

**Geographic variation in *J. phoenicea* var. *phoenicea* from throughout its range:
Analysis of nrDNA and the petN-PsbM cp region.**

Robert P. Adams

Biology Department, Baylor University, Box 97388, Waco, TX 76798, USA Robert_Adams@baylor.edu

Joaquín Altarejos

Departamento de Química Inorgánica y Orgánica, Facultad de Ciencias Experimentales,
Universidad de Jaén, E-23071 Jaén, Spain

Montserrat Arista

Departamento Biología Vegetal y Ecología, Universidad de Sevilla, Apdo. 1095, E-41080 Sevilla, Spain

and

Andrea E. Schwarzbach

Department of Biomedicine, University of Texas at Brownsville, Brownsville, TX 78520, USA.

ABSTRACT

Juniperus phoenicea (var. *phoenicea*) was examined from five populations throughout its range by sequencing nrDNA and the petN-psbM cp region. In addition, data from seventeen populations of *J. turbinata* (*J. phoenicea* var. *turbinata*) were included in a Bayesian analysis. All populations of *J. phoenicea* were found in a well-supported clade, separate from *J. turbinata*. Little infra-specific variation was found in *J. phoenicea*, with only one substitution present in France, one in Zaragoza and one in El Penon populations. The divergence of the El Penon correlates with a previous report of differences in the leaf volatile oils. Published on-line www.phytologia.org *Phytologia* 96(4): 247-251 (Oct. 1, 2014).

KEY WORDS: *Juniperus phoenicea* var. *phoenicea*, Cupressaceae, nrDNA, petN-psbM, geographic variation, *J. turbinata*.

Adams et al. (2013) analyzed nrDNA and petN-psbM sequences for *J. phoenicea* L. (*sensu stricto*) from throughout the Mediterranean region (Fig. 1). They found *J. phoenicea* var. (or subsp.) *phoenicea* to be restricted to Spain and France, whereas *J. phoenicea* var. *turbinata* (Guss.) Parl. (*J. turbinata* Guss.) was widely distributed from the Canary Islands to the Sinai. No differentiation was found between the typical Mediterranean and Canary Island populations, offering no support for the recognition of *J. phoenicea* subsp. *canariensis* (Guyot) Rivas-Martínez (Fig. 1). *Juniperus turbinata* is widespread, ranging from Madeira - Canary Islands to the Sinai with few DNA differences among most populations. However, some populations (Grazalema, Madeira, Sinai, central Italy) displayed (Fig. 1) moderate amounts of divergence (3-4 mutations).

In a broad phylogenetic study of *Juniperus*, Adams and Schwarzbach (2013) found that *J. phoenicea* was not part of a clade of serrate-leaf junipers occurring in the western hemisphere, leading them to denote *J. phoenicea* as a 'pseudoserrate' juniper. In addition, they found *J. p.* var. *phoenicea* and var. *turbinata* to be as different in their DNA sequences as several other recognized species of *Juniperus*. Based on these data, they recognized *J. turbinata* Guss., as had been proposed by Lebreton and Pérez de Paz (2001) based largely on the concentration of prodelphinidin, a polymeric tannin. The prodelphinidin

data indicated that *J. p.* var. *phoenicea* was confined to the Iberian Peninsula, while var. *turbinata* occurred throughout the Mediterranean region. Lebreton and Pérez de Paz (2001) found a clear separation between *J. phoenicea* (Spain and France) and all other populations examined (*J. turbinata*).

Adams, Altarejos and Arista, (2014) examined the volatile leaf oil of *J. phoenicea* (*sensu stricto*) from throughout its range (Spain and France). They reported the composition of the volatile leaf oils of four of the five populations varied very little except for a chemotype (one tree) in the Zaragoza population that was high in cedrol and other cedarwood terpenoids. However, all five trees sampled in the Grazalema population had the cedarwood chemotype and were high in cedrol. The oil from the high cedrol plants at Grazalema seems quite different due to the presence of cedarwood oil components, but the oil is actually not very different, if one removes the heartwood terpenoids and re-normalizes the remaining terpenoids (Table 1, Adams et al., 2014).

It is of interest to examine variation in DNA sequence data from the same populations (Adams et al., 2014) in France and Spain.

The purpose of the present study is to present analyses of nrDNA and petN-psbM sequences from populations of *J. phoenicea* var. *phoenicea* throughout its range.

