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## Geographic variation in the Random Amplified Polymorphic DNAs (RAPDs) of *Juniperus phoenicea*, *J.p.* var. *canariensis*, *J.p.* subsp. *eu-mediterranea*, and *J.p.* var. *turbinata*

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### Abstract

Random Amplified Polymorphic DNAs (RAPDs) were analyzed for *J. phoenicea* var. *phoenicea* from Spain, *J.p.* var. *canariensis* from the Canary Islands, and *J.p.* subsp. *eu-mediterranea* from Portugal, and *J.p.* var. *turbinata* from Corsica, Greece, and Spain. *Juniperus phoenicea* was clearly divided into *J.p.* var. *phoenicea* and *J.p.* var. *turbinata* and affiliated populations. *J.p.* subsp. *mediterranea* Lebr. and Thiv. from Portugal was confirmed to be conspecific with *J.p.* var. *turbinata*. Thus, by nomenclatural priority, *J.p.* subsp. *mediterranea* must be recognized as *J.p.* var. *turbinata* (Guss.) Parl. The *J.p.* var. *canariensis* from the Canary Islands showed a strong relationship to plants from southern Greece and to *J.p.* var. *turbinata* plants. The major trend in the population affinities was east–west (Canary Islands, Portugal, Spain to Greece). The plants from near Delphi, Greece were found to more like var. *turbinata* whereas, those from near Nea Epidavios, Greece were more like the plants from the Canary Islands. Based on RAPD data and morphological observations, only two infraspecific taxa are recognized: *J.p.* var. *phoenicea* and *J.p.* var. *turbinata* until additional research is completed in Morocco and Turkey. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** *Juniperus phoenicea*; *Cupressaceae*; Geographic variation; RAPDs

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## 1. Introduction

*Juniperus phoenicea* L. is a small tree that is native to the northern lands bordering the Mediterranean Sea from Portugal to Israel. It is also native to N. Africa in Algiers and Morocco as well as the Canary Islands (1). Gaussen (1968) discussed several infraspecific taxa: var. *turbinata* (Guss.) Parl.(=var. *oophora* Kunze) with female cones elongated (turbinate) in littoral sites throughout the Mediterranean; 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, Morocco. It is most likely that these varieties are not distinct taxa but reflect the lack of access to the type specimens by botanists who described local populations as new varieties of *J. phoenicea*. More recently, 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. Later, 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 proanthocyanidines (implying *J.p. eu-mediterranea*). He did not have samples from the Gibraltara (Tarifa) region of Spain and indicated uncertainty by use of a question mark (?) on his map (LeBreton, Fig. 1). Tarifa is an area where *J.p. var. turbinata* occurs and LeBreton's population 70 (LeBreton, 1983), west of Setubal, is in the middle of his distribution for *J.p. eu-mediterranea*.

Adams et al. (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 et al. (1996) concluded that *J.p. var. turbinata* and *J.p. subsp. eu-mediterranea* were conspecific and that the name *J.p. var. turbinata* would take precedence over *J.p. subsp. eu-mediterranea*.

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

Random Amplified Polymorphic DNAs (RAPDs) have been used in several *Juniperus* studies and have proved to yield excellent results (Adams 1999, 2001; Adams, 2000a,b,c,d). In this study, we have analyzed RAPDs from the aforementioned (Adams et al., 1996) populations and taxa, plus new collections from Corsica Island (Cluster I and Cluster II plants), El Penon, Spain (typical, inland *J.p. var. phoenicea*), and the Canary Islands (*J.p. var. canariensis*).

## 2. Experimental

Specimens collected: *J. phoenicea*: Nea Epidavios, southern Greece, R.P. Adams, 5653–5654; Tenerife, Canary Islands, R.P. Adams 8147–8149; El Penon, Spain, R.P.

