TAXONOMY OF *JUNIPERUS* IN IRAN:
INSIGHT FROM DNA SEQUENCING

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

and

Fatemeh Hojjati
Dept. of Plant Sciences, University of Tehran, Box 14155-6455,
Tehran, Iran

ABSTRACT

Sequence data from four gene regions (nrDNA, petN-psbM, trnD-trnT, trnS-trnG, 3,705 bp) revealed that junipers from the southern mountain ranges of Iran are very diverse. The combined NJ tree (3,705 bp) showed strong support for the distinct nature of *J. excelsa*, *J. polycarpos*, *J. p*. var. *turcomanica*, and *J. p*. var. *seravschanica*. The samples from NW Iran are *J. polycarpos* and samples from NE Iran are clearly *J. p*. var. *turcomanica*, as are the samples from Fasa in SW Iran. The samples from nearby south central Iran (Khabr) are part of a clade with *J. p*. var. *seravschanica.* However, the divergence of the Khabr and Kuhbanan junipers from *J. p*. var. *seravschanica* (9 bp or more) is so great that a new taxon may be present in southern Iran. *Phytologia 94(2): 219-227 (August 1, 2012).*

KEY WORDS: *Juniperus polycarpos* var. *polycarpos, J. p.* var. *seravschanica, J. p. var. turcomanica, J. excelsa,* Cupressaceae, Iran, nrDNA, petN-psbM, trnD-trnT, trnS-trnG.

The distributions of *J. excelsa* M.-Bieb. and *J. polycarpos* K. Koch in Iran and the surrounding region are not well understood. Adams (2011) noted the occurrence of *J. excelsa* in Turkey and thence eastward into Armenia with the *J. p*. var. *polycarpos* mapped into the southern Iran mountains (Fig. 1). However the taxa are very similar in

morphology and specimens in southern Iran do not seem typical of *J. p.* var. *polycarpos* (Adams, 2011).
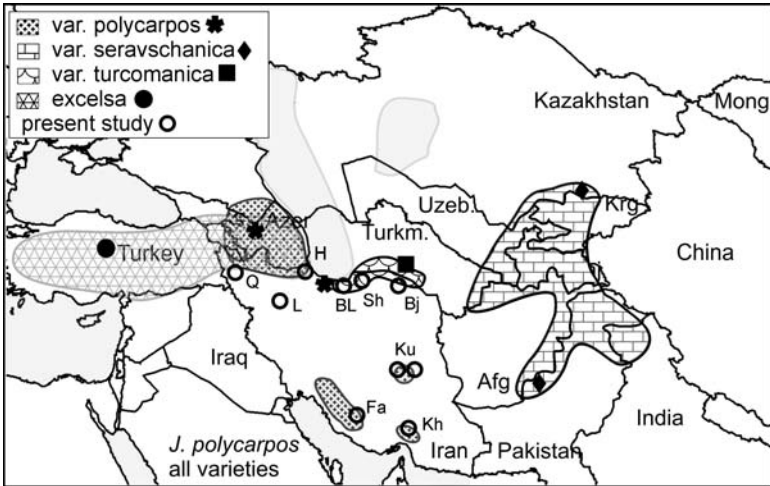

Figure 1. Distributions of *J. excelsa* (Greece not shown), *J. polycarpos* var. *polycarpos*, *J. p.* var. *seravschanica*, *J. p.* var. *turcomanica.* (adapted from Adams, 2011). Symbols indicate the populations sampled for each taxon.

Recently, Hojjati et al. (2009) reported on variation in isozymes of *Juniperus* from throughout Iran (Fig. 2). They concluded that their samples from northwestern Iran were *Juniperus polycarpos* (group C, Fig. 2); *J. p.* var. *turcomanica* (B. Fedtsch.) R. P. Adams (group D, Fig. 2) in northeastern Iran, and an undescribed, cryptic species from southern Iran (group A, Fig. 2). In addition, they concluded *J. excelsa* (*sensu stricto*) was not present in Iran. However, they did not include typical *J. excelsa* (cf. Turkey, Fig. 1) nor *J. p.* var. *seravschanica*, Fig. 1). In an early study of the taxonomic utilization of isozymes in *Juniperus*, Kelley and Adams (1978) found that isozymes were not useful in detecting known geographic variation in *J. ashei* Buch. nor for use in the taxonomy of *Juniperus*. They concluded that the homology of co-migrating bands is a potentially serious problem for the taxonomic use of isozymes.
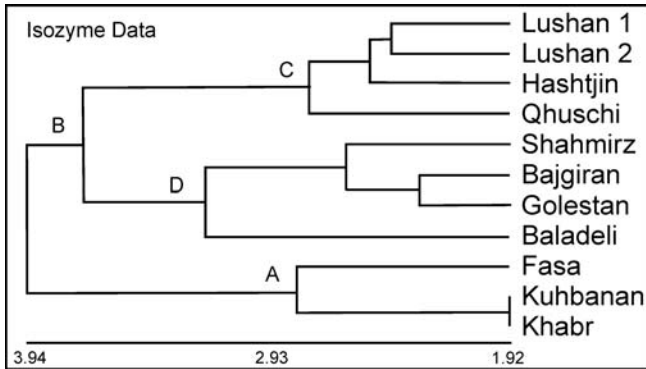
Figure 2. Phenogram of 11 populations of Juniperus from Iran based on isozymes using Euclidean distances. Adapted from data in Hojjati et al. (2009).

