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Geographic variation in nrDNA and four cpDNA regions of *Juniperus excelsa*: Analysis of new records from Bulgaria, Cyprus and southwestern Turkey

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ABSTRACT

Sequencing of nrDNA, plus four cp DNA regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF of newly acquired samples of *J. excelsa* from Bulgaria, Cyprus and Turkey showed little variation in *J. excelsa* (sensu stricto), except for the unusual situation in Lebanon, where *J. excelsa* and *J. polycarpos* (and likely *J. p.* var. *turcomanica*) grow near each other and may be hybridizing. The genetic composition of the eastern-most populations of *J. excelsa* in Turkey is unknown and deserves further study. Published on-line www.phytologia.org *Phytologia* 98(1): 1-7 (Jan. 5, 2016). ISSN 030319430

KEY WORDS: *Juniperus excelsa*, *J. polycarpos* var. *polycarpos*, *J. polycarpos* var. *turcomanica*, *J. seravschanica*, DNA sequencing, nrDNA, petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF.

Recently, Adams et al. (2014a) examined *J. excelsa* and putative *J. polycarpos* from the eastern Mediterranean eastward into Azerbaijan. They reported that putative *J. excelsa* from Azerbaijan is *J. polycarpos* (= *J. excelsa* subsp. *polycarpos*). Two Lebanon *Juniperus* populations from Afqa (1306 m) and Wadi El Njass (2287 m), previously shown to be divergent in their microsatellites, were shown to be *J. excelsa* and *J. polycarpos*, respectively. This was the first report of the occurrence of *J. polycarpos* in Lebanon.

Analyses of the volatile leaf oils of *J. excelsa* (Adams et al. 2014b) revealed that the oils from Bulgaria and Greece were higher in α -pinene, limonene and β -phellandrene than populations from Turkey, Cyprus and Lebanon. Otherwise, there was little variation in the oils between these populations. Cedrol was a major component in each of the populations, ranging from 22.6 to 29.3%. Analysis of *J. polycarpus* var. *polycarpus* from Azerbaijan revealed the presence of high cedrol and zero cedrol chemotypes. The high cedrol chemotype was similar to the oil from Armenia. The Azerbaijan zero cedrol chemotype was similar to the oil from El Njass, Lebanon.

The aforementioned reports were preceded by the Douaihy et al. (2011) study in which 3 microsatellites of putative *J. excelsa*, reported that the Nei genetic distance separated their 12 populations into 3 groups: Lebanon (Leb 1, 2, 4, 5), Lebanon (Leb 3, 6) and the Crimea, Cyprus, Greece and Turkey populations. PCO of the data removed 38.8% and 27.5% on the first two axes. Ordination clearly showed: Lebanon (Leb 1, 2, 4, 5), Lebanon (Leb 3, 6) and the Crimea, Cyprus, Greece and Turkey populations. El Njass (Leb 3, 2287 m) and Aarsal (Leb 6, 2180 m) are from higher elevations in Lebanon.

Examination of plants (RPA) from Afqa, 1300 m and El Njass, 2287 m, found that Afqa plants had very fine, small leaves. The Afqa leaves were bluish green similar to *J. excelsa* from Greece. The leaves of the

El Njass plants were larger, coarse and yellowish green similar to *J. polycarpus* from Armenia and *J. p.* var. *turcomanica* from Turkmenistan.

In a comprehensive study of the reproductive ecology of *Juniperus* in Lebanon, Douaihy et al. (2013) reported differences between the higher (El Njass, Aarsal) and lower (Afqa, etc.) populations in cones density classes, frequencies of adult and juvenile trees, and dioecious (El Njass, Aarsal) vs. monoecious (Afqa, etc.) individuals. Interestingly, Adams (2014) describes *J. excelsa* as monoecious or dioecious and *J. polycarpus* as dioecious.