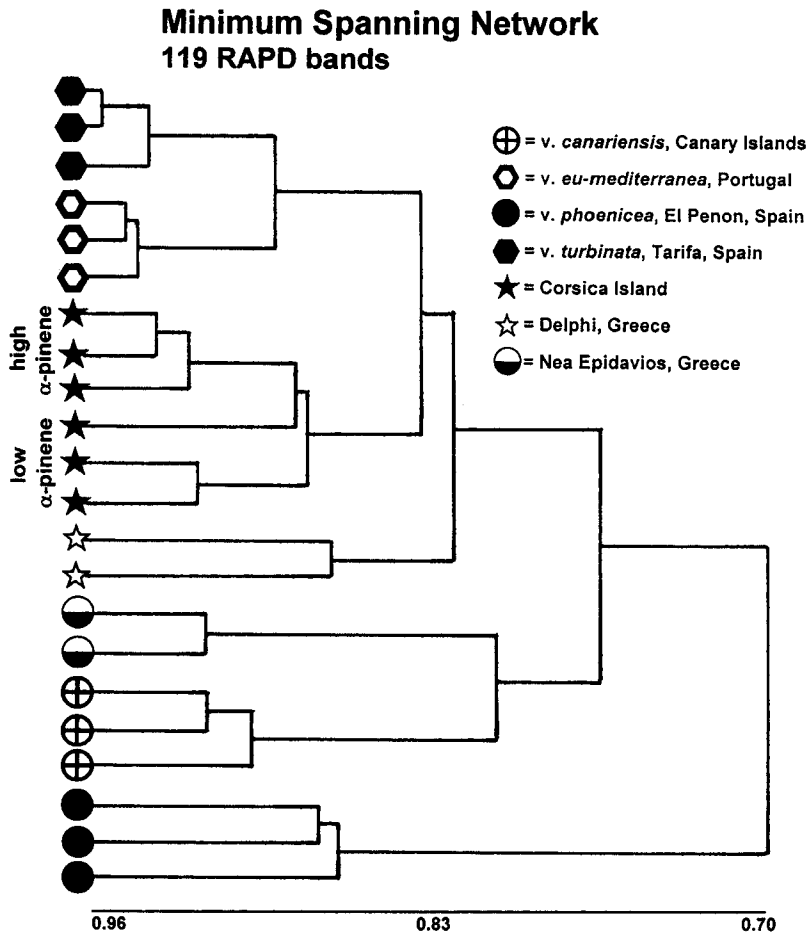


Fig. 1. Minimum spanning network based on 119 RAPD bands. Notice the major separation of *J.p.* var. *phoenicea* plants from El Penon, Spain and close affinity of the *J.p.* var. *turbinata* plants from Tarifa, Spain and those from Portugal (= *J.p.* subsp. *eu-mediterranea*).

Adams, 7077–7079; Delphi, Greece, R. P. Adams 8793–94; *J.p.* var. *turbinata*: w. of Setubal, Portugal, R. P. Adams, 7074–7076; and w. of Tarifa, Spain, R. P. Adams, 7302–7304 and putative *J.p.* var. *turbinata*, Corsica Island, France, high  $\alpha$ -pinene type, S. Rezzi #1–3 (=Lab #: Adams 8890–92): low  $\alpha$ -pinene type, S. Rezzi #4–6 (=Lab #: Adams 8893–95). Voucher specimens are deposited at BAYLU and Universite de Corse.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at  $-20^{\circ}\text{C}$  until the DNA was extracted. DNA was extracted from juniper leaves by the hot CTAB protocol (Doyle and Doyle, 1987) with 1% (w/v) PVP added to the extraction buffer. The RAPD analyses follow that of Adams and Demeke (1993). Ten-mer primers were purchased from the Uni-

versity of British Columbia (5'–3'): 153: GAG TCA CGA G; 184: CAA ACG GAC C; 204: TTC GGG CCG T; 212: GCT GCG TGA C; 218: CTC AGC CCA G; 239: CTG AAG CGG A; 244: CAG CCA ACC G; 249: GCA TCT ACC G; 250: CGA CAG TCC C; 265: CAG CTG TTC A; 268: AGG CCG CTT A; 327: CTA GAG GTC C; 338: CTG TGG CGG T; 346: TAG GCG AAC G.

PCR was performed in a volume of 15  $\mu$ l containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM MgCl<sub>2</sub>, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36  $\mu$ M primers, 0.3 ng genomic DNA, 15 ng BSA and 0.6 unit of Taq DNA polymerase (Promega). A 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.). The thermal cycle was: 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.

