# Taxonomy of *Juniperus* in Iran: DNA sequences of nrDNA plus three cpDNAs reveal *Juniperus polycarpos* var. *turcomanica* and *J. seravschanica* in southern Iran

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## ABSTRACT

Sequence data from four gene regions (nrDNA, petN-psbM, trnD-trnT, trnS-trnG, 3,708 bp) showed strong support for the plants from Fasa in SW Iran being *J. p*. var. *turcomanica*. The samples from nearby south central Iran (Khabr) are part of a clade with *J. seravschanica.* Nine samples from Kuhbanan are in a clade with *J. seravschanica*, but 2 samples are in a clade with *J. p*. var. *turcomanica*. A minimum spanning network revealed that the Kuhbanan trees are genetically diverse and differentiated from more typical *J. seravschanica.* Nearly all samples from Kuhbanan were polymorphic in their nrDNA (ITS) chromatograms, implying either hybridization or incomplete lineage sorting. Additional research utilizing other nuclear genes (cf. ABI3, 4CL, etc.) will aid in understanding this complex. Published on-line **www.phytologia.org** *Phytologia 96(1): 19-25 (Jan. 8, 2014).* ISSN 030319430

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

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Previously we reported (Adams and Hojjati, 2012) that *Juniperus* from Iran has a complex mixture of taxa in southern Iran (Fig. 1); *J. polycarpos* var. *polycarpos* from Hashtjin (H), Lushan (L), and Qushchi, Q) were confirmed, as was *J. p.* var. *turcomanica* from Baladae (BL), Bajgiran (Bj) and Shahmirzad (Sh) in northern Iran. In southern Iran, the two plants from Fasa (F) were found to be *J. p*. var. *turcomanica* (Fig. 2). Two samples from Khabr were separated by 9 SNPs

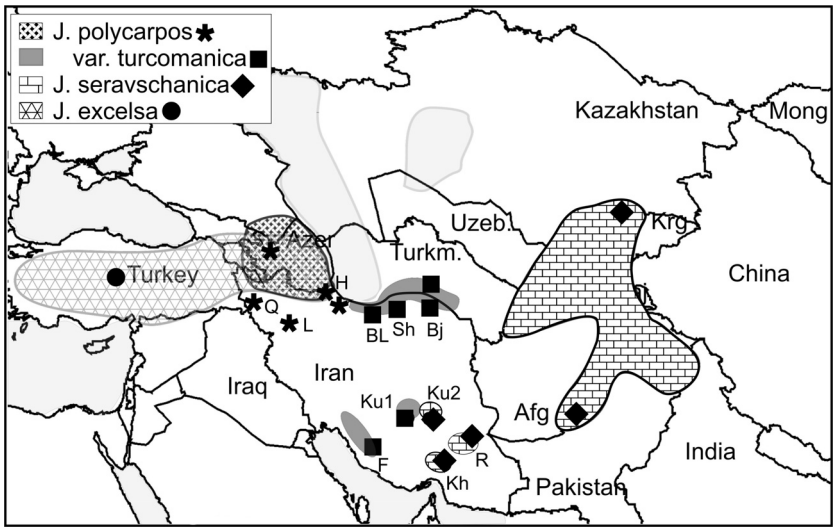


Figure 1. Dist. of *J. excelsa* (Greece not shown), *J. polycarpos*, *J. seravschanica*, and *J. p.* var. *turcomanica* (from Adams and Hojjati, 2012). Symbols indicate the popns. sampled for each taxon.