Juniperus excelsa M.-Bieb. grows from Greece (Fig. 2). Farjon (2005, 2010) treated *J. polycarpus*, *J. p.* var. *seravschanica* and *J. p.* var. *turcomanica* as *J. excelsa* subsp. *polycarpus*. However, Adams et al. (2008), Adams and Schwarzbach (2012) and Adams (2013), utilizing DNA sequence data, recognized *J. excelsa*, in addition to *J. polycarpus*, *J. p.* var. *turcomanica* and *J. seravschanica*. Adams

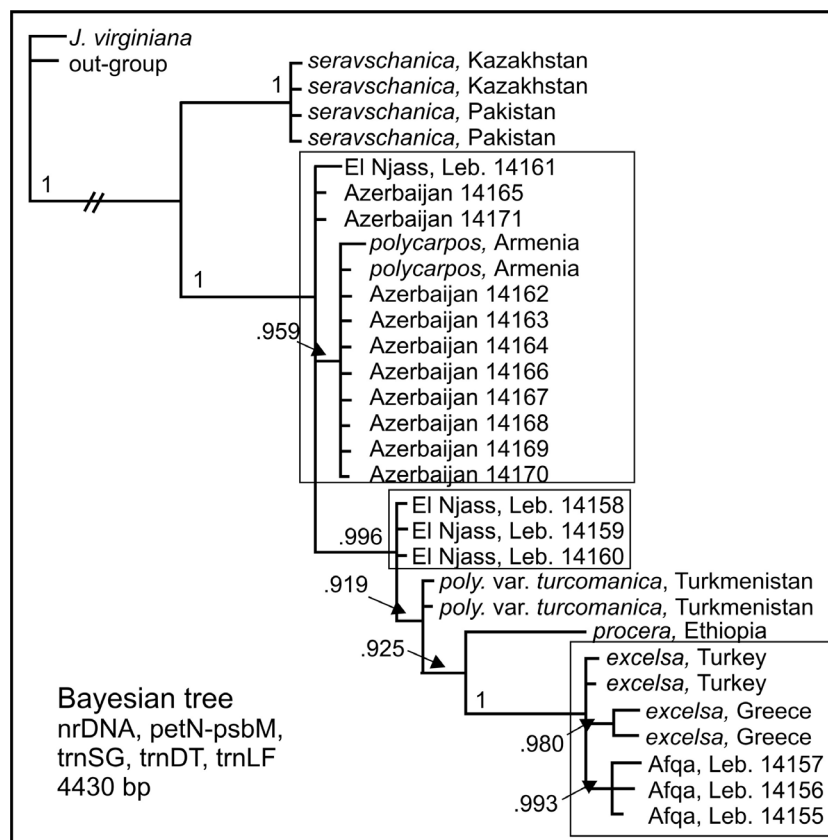


Figure 1. Bayesian tree based on nrDNA (ITS) and four cp regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF (4430 bp). Numbers at the branch points are posterior probabilities.

and Hojjati (2012) and Adams, Hojjati and Schwarzbach (2014), using sequences from 4 gene regions, did not find *J. excelsa* in Iran, but did confirm *J. polycarpus*, *J. p. var. turcomanica* and *J. seravschanica* in Iran. Putative *J. excelsa* from Qushchi, in extreme northwest Iran, had none or only one SNP difference compared with *J. polycarpus* var. *polycarpus* from Armenia and was concluded to be *J. polycarpus* (Adams and Hojjati, 2012). Adams et al. (2014a) found that putative *J. excelsa* in Azerbaijan was, in fact, *J. polycarpus* or in one case, a putative hybrid.

The distribution of *J. excelsa* in Bulgaria, Cyprus and southwestern Turkey has proved difficult to determine by modern methods of DNA sequencing and leaf essential oil data, due to the lack of samples from these regions. Recently, materials were obtained of *J. excelsa* from Bulgaria, Cyprus and southwestern Turkey. This afforded the opportunity to further examine geographic variation in the DNA sequences of *J. excelsa*.

The purpose of the paper is to examine nrDNA, and 4 cp DNA regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF and report on variation in *J. excelsa*.



Figure 2. Distribution of *J. excelsa*, *J. polycarpus* var. *polycarpus* (P) and *J. p. var. turcomanica* (T). Questionable locations of *J. excelsa* and *J. polycarpus* are indicated by E? and P? (modified from Adams, et al., 2014a).

MATERIALS AND METHODS

Plant material -

J. excelsa:

Bulgaria, Central Rhodopes, above the town of Kritchim, Reserve "Izgorialoto Gune", 42° 01' 22.0" N; 24° 28' 03.1" E, 356 m, Alex Tashev, 2012-1-JE -5-JE, 1 Sep 2012, Lab Acc. Adams 13720-13724,; **Cyprus**: 34° 57' 45.82" N, 33° 59' 55.33" E, elev. 1461m, Salih Gucl ns, 3 July 2015, Lab Acc. Adams 14570-14574; **Greece**: Lemos, ca 40° 49' N, 21° 03' E, 1100m, Adams 5983-5985, 5987; **Lebanon**: Afqa, 34° 04' 58.12"N, 35° 53' 08.52" E, 1306 m, Bouchra Douaihy 1-3, 4 Nov 2013, Lab Acc. Adams 14155-14157; **Turkey**: Antalya-Manavgat, Köprülü Canyon National Park, 37° 20' N; 31° 16' E, elev. 550 m,