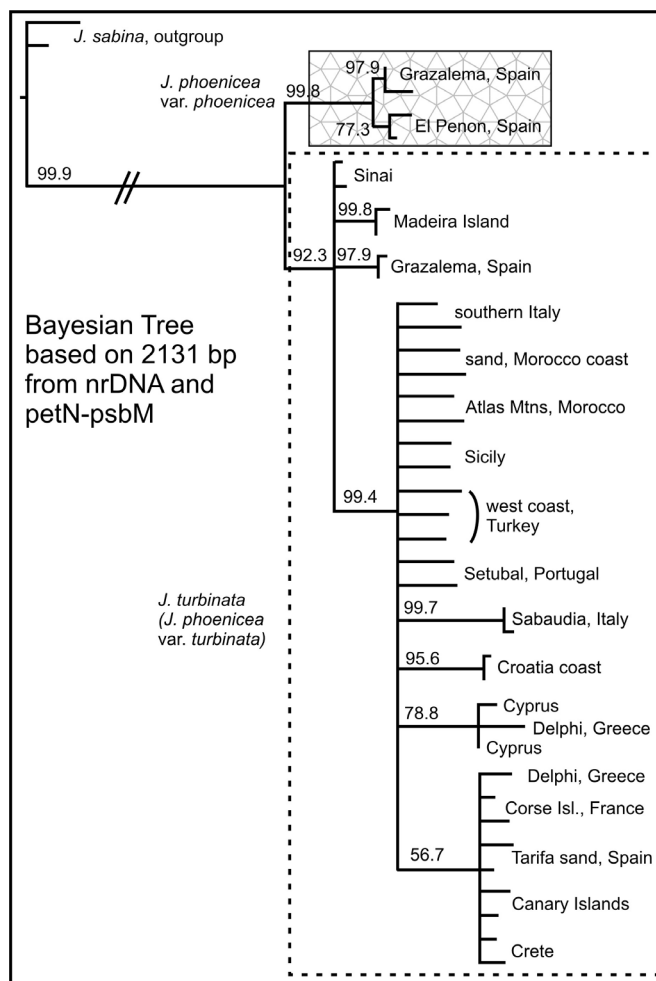


Figure 1. Bayesian tree of *J. phoenicea* and *J. turbinata* (*J. p.* var. *turbinata*) from throughout the species ranges. (from Adams et al. , 2013).

MATERIALS AND METHODS

Figure 2 shows the distributions of *J. phoenicea* var. *phoenicea* and populations sampled in this study.

Specimens used in this study: ***J. p.* var. *phoenicea*:**

France, Narbonne, near St. Pierre sur Mer, 43° 10' 0.2" N, 3° 09' 57.6" E, 23 m, *J. Altarejos* 1-5, Baylor specs. Adams 14123-14127.

Andorra, Coll de Jou near Sant Julià de Lòria, 42° 26' 56.8" N, 1° 28' 04.6" E, 1426 m, *J. Altarejos* 6-10, Baylor specs. Adams 14128-14132.

Spain, Zaragoza, Montes de la Retuerta de Pina W of Bujaraloz, 41° 28' 59"N, 0° 19' 31.2"W, 317 m, *J. Altarejos* 11-15, Baylor specs. Adams 14133-14137.

Spain, El Peñón, approx. 25 km west of Guadahortuna, 37° 35' 38" N, 3° 31' 22" W, 760 m, Adams 7077-7079,

Spain, Cádiz, Sierra de Grazalema, 36° 47' 51.5" N, 5°24' 43.7"W, 835 m; *M. Arista* 1-5, Baylor specs. Adams 13813-13817.

Locations of populations of *J. turbinata* are listed in Adams et al. (2014). Voucher specimens are deposited at BAYLU herbarium Baylor University.

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° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 μ l reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 μ l 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 μ M each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 μ M each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized.

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R6-1 (Biomatters. Available from <http://www.geneious.com/>) and the MAFFT alignment program. Analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck, 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall, 1998) and Akaike's information criterion.