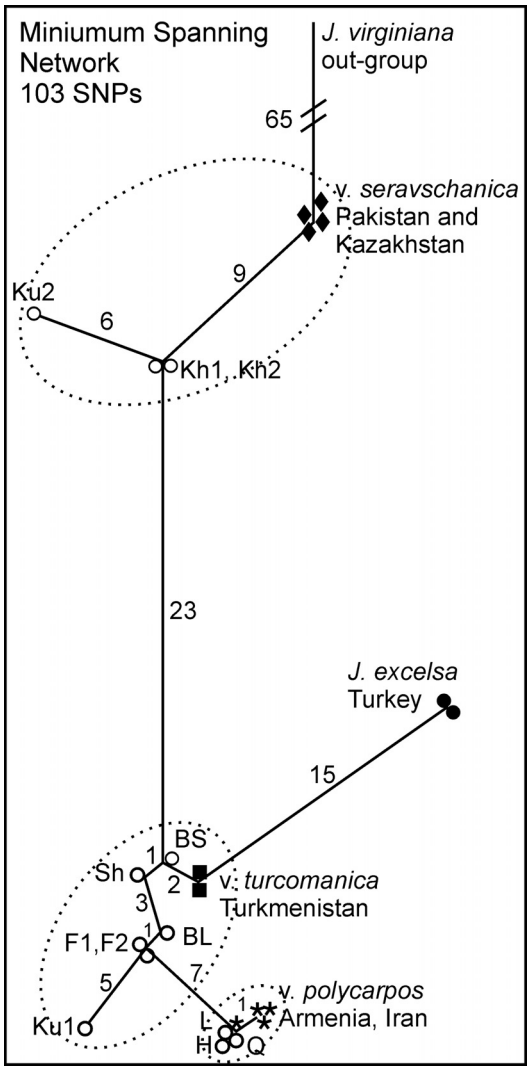


Fig. 2. Minimum spanning network. Numbers on lines are the number of SNPs for that link (from Adams and Hojjati, 2012).

(substitutions + indels) from *J. seravschanica* (Pakistan and Kazakhstan, Fig. 2). One sample from Kuhbanan (Ku1) was found to be *J. p*. var. *turcomanica* and the other (Ku2) was like *J. seravschanica* (Fig. 2). The Ku2 sample differed by 6 SNPs from Kh1 and Kh2, Suggesting that another infraspecific taxon of *J. seravschanica* might be present southern Iran.

To gather additional data, more samples were gathered from southern Iran and the leaf volatile oils analyzed (Adams and Hojjati, 2013).

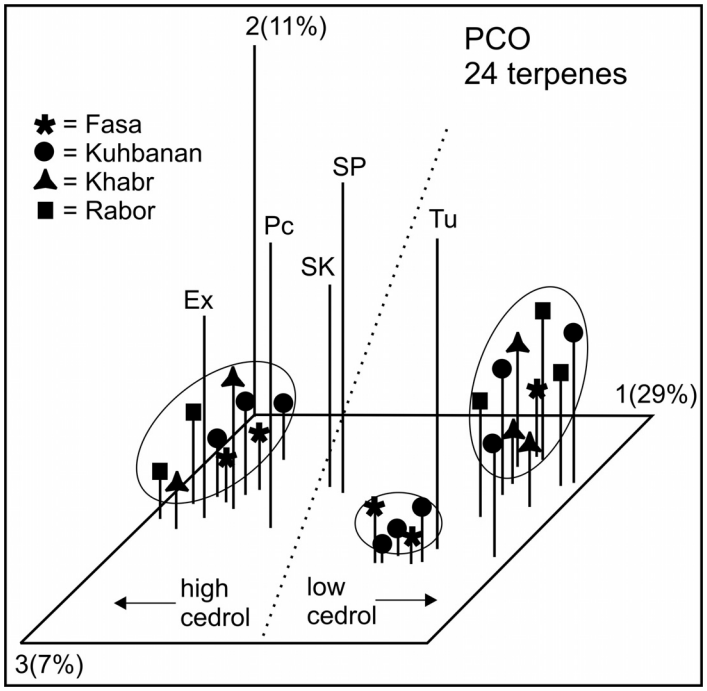


Fig. 3. PCO based on 24 terpenes of junipers of southern Iran. Ex= *J. excelsa*, Pc= *J. polycarpos*, SP, SK= *J. seravschanica*, from Pakistan and Kazakhstan (from Adams and Hojjati, 2013).

