum spanning network (Fig. 1) is the separation of *J. phoenicea* var. *phoenicea* (El Penon, Spain) from *J. p.* var. *turbinata* (Tarifa sand), Morocco and Canary Island populations. The Tarifa, Canary Islands, and Morocco populations are each distinct and relatively uniform within populations (Fig. 1).

Factoring the association matrix resulted in four eigenroots of 40.6%, 20.3%, 13.5% and 10.35% that accounted for 84.7% of the variance among samples. A major portion of the variation (40.6%) is due to the separation of *J. p.* var. *phoenicea* (El Penon, Spain) from the other four populations (Fig. 2). The other four populations (Fig. 2) form a loose assemblage with no apparent subgroups.

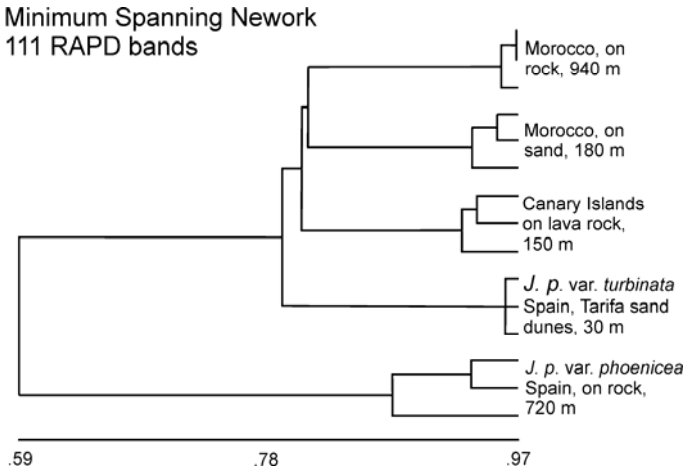

Figure 1. Minimum spanning network based on 111 RAPD bands. Two major groups are apparent: *J. p*. var. *phoenicea* and *J. p*. var. *turbinata*. The Tarifa, Canary Islands and two Moroccan populations are each distinct.

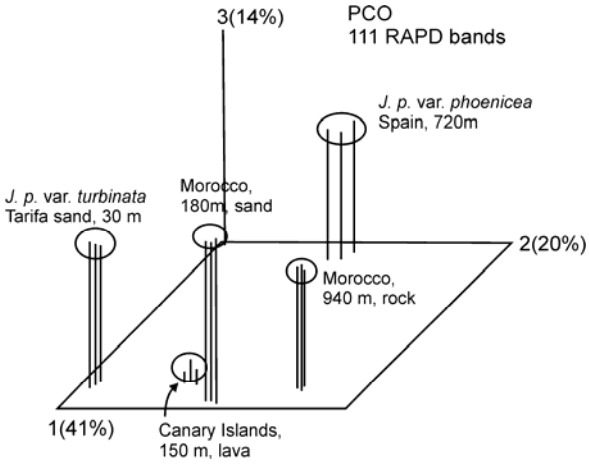

Figure 2. PCO for *J. p*. var. *phoenicea*, *J. p*. var. *turbinata* and associated populations. See text for discussion.

Contouring the similarities among populations shows a similar trend (Fig. 3) in which the southernmost populations (C, Canary Islands; E, Essaouira, Morocco; A, Atlas Mtns., Morocco) cluster at 0.808 similarity. Next, the *J. p*. var. *turbinata* population from the Tarifa sand dunes, Spain (T, Fig. 3) joins the cluster at 0.793. *Juniperus p*. var. *phoenicea* (P, El Penon, Spain, Fig. 3) is loosely associated at 0.590. Clearly, the Canary Island and Moroccan populations have strong affinities to var. *turbinata* (T, Fig. 3), rather than to var. *phoenicea* (P, Fig. 3).