Bands that occurred once or did not show fidelity within the two replicated samples of each taxon were eliminated. It should be noted that these bands contain very useful information for the study of genetic variance and individual variation, but are merely "noise" in the present taxonomic study. Bands were scored in four classes: very bright (=6); medium bright (=5), faint (=4) and absent (=0). See Adams and Demeke (1993) for details on electrophoresis and RAPD band scoring.

Similarity 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, 1975a,b). For the terpenoid data, similarities were computed as quantitative matches as well as simple presence/absence matches. The presence/absence ( $\pm$ ) matching was found to be more similar to the DNA data. Principal coordinate analysis (PCO) of the similarity matrices follows Gower (1966). Program PCO3D is available for MS DOS IBM compatible computers with a math co-processor (correspond to RPA for distribution details).

### 3. Results and discussion

There is obviously considerable infraspecific genetic variation within *J. phoenicea* (Fig. 1). The Portugal population of *J.p. var. turbinata* (previously recognized (LeBreton and Thivend, 1981) as *J.p. subsp. eu-mediterranea*) is closely related to the Tarifa, Spain, *J.p. var. turbinata* individuals (Fig. 1). The chemical types (high and low -pinene) of *J.p. var. turbinata* from Corsica form a distinct cluster and loosely cluster by oil type (Fig. 1). This cluster is loosely associated with *J.p. var. turbinata* from Portugal and Tarifa, Spain and the plants from Delphi, Greece (Fig. 1). All of these populations have turbinate female cones.

A second cluster involves a loose association between Nea Epidavios, Greece and the Canary Islands (Fig. 1) and these populations have large (9–11 mm), dark red female cones. And finally, the most distinct cluster is the El Penon, Spain plants (typical, inland, *J.p. var. phoenicea* plants) which has small (~8 mm) copper-red female cones.

Principal coordinate analysis was used to factor this similarity matrix and the eigenroots appeared to asymptote after eigenroot seven. This indicates considerable amounts of independent variation among these seven populations (22 samples). The first seven eigenroots extracted accounted for: 26.8, 14.4, 14.1, 7.9, 5.8, 5.1, and 4.6% of the variance (sum=78.7% of the total variance among the 22 samples).

Ordination using the first three principal coordinate axes is shown in Fig. 2. This gives a clearer picture of the relationships among the groups than the minimum spanning network (but not the fine structure within groups seen in Fig. 1). Notice the clear separation of *J.p.* var. *phoenicea* from El Penon, Spain. The Corsica *J.p.* var. *turbinata* plants cluster closely with the plants from Delphi, Greece.

The clustering of the Nea Epidavios, Greece with the plants from the Canary Islands (Fig. 2) goes along with the large, round, dark red cones. These same type cones have recently been observed on *J. phoenicea* in Morocco (R.P. Adams). It is likely that because junipers seeds are disseminated by birds, that *J. phoenicea* was transported from the Greek Islands to n. Africa and thence to the Canary Islands. A reexamination of the female cones from various populations revealed that the “turbinate” fruits are quite variable, often ranging from turbinate to globose on a single specimen. So the morphology is not very clear to distinguish var. *phoenicea* from var. *turbinata*.

Examination of the geography of the genetic linkage between populations reveals a chiefly east–west pattern (Fig. 3). It is interesting to note that the second highest genetic similarity to the Corsica plants is with the Delphi, Greece population (Fig.