Recently, Adams and Shanjani (2011) have shown that DNA sequencing is very useful to elucidate these difficult central Asia taxa.

The purpose of this study was to utilize DNA sequence data from nrDNA, petN-psbM, trnD-trnT, trnS-trnG regions to analyze *Juniperus* from throughout Iran.

MATERIALS AND METHODS

In order to address variation in Iran junipers, samples were selected from specific populations (Fig. 2) that were examined by Hojjati et al. (2010). DNA was extracted from plant materials from the following Hojjati populations (Popn. # and symbols are compatible with Hojjati et al., 2009):
Popn 1 L1, Lushan: *Adams 12789-12791*, 36° 40' 27"N, 49° 38' 49.5" E, Oct, 2006, Popn 2:L2, Lushan *Adams* 12792-94 (3), 36° 40' 50" N, 49° 42' 24" E, Oct. 2006, Popn 3: H, Hashtjin *Adams 12795-12797*, 37° 26' 59" N, 48° 24' 13" E, Oct., 2006, Popn 4: Q, Qushchi *Adams 1*2798, 38° 01' 20.3" N, 44° 57' 45.5" E, Oct., 2006, Popn 5: Sh, Shahmirzad *Adams 12799-12801*, 35° 50' 55" N, 53° 26' 24.2" E, Nov, 2006, Popn 6: Bj, Bajgiran *Adams 12802-12804*, 37° 25' 9.8" N, 58° 32' 0.2" E,

Nov, 2006, Popn 7: G, Golestan *Adams 12805-12807*, 37° 19' 46.3" N, 56° 02' 34.2" E, Nov, 2006, Popn 8: BL, Baladae *Adams 12808*, 36° 14' 34.4" N, 51° 50' 20.4" E, Oct, 2006, Popn 9: F, Fasa *Adams 12809-12811*, 29° 09' 57.8" N, 53° 40' 7.8" E, Dec, 2006, Popn 10: Ku, Kuhbanan Adams 12812-12814, 31° 28' 21.5" N, 55° 52' 58.9" E, Jan, 2007, Popn 11: Kh, Khabr *Adams 12815-12817*, 28° 51' 8.4" N, 56° 22' 51.7" E, Jan, 2007.

       Authentic, typical taxonomically identifiable reference taxa, were included from *J. excelsa,* n of Eskisehir, Turkey, *Adams 9433-9435*, *J. polycarpos* var. *polycarpos*, Lake Sevan, Armenia, *Adams 8761-8763*, *J. p.* var. *seravschanica*, Quetta, Pakistan, *Adams 8483-8485*, Dzhabagly, Kazakhstan, *Adams 8224-8226*; Elburz Mtns., Iran, *Shanjani s. n.*, [=*Adams 12603, 12604*].   Voucher specimens are deposited at Baylor University (BAYLU).

        DNA Analysis - 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 using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). PCR 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-psbM, trnDT, trnSG) 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 $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams et al. (2011) for the ITS, petN-psbM, trn D-trnT and trnS-trnG primers utilized. The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.).   In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit. The gel purified DNA band with the appropriate primer was sent to McLab Inc. (South San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.). Alignments and NJ trees were made using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/). Minimum spanning networks were constructed from SNPs data using PCODNA software (Adams et al., 2009). Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the maximum observed value for that compound over all taxa (= Gower