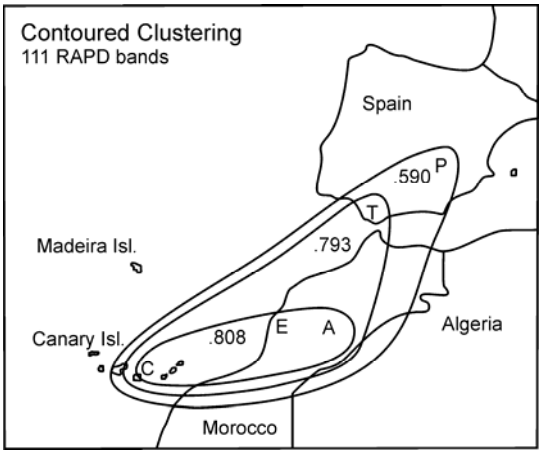

Figure 3. Contoured clustering shows that the southern populations (C = Canary Islands, E = Essaouira, Morocco, A = Atlas Mtns., Morocco) form a cluster followed by the Tarifa (T) population (*J. p*. var. *turbinata*) with *J. p.* var. *phoenicea* (P = El Penon, Spain) loosely associated.

A similar trend is seen in the minimum spanning network (Fig. 4). Notice the same similarity between Essaouira (E) - Atlas Mtns. (A) as for Essaouira (E) - Canary Islands (C) populations (E-A, E-C, each 0.808). The link from the Canary Islands to *J. p*. var. *turbinata*, Tarifa sands, Spain is only slightly less (C-T, 0.793) than the other linkages. *Juniperus p*. var. *phoenicea* (P, El Penon, Spain) links at a much lower level to the Canary Islands population (P-C, 0.590). To examine the divergence of

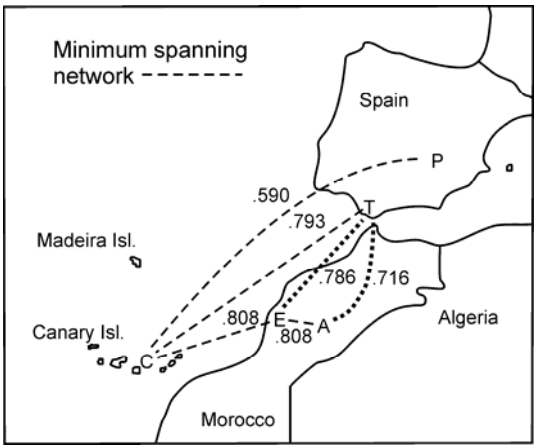

Figure 4. Minimum spanning (dashed lines) show the linkage order among populations. The dotted lines show the secondary linkages between Tarifa - Essaouira, and Tarifa - Atlas Mtns. See text for discussion.

the Moroccan and Canary Islands populations from typical *J. p*. var. *turbinata*, the linkages from Tarifa (T) to the Moroccan populations were mapped (Fig. 4, dotted lines). The Tarifa population is much less similar to the Atlas Mtns. (T-A, 0.716), than to Essaouira (T-E, 0.786). So, although Essaouira has the same similarity to both the Canary Islands and Atlas Mtns. populations (0.808), Essaouira plants are more similar to *J. p*. var. *turbinata* and less similar to the Atlas Mtns. plants.

CONCLUSIONS

Adams et al. (2002), in a the previous study involving *J. p*. var. *phoenicea*, *J. p.* var. *canariensis*, and *J. p.* var. *turbinata*, concluded that although *J. p*. var. *canariensis* had some distinct DNA differences, it did not merit recognition as a variety. However, they did not include materials from Morocco. Gaussen (1968) recognized var. *canariensis* on the Canary Islands; var. *mollis*, common in Morocco; and var. *megalocarpa*, on dunes near Mogador (= Essaouira), Morocco. In this study, we have collected materials from var. *megalocarpa* (Essaouira)

and var. *mollis* (Atlas Mtns.) and analyzed the DNA fingerprints from these plants. The Canary Islands, Essaouira and Atlas Mtns. populations were found to be somewhat divergent from var. *turbinata*, but basically there seem to be two meaningful entities, var. *phoenicea* and var. *turbinata*. We conclude that there is insufficient support for the recognition of *J. p*. var. *canariensis*, var. *megalocarpa*, or var. *mollis*. It is surprising to find var. *turbinata* in the high Atlas Mtns. (940 m), as it is has traditionally been associated with coastal habitats. It is likely that the Moroccan populations were part of a refugium for *J. phoenicea* during the Pleistocene glacial ages.

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