However, the oils were dominated by two chemotypes: high cedrol (wood oil sesquiterpenes) and low cedrol (leaf oil terpenoids). These chemotypes appeared to group irrespective of locations (Fig. 3). Interestingly, none of the oils from recognized species was closely grouped with the oils of junipers from southern Iran except *J. excelsa* (Fig.3).

Because the original DNA study (Adams and Hojjati, 2012) utilized only 2 samples from each of three southern Iran populations, additional samples were analyzed. The purpose of the present study was to utilize DNA sequence data from nrDNA, petN-psbM, trnD-trnT, trnS-trnG regions to analyze additional samples of *Juniperus* from southern Iran so as to better discern the taxonomic patterns in that region.

## MATERIALS AND METHODS

In order to address variation in Iranian junipers, samples were selected from the same populations (Fig. 1) examined by Hojjati et al. (2009). 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;* Dec, 2006, *Adams 13754-13758*, Oct. 2012, 29° 09' 57.8" N, 53° 40' 7.8" E, Popn 10: Ku, Kuhbanan *Adams 12812-12814,* Jan, 2007, *Adams 13759-13767,* Oct. 2012, 31° 28' 21.5" N, 55° 52' 58.9" E, Popn 11: Kh, Khabr *Adams 12815-12817,* Jan, 2007, *Adams 13768-13772*, Oct. 2012, 28° 51' 8.4" N, 56° 22' 51.7" E, R, Rabor *Adams 13773-13777*, Oct. 2012, 28° 49' 06.7" N; 56° 21' 21.7" W. elev. 2086 m.

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. *turcomanica*, Kopet Mtns., Turkmenistan, *Adams 8757-8760, J. seravschanica*, Quetta, Pakistan, *Adams 8483-8485*, Dzhabagly, Kazakhstan, *Adams 8224-8226*; Elburz Mtns., Iran, *Shanjani s. n.*, (see Adams and Shanjani, 2011) [=*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$^{o}$ 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 and Schwarzbach (2013) for the ITS, petN-psbM, trn D-trnT and trnS-trnG primers utilized. The PCR 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). Sequence data sets were analyzed using Geneious v. R6-4 (Biomatters). Available from http://www.geneious.com/), and the MAFFT alignment program. Further analyses utilized the Bayesian tree 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.

## RESULTS AND DISCUSSION

The Bayesian tree based on 3,708 bp shows (Fig. 4) strong support for the distinct nature of *J. excelsa*, *J. polycarpos*, *J. p.* var. *turcomanica*, and *J. seravschanica*. as found in other studies (Adams,

Morris and Schwarzbach, 2008; Adams, 2011; Adams and Schwarzbach, 2013). The samples from north- western Iran (I1, I2, L, H, Q) are *J. polycarpos* (Fig. 4). Interestingly, one tree (F5) from Fasa in southern Iran is in the clade with *J. polycarpos*. The other 6 trees from Fasa are in a group with *J. p.* var. *turcomanica* (Fig.4) with 2 trees from Kuhbanan (U0, U1). However, all of these trees seem to be a little different from typical *J. p.* var. *turcomanica* from the Kopet Mtns., Turkmenistan (Fig. 4. There is a large group of trees from southern Iran in the large clade with *J. seravschanica* (Kazakhstan and Pakistan). However, there are lots of differences within this clade. Three trees from Rabor [R3(R2, R4)] are well supported as a distinct clade (Fig. 4). All 5 trees from Khabr form a well-supported clade.