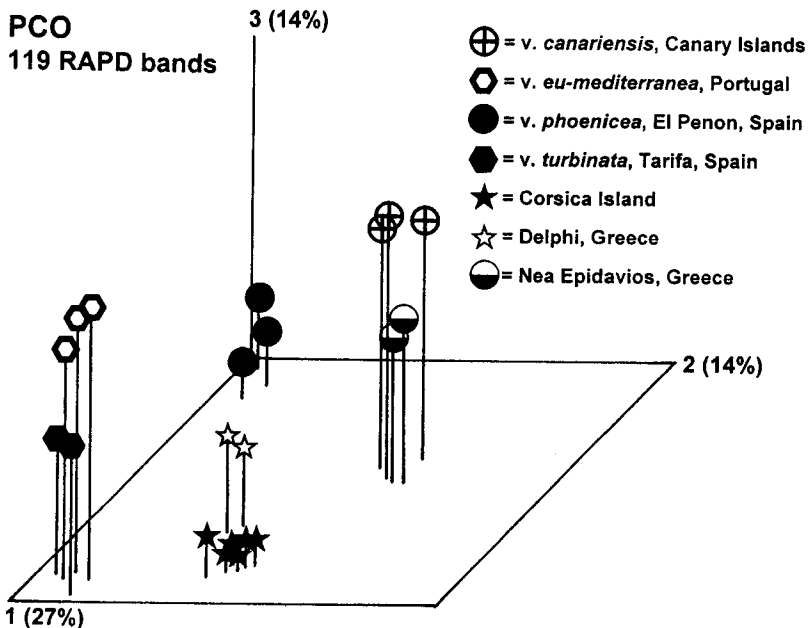


Fig. 2. Principal coordinates analysis (PCO) based on 119 RAPDs. *J.p.* var. *phoenicea* is clearly separated on the first axis. See text for discussion.

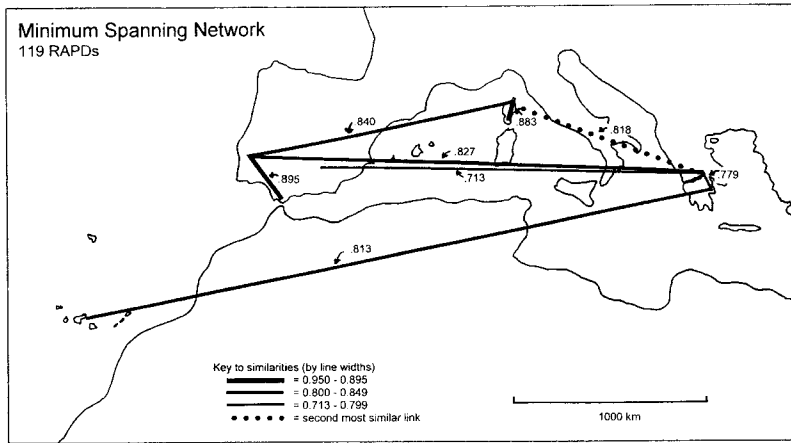


Fig. 3. Minimum spanning network superimposed on a map of the populations sampled. Note the major east-west links. The dotted line shows the second most similar population to the Corsica plants is the Delphi, Greece population.

3). There is a very large dissimilarity between the two Greek populations (Fig. 3, 0.779). Examination of the specimens revealed that the Delphi population has generally turbinate cones and the Nea Epidavios plants had large, dark red, globose cones. So the cone characters appear to be concordant with the RAPD data.

The distinctness of the *J.p.* var. *phoenicea* from El Penon is shown in its linkage to the Delphi, Greece population (Fig. 3, 0.713, the lowest level in the study). The expected linkage between Spain/Portugal populations and the Canary Islands was not seen, but rather a linkage between the Canary Islands and southern Greece (Fig. 3, 0.813).

This study using RAPDs data confirms the previous study based on leaf essential oils (Adams et al., 1996) that *J.p.* subsp. *mediterranea* Lebr. and Thiv. from Portugal is clearly a part of *J.p.* var. *turbinata*. So by nomenclatural priority, this infraspecific taxon must be recognized as *J.p.* var. *turbinata* (Guss.) Parl.

There is some support for recognizing *J.p.* var. *canariensis* Guyot, endemic to the Canary Islands, but additional work in Morocco (in progress) is needed as the plants of the Canary Islands are very similar to those in Morocco. It may be that *J.p.* var. *mollis* or *J.p.* var. *megalocarpa* in Morocco is the same as *J.p.* var. *canariensis*. This work is in progress.

Thus, we are found a major division between inland *J.p.* var. *phoenicea* plants (El Penon, Spain) and all the other “var. *turbinata* and affinities” plants.

Hopefully, additional population sites may clarify this pattern. Additional collections are needed to understand this species complex. Particularly important will be the addition of samples from Greece, Italy, the Mediterranean Islands, Morocco and Turkey.

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