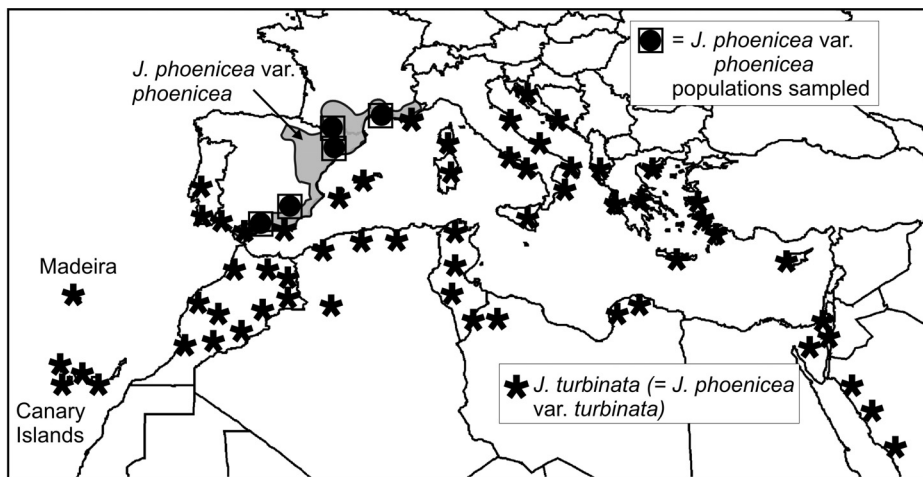


Figure 2. Distributions of *J. phoenicea* and *J. turbinata* (adapted from Adams, 2014; Lebreton and Pérez de Paz, 2001). Gray shaded area shows the general distribution of *J. phoenicea* (var. *phoenicea*) and solid circles inside squares show the five populations of *J. phoenicea* sampled in the present study.

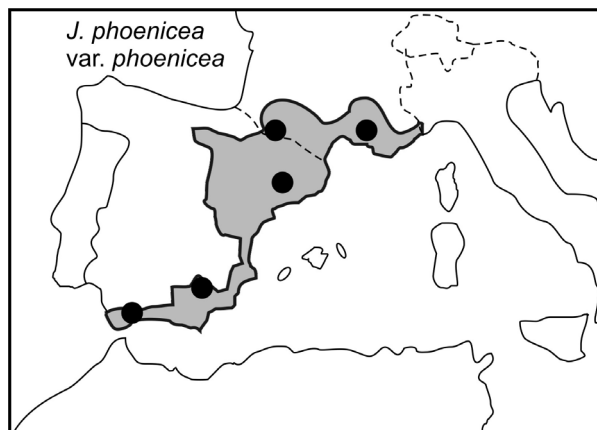


Figure 3. Distribution of *J. phoenicea* var. *phoenicea* showing populations sampled in this study (closed circles)

RESULTS AND DISCUSSION

Sequencing nrDNA and petN-psbM resulted in 2131 bp of data. The Bayesian tree shows (Fig. 4) all populations of *J. phoenicea* (var. *phoenicea*) in a well-supported clade, separated from *J. turbinata* (= *J. phoenicea* var. *turbinata*).

The El Penon population shows the largest differentiation (Fig. 4). France and Zaragoza populations show small differences (Fig. 4). No substitutional differences were found between the Andorra and Grazalema populations (Fig. 4).

To examine this geographical pattern, the order of linkage in the Bayesian tree (Fig. 4, *J. phoenicea* clade) was contour-mapped (Fig. 5). This shows the Andorra (AN) population linked to the Grazalema (GR) population (no substitutional differences found). Next, the Zaragoza (ZA) population enters the tree; then, the France (FA) population enters (Fig. 5). The most divergent population (El Penon, EP) enters the tree as the last member of the *J. phoenicea* clade (Fig. 5).

The uniformity of the *J. phoenicea* populations is shown in their petN-psbM sequences that had only one substitution (in the France population) and two indels. One 19 bp indel was present in both samples from Grazalema and one sample from El Penon. The other indel, a poly A 10-mer, was in all samples, except for an 11-mer in one sample from France and El Penon and found as a 12-mer in the second sample from France.