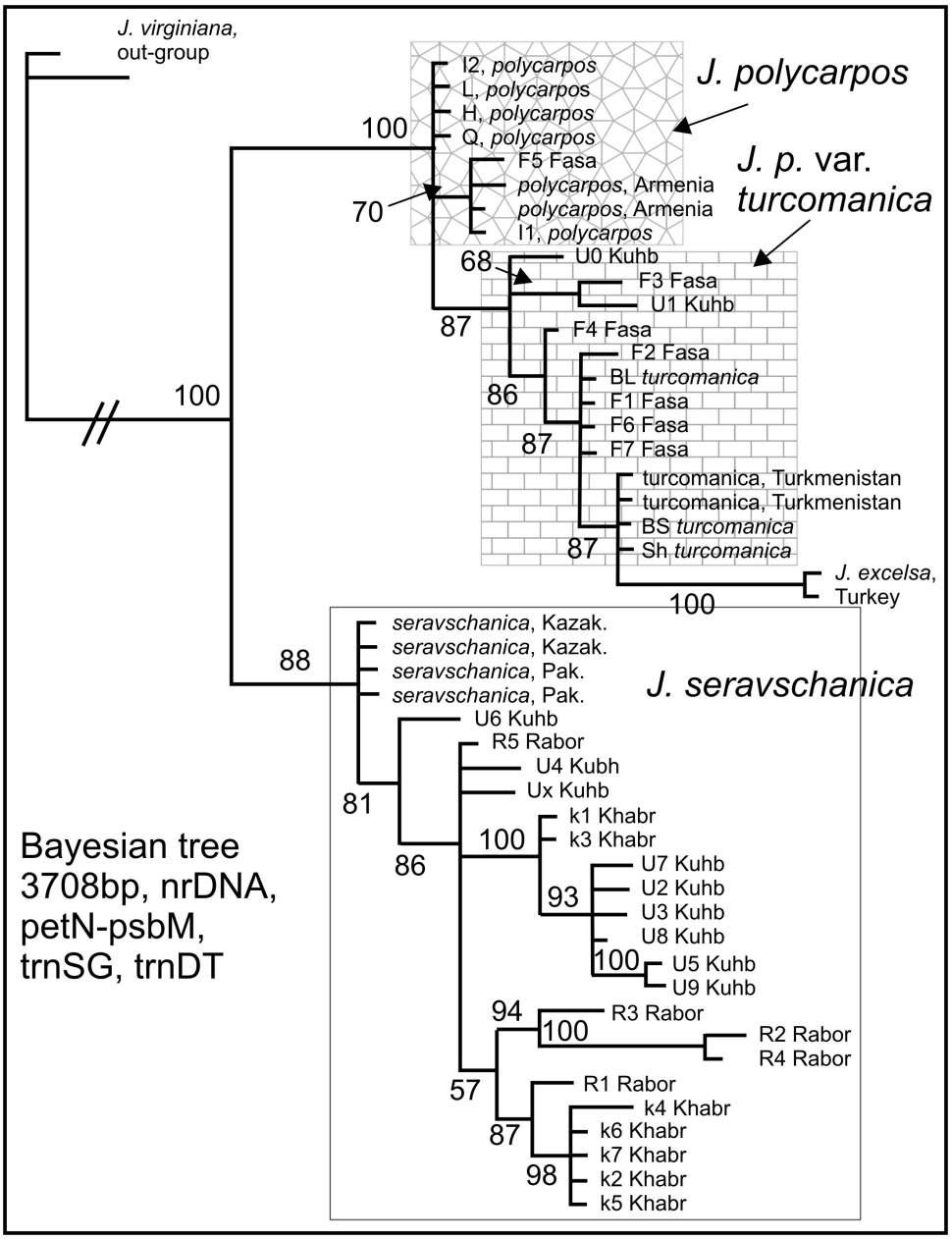


Figure 4. Bayesian tree of *Juniperus* from Iran plus exemplars of *J. excelsa*, *J. polycarpos*, *J. p*. var. *turcomanica*, and *J. seravschanica*. Numbers at branch points are posterior probabilities as percent.