metric, Gower, 1971; Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

The NJ tree based on 3,705 bp shows (Fig. 3) strong support (100%) for the distinct nature of *J. excelsa*, *J. polycarpos*, *J. p.* var. *turcomanica*, and *J. p.* var. *seravschanica*. The samples from NW Iran (L, H, Q) are nested with *J. polycarpos* as found in the isozyme data (Fig. 2). Samples from NE Iran (Sh, BS, BL) are clearly *J. p.* var. *turcomanica* (Fig. 3) as are the samples from Fasa in SW Iran. The
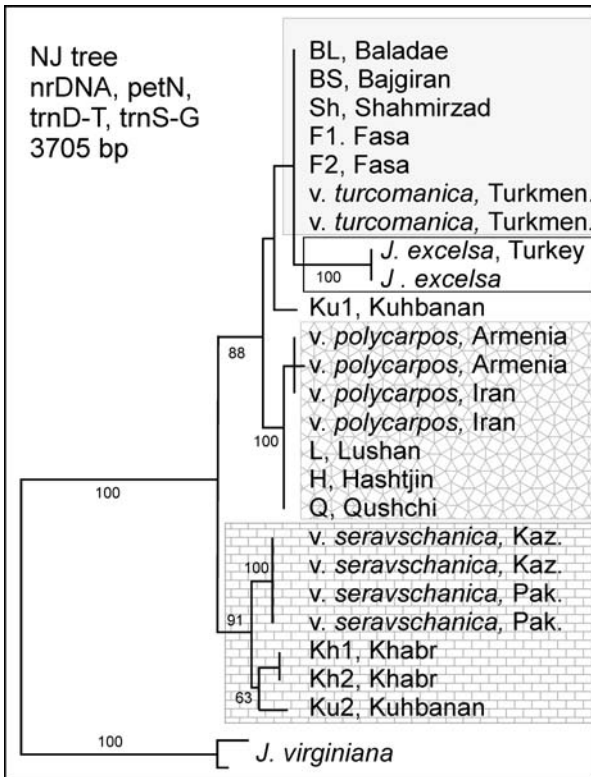

Figure 3. NJ tree based on four gene regions. See text for discussion.

samples from nearby south central Iran (Khabr) are part of a clade with *J. p.* var. *seravschanica* from Pakistan and Kazakhstan. There is support (63%) for a clade composed of Khabr (Kr1, Kr1) and Kuhbanan (Ku2). The Kuhbanan region seems to contain two taxa, as represented by Ku1 and Ku2 (Fig. 3).

To examine the amount of differentiation in DNA, both substitutions and indels were coded and a minimum spanning network was constructed (Fig. 4). The vast majority (65/103) of SNPs separate the out-group (*J. virginiana*). No variation was found in v. *seravschanica* from Pakistan and Kazakhstan (Fig. 4). The Khabr plants are separated by 9 SNPs from var. *seravschanica* and by 6 SNPs from the KU2 individual. The *seravschanica* complex is the most distinct (23 SNPs, Fig. 4), followed by *J. excelsa* (Turkey, 15 SNPs, Fig. 4). The NW Iran samples (L, H. Q) differ by zero or one SNP from var. *polycarpos*, Armenia (Fig. 4). The samples from NE Iran (BL, BS, Sh) are closely linked to var. *turcomanica*, Turkmenistan, along with plants from Fasa in SW Iran (F1, F2, Fig. 4). However, an individual (Ku1) from Kuhbanan differs by only 5 SNPs from Fasa.

To visualize the geographical trends, the minimum spanning network was overlaid on a geographic map (Fig. 5). This visual image highlights the uniformity of var. *polycarpos* from Armenia into NW Iran and var. *turcomanica* in NE Iran and thence into the Fasa population in SW Iran (Fig. 5). Note the area in N Iran, where possibly sympatric var. *polycarpos* (Sj) and var. *turcomanica* (BL) differ by 9 bp. The Fasa population is large (see distribution map, Fig. 1). It is not known at this time if this large region is composed of only var. *turcomanica*.