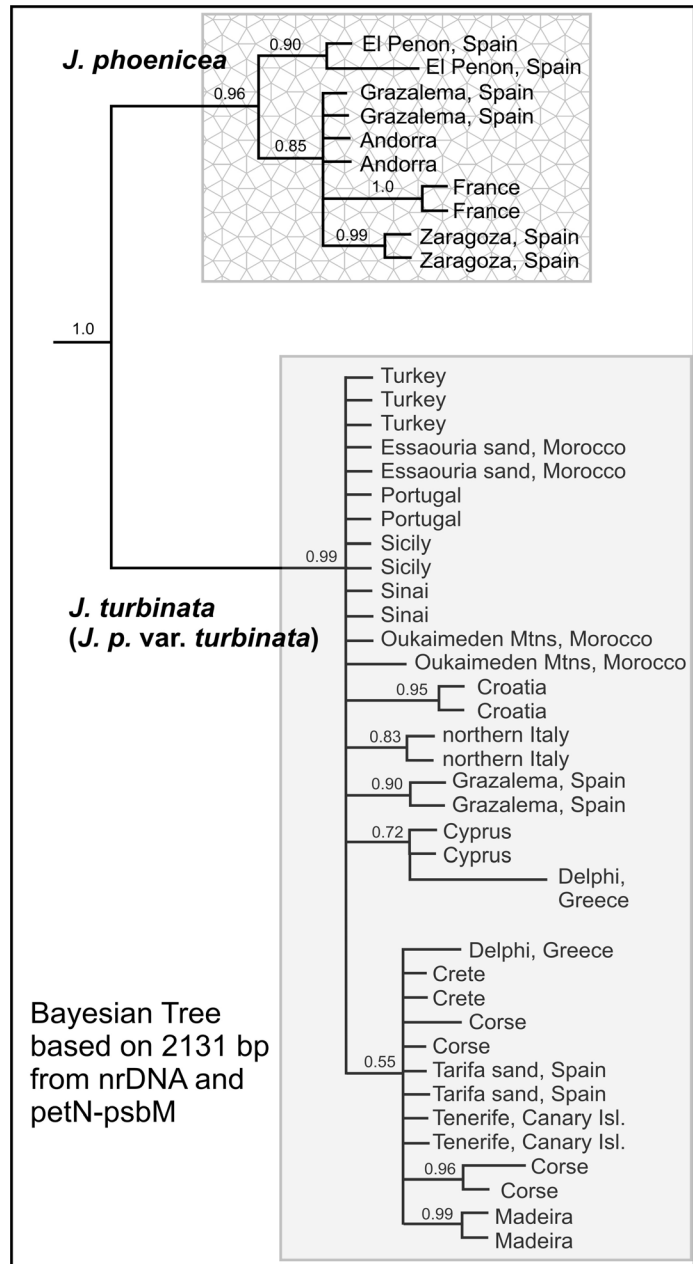


Figure 4. Bayesian tree for *J. phoenicea* populations (upper clade). *Juniperus turbinata* samples included for reference. Numbers at the branch points are posterior probabilities (1.0 - 0.0).

The *J. phoenicea* populations were a little less uniform in their nrDNA with 5 substitutions and one indel. One substitution event was found in both Zaragoza individuals. Another substitution was present in both El Penon samples and the third substitution event was present in both trees from France. The final two substitution events were present in only one tree: 7078 from El Penon. The 3-bp indel was absent in both trees from El Penon and one sample from Grazalema.

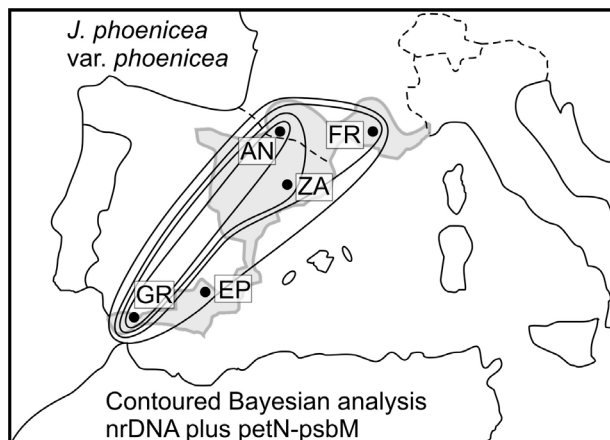


Figure 5. Contoured Bayesian analysis of *J. phoenicea* populations based on nrDNA and petN-psbM. AN = Andorra, FR = France, ZA = Zaragoza, GR = Grazalema, EP = El Penon.

In our study of geographic variation in the leaf essential oil from these same five populations of *J. phoenicea* (Adams et al., 2014), the leaf oils of one individual from Zaragoza and all the trees from Grazalema contained large amounts of typical heartwood oil components (α -cedrene, cis-thujopsene, α -alaskene, allo-cedrol, cedrol, etc.). When we corrected the leaf constituents (by removing the heartwood components and re-normalizing the percentages), there was little variation among the five populations, except for four compounds in the El Penon population: myrcene, β -phellandrene, α -terpineol were in higher concentrations, whereas, (E)-caryophyllene was present in a lower concentration. The divergence of the El Penon trees is congruent with the divergence in the DNA data presented in this study. Additional research is needed to understand the divergence of the El Penon population.

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