To examine the magnitude of DNA differences among OTUs, a minimum spanning network was constructed using 124 MEs (mutational events = substitutions + indels). Five trees from Fasa appear to be the very uniform (Fig. 6) and differ by only 1 or 2 MEs from *J. p*. var. *turcomanica*. But F5 differs by 5 MEs from *J. p.* var. *turcomanica* and only 3 MEs from *J. polycarpos* (Fig. 5). F3 differs by 4 MEs . These trees may be relicts of ancient (or modern) hybridization between *J. polycarpos* and *J. p*. var. *turcomanica* in the Fasa area. Whether typical *J. polycarpos* occurs in the Fasa area has not been determined. Although tree F5 is in a clade with typical *J. polycarpos* from Armenia (Fig. 4), support is only 70%. The minimum spanning network indicates that F5 might be a hybrid.

Two of the trees from Kuhbanan (U0, U1) are grouped with the *J. turcomanica* from Fasa (Fig. 5), whereas 7 Kuhbanan trees are linked to *J. seravschanica* (Fig. 6). Two Kuhbanan trees (Ux, U4) are linked with Khabr samples (Fig. 5). The Kuhbanan population appears to be very diverse. This may be due to hybridization. The nrDNA (ITS) sequences indicated that most of the trees from Kuhbanan appear to be hybrids (see boxes in Fig. 5) or multi-copy due to incomplete lineage sorting. The Rabor trees are somewhat different and 2 trees (R4, R2) differ by 10 MEs from other Rabor trees (Fig. 5). This is also reflected in the Bayesian tree (Fig. 4) where R4 and R2 form a distinct clade.

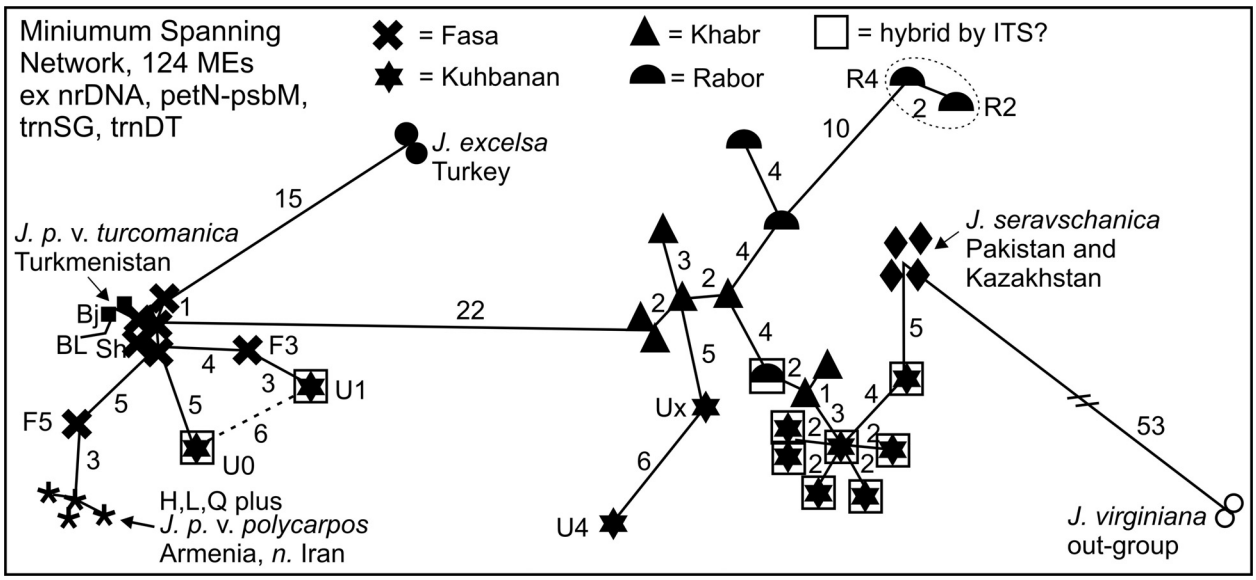


Figure 5. Minimum spanning network based on 124 MEs (mutational events = substitutions + indels). The numbers next to the lines are the number of MEs for that link.