It is interesting the Khabr plants and one Kuhbanan plant differ by only 9 SNPs from var. *seravschanica* in nearby Pakistan. It appears that two taxa are present in the Kuhbanan region. Our two samples (Ku1, Ku2) differ by 24 SNPs (Fig. 5), comparable to the difference between Fasa - Khabr (30 SNPs, Fig. 5). Additional studies are needed to more fully resolve these taxonomic and distributional questions (in progress by authors).
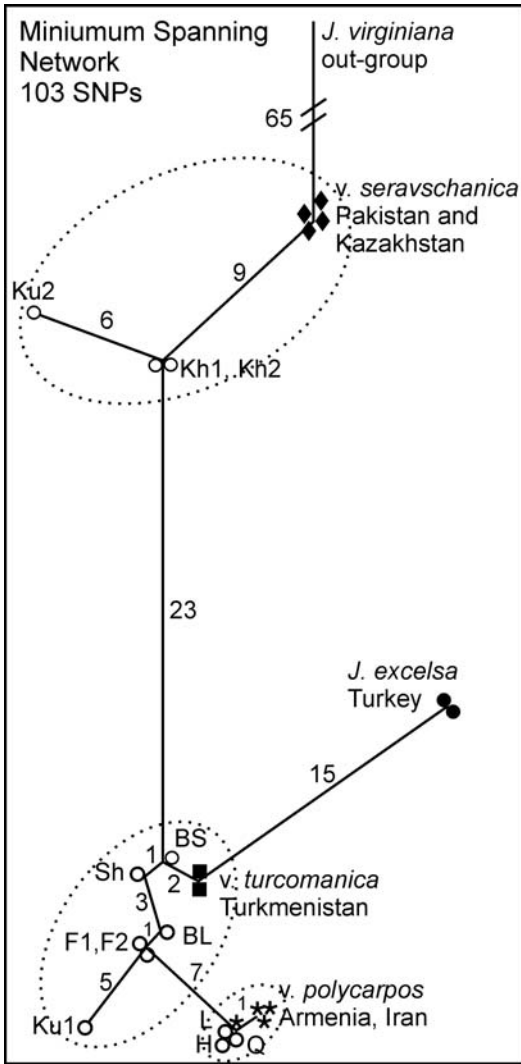
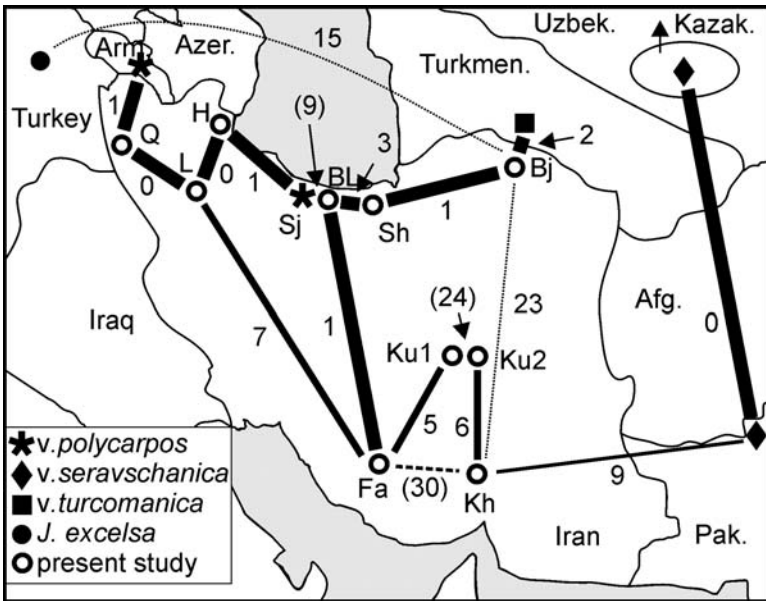Figure 4. Minimum spanning network based on 103 SNPs. The numbers next to the links are the number of SNPs.

Figure 5. Minimum linkage map based on 37 SNPs. Line widths are proportional to similarity. Numbers next to links are the number of SNPs differing adjacent nodes. Notice the southern Iran populations of Khabr (Kh) and Kuhbanan (Ku2) are loosely linked var. *seravschanica* (diamonds) whereas the other S Iran population (Fasa, Fa) is most closely linked to var. *turcomanica* (Turkmenistan)differs by on 1 SNP from BL and.

ACKNOWLEDGEMENTS

LITERATURE CITED

Adams, R. P. 1975. Statistical character weighting and similarity
    stability. Brittonia 27: 305-316.
Adams, R. P. 2011. The junipers of the world: The genus *Juniperus*.
    3rd ed. Trafford Publ., Victoria, BC.

Adams, R. P., J. A. Bartel and R. A. Price. 2009. A new genus, *Hesperocyparis*, for the cypresses of the new world. Phytologia 91: 160-185.

Adams, R. P., J. A. Morris and A. E. Schwarzbach. 2008. Taxonomic study of *Juniperus excelsa* and *J. polycarpos* using SNPs from nrDNA and cp trnC-trnD plus essential oils and RAPD data. Phytologia 90: 208-225.

Adams, R. P. and P. S. Shanjani. 2011. Identification of the Elburz Mountains, Iran juniper as *Juniperus polycarpos* var. *polycarpos*. Phytologia 93: 316-321.

Gower, J. C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53: 326-338.

Gower, J. C. 1971. A general coefficient of similarity and some of its properties. Biometrics 27: 857-874.

Hojjati, F., S. Zarre and M. Assadi. 2009. Isoenzyme diversity and cryptic speciation in Juniperus excelsa (Cupressaceae) complex in Iran. Biochem. Syst. Ecol. 37: 193-200.

Kelley, W. A. and R. P. Adams. 1978. Analysis of isozyme variation in natural populations of *Juniperus ashei*. Rhodora 80:107-134.

Shanjani, P. S., M. Mirza, M. Calagari and R. P. Adams. 2010. Effects of drying and harvest season on the essential oil composition from foliage and berries of *Juniperus excelsa*. Industrial Crops and Products 32: 83-87.

Veldman D. J., 1967. Fortran programming for the behavioral sciences. Holt, Rinehart and Winston Publ., NY.