Tuğrul Mataraci 2015-18, 24 May 2015, Lab Acc *Adams 14569*; Isparta-Eğirdir, junction of Kasımlar-Sütçüler road, 37° 28' N; 30° 59' E, elev. 1180 m, *Tuğrul Mataraci, 2015-7*, 24 May 2015, Lab Acc. *Adams 14596*; ~40 km north of Eskişehir, with Oaks, 39° 58.307'N; 30° 41.045' E, Turkey, 820m, *Adams 9433-9435*;

J. polycarpus:

Armenia: Lake Sevan, 1900m, *Adams 8761-8763*; **Azerbaijan**, 40° 44' 41.05" N; 47° 35' 19.14" E, 177-231m, *Vahid Farzaliyev 1-10*, Dec 2013, Lab Acc. *Adams 14162-14171*; **Lebanon**:, Wadi El Njass, 34° 20' 47.79"N, 36° 05' 45.54"E, 2287m, *Bouchra Douaihy 4-7*, 14 Nov 2013, Lab Acc. *Adams 14158-14161*;

J. polycarpus var. *turcomanica*: **Turkmenistan**: Kopet Mtns., 38° 25.12'N, 56° 58.80'E, 1535 m, 22 May 1999, *Adams 8758-8790*;

J. procera: **Ethiopia**: on the road to Guder, ca. 40 km w of Addis Abba, ca. 9° 02'N, 38° 24' W, 2400 m, *Adams 6184-6188*;

J. seravschanica: **Pakistan**: near Quetta, Baluchistan, *A. Hafeez Buzdar ns*, 6 Apr 1998, Lab Acc. *Adams 8483-8485*; **Kazakhstan**: west end of Talasskiy Ala-Tau Range, ca. 2 km S. of Dzhabagly, 42° 24.53'N, 70° 28.50'E, 1770m, 12 Sept 1997, *Adams 8224-8226*.

Voucher specimens deposited in the Herbarium, Baylor University (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° 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 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 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 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM MgCl₂ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010).

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. R7 (Biomatters. Available from <http://www.geneious.com/>), the MAFFT alignment program. Further 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. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975; Veldman, 1967).

RESULTS AND DISCUSSION

Sequencing nrDNA (ITS) and four cp regions petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF yielded 4430 bp of data. The Bayesian consensus tree shows *Juniperus seravschanica*, *J. polycarpus*, *J. p.* var. *turcomanica*, *J. procera* and *J. excelsa* in well-supported clades. *J. excelsa* samples, newly

collected from Bulgaria, Cyprus, and sw Turkey, are in a clade with other *J. excelsa* (Fig. 3). There is some minor variation among the *J. excelsa* samples, mostly notably in the Afqa, Lebanon population.

All of the *J. polycarpus* samples from Azerbaijan are closely related with *J. polycarpus*, Armenia along with the El Njass, Lebanon (Adams 14161) sample (Fig. 3). Three other El Njass samples (Adams 14158, 14159, 14160) appear to be intermediate between *J. polycarpus* and *J. p.* var. *turcomanica* (Fig. 3).