As previously mentioned, the leaf oils of the new samples were found to have 2 chemotypes: high and low cedrol. In *Juniperus* (and most Cupressaceae), the leaf essential oils and the heartwood oils are usually very different in composition. The wood oil composition is conserved and composed of only sesquiterpenes: cf. cedrol, widdrol, α-cedrene, β-cedrene and cis-thujopsene (Adams, 1991, 2011). The leaf oils are very diverse in *Juniperus* with large amounts of monoterpenes: cf. α-pinene, sabinene, δ-3-carene, limonene, camphor, 4-terpineol, bornyl acetate; sesquiterpenes: caryophyllene, elemol, cadinenes, muurolenes, and diterpenes: cf. manool, manool oxide, abietadiene, abietatriene, trans-totarol, etc. In the western hemisphere, the wood and leaf terpene synthesis pathways appear to be almost exclusively expressed in different tissues. However, in the multi-seeded junipers of section Sabina in the eastern hemisphere (*J. excelsa, J. foetidissima, J. polycarpos, J. procera, J. seravschanica, J. thurifera*), it is common to find wood oil components in the leaf oil (but not *vice versa*). In fact, cedrol can be the major component in the leaf oils as reported by Adams and Hojjati (2013) for: *J. excelsa* - 25.4%; *J. polycarpos* - 30.3% and *J. seravschanica* - 22.7 - 13.8%. To examine the correlation of high cedrol with taxonomy, the individuals with high cedrol are circled in Fig. 6. The individuals with high cedrol are scattered

among all four populations. It seems likely the expression in different tissues may be controlled by simple mechanisms and this could be responsible for the mosiac of terpene profiles found among the taxa.