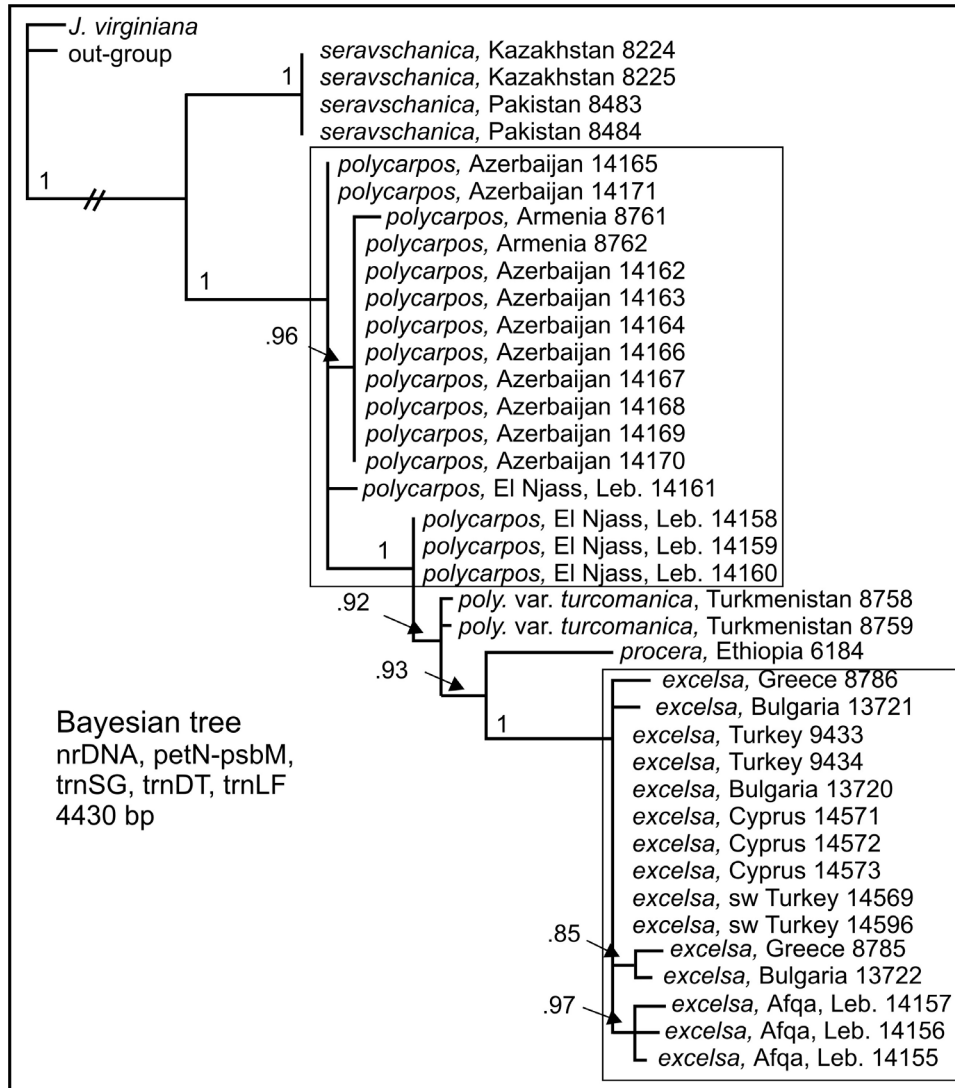


Figure 3. Bayesian analysis based on nrDNA, petN-psbM, trnSG, trnDT and trnLF. Numbers at the branch points are posterior probabilities.

Overlaying a minimum spanning network onto a distribution map gives one a perspective of the geographic trends (Fig. 4). The newly sampled *J. excelsa* populations from Bulgaria, Cyprus and sw Turkey are identical or nearly identical to *J. excelsa* of Eskisehir, Turkey (Fig. 4). Both the Cyprus and southwestern Turkey populations of *J. excelsa* showed no differences (Fig. 4). The Bulgaria *J. excelsa* differed by none or one difference from Eskisehir, Turkey (Fig. 4).

As previously reported (Adams et al., 2014a), the Afqa, Lebanon *J. excelsa* population differs by 2 MEs from Eskisehir, Turkey, which in turn, differs by only 1 ME from the Lemos, Greece population (Fig. 4). The other Lebanon populations that group with Afqa are probably *J. excelsa*.

However, the Wadi El Njass, Lebanon (2287 m) population, although near Afqa, is *J. polycarpus* and differs by 1 to 3 MEs from *J. p. var. turcomanica*, Turkmenistan and by 1 to 2 MEs from *J. polycarpus*, Armenia (Fig. 4). The *J. excelsa*, Afqa population is only about 100 - 150 km from other *J. excelsa* populations (Fig. 4), but the Wadi El Njass, *J. polycarpus* population is 700 to 1000 km from the nearest *J. polycarpus* population, still, it differs by only 1 to 3 MEs.



Figure 4. Minimum spanning network mapped onto the distributions of *J. excelsa* and *J. polycarpus*. Numbers next to lines are the number of MEs (Mutational Events = base substitutions plus indels).

Clearly, DNA sequencing shows that *J. excelsa*, as sampled in this study, is a fairly uniform species, except for the unusual situation in Lebanon, where *J. excelsa* and *J. polycarpus* (and likely *J. p. var. turcomanica*) grow near each other and may be hybridizing. The genetic composition of the easternmost populations of *J. excelsa* in Turkey is unknown and deserves study.

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