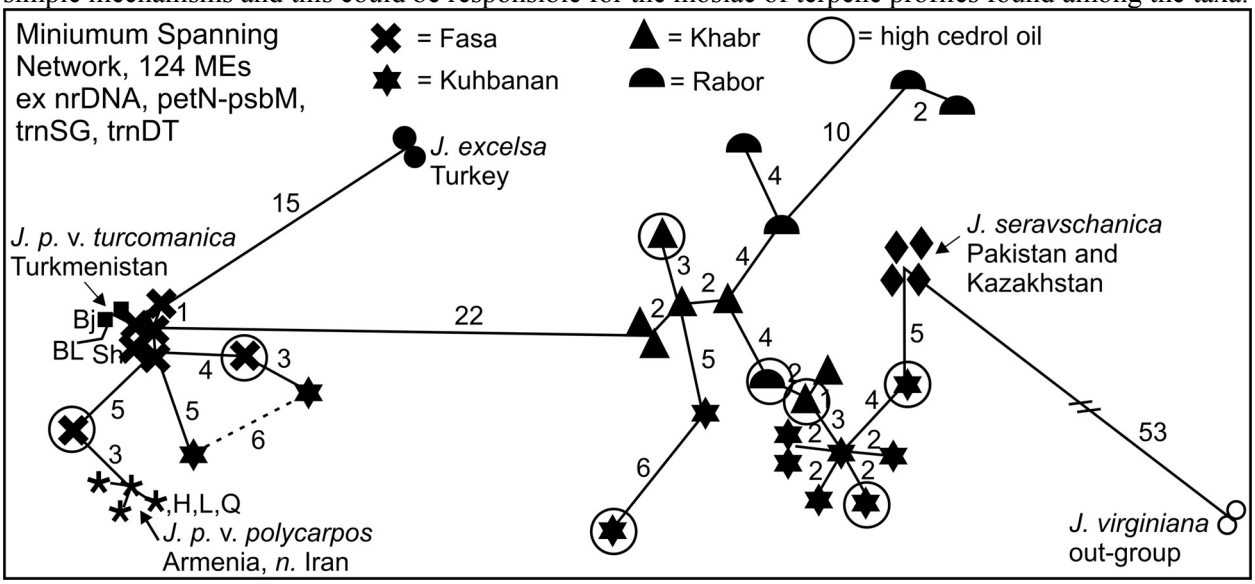


Figure 6. Minimum spanning network with individuals high in cedrol circled.

A summary figure (Fig. 7), shows that all the samples from Fasa are *J. p*. var. *turcomanica,* except F5 that is intermediate to *J. polycarpos* (Figs. 5, 7). All the other samples from Rabor and Khabr are part of *J. seravschanica* (Fig. 7). The Kuhbanan population contains 2 samples of *J. p.* var. *turcomanica* and 9 samples affiliated with *J. seravschanica.*

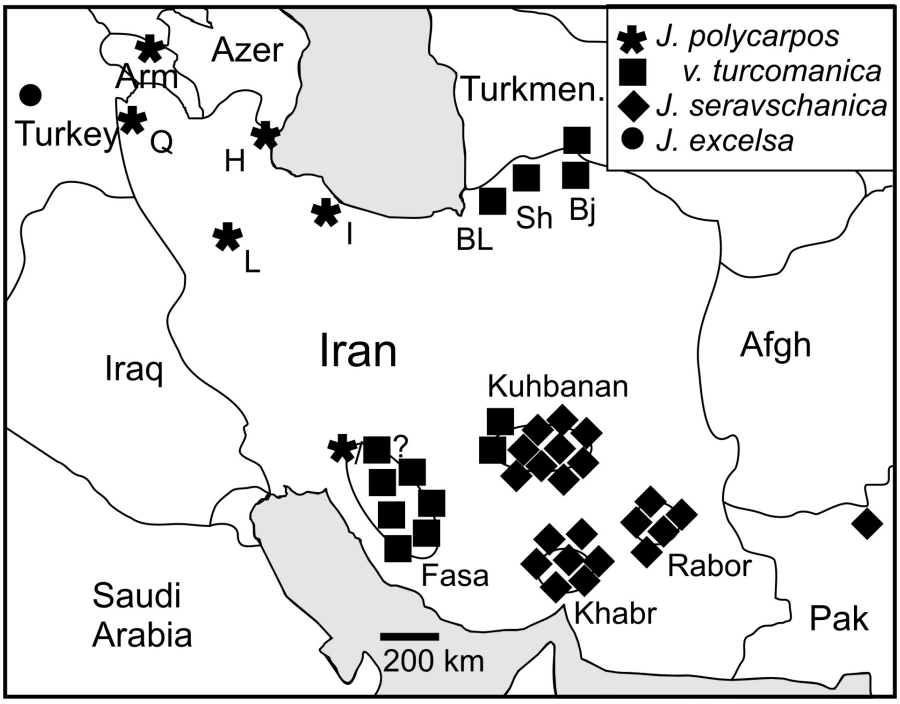


Figure 7. Distribution of *Juniperus* in Iran as per current sample analyses. The presence of *J. polycarpos* in the Fasa population is postulated based on a sample (F5) that is intermediate between *J. polycarpos* and var. *turcomanica*. The */box? in the Fasa population is tree F5 that may be a hybrid.

Nearly all samples from Kuhbanan were polymorphic in their nrDNA (ITS) chromatograms, implying either hybridization or incomplete lineage sorting with mixed nrDNA. Clearly the dynamics of *Juniperus* taxa in southern Iran are complex. Additional research utilizing other nuclear genes (ex. ABI3, 4CL, etc.) should aid in understanding this complex.

## ACKNOWLEDGEMENTS

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