

Chloroplast capture in *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev, from the Balkan peninsula: A new variety with a history of hybridization with *J. thurifera*

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ABSTRACT

An example of chloroplast capture has been identified in *Juniperus sabina* from Bulgaria and Greece in the Balkan peninsula. Nuclear DNA and overall morphology clearly indicate a close relationship to *Juniperus sabina*, whereas the cpDNA from these populations is very uniform and is nearly identical to that of *J. thurifera*, an unrelated species currently growing in France, Spain and Morocco. The new taxon is recognized as *Juniperus sabina* var. *balkanensis* R. P. Adams and A. Tashev. At present, this new variety is known only from locations in Bulgaria and Greece. Published on-line www.phytologia.org *Phytologia* 98(2): 100-111 (Apr 4, 2016). ISSN 030319430.

KEY WORDS: *Juniperus sabina* var. *balkanensis* var. nov., *J. sabina*, *J. thurifera*, relictual hybrid, nrDNA, petN-psbM, trnSG, trnDT, trnLF, leaf terpenoids, morphology, chloroplast capture.

For the past decade, the use of nrDNA and chloroplast (cp) markers has dominated phylogenetic research. However, even two decades ago, Rieseberg and Soltis (1991) warned of chloroplast capture (both recent or ancient via hybridization) that provides incongruent topologies in phylogenetic trees between nuclear and cp data. They found evidence of chloroplast capture in 37 cases and, of those, 28 were thought to be conclusive (Table 1, Rieseberg and Soltis, 1991). With the explosion of the use of nrDNA and cp markers, there are hundreds of examples of chloroplast capture today. A few recent examples of putative chloroplast capture include *Heuchera* (Soltis and Kuzoff, 1995), *Brassica napus* - *B. rapa* (Haider et al. 2009), and *Osmorhiza* (Yi et al., 2015).

There are fewer examples of chloroplast capture in conifers. In *Pinus* and other conifers, Hipkins et al. (1994) concluded that "past hybridization and associated 'chloroplast capture' can confuse the phylogenies of conifers." Bouille et al. (2011) found significant topological differences in phylogenetic trees based on cpDNA (vs. mtDNA sequences) in *Picea* that suggested organelle capture.

In *Juniperus*, Terry et al. (2000) suggested that chloroplast capture was involved in the distribution of cp haplotypes in *J. osteosperma* in western North America. More recently, Adams (2015a, b) found widespread hybridization and introgression between *J. maritima* and *J. scopulorum* in the Pacific northwest, with introgression from *J. maritima* into *J. scopulorum* eastward into Montana. The disparity between cpDNA and nuclear markers (nrDNA and maldehy) suggested that cp capture had occurred.

Although chloroplast capture, on its face, seems unlikely, Tsitrone et al. (2003) proposed a model of chloroplast capture that provides some basis for the concept.

The genus *Juniperus* consists of approximately 75 species (Adams, 2014), all of which grow in the northern hemisphere, although, *J. procera* Hochst. ex Endl. also grows southward along the rift mountains in East Africa into the southern hemisphere (Adams, 2014). The recent molecular phylogeny of the genus (Adams and Schwarzbach, 2013b) divides *Juniperus* into three sections: *Caryocedrus* (one species, *J. drupacea* Labill.); *Juniperus* (= *Oxycedrus*) with 14 species and *Sabina* (the remaining 60 species).

Section *Sabina* can be further divided into junipers with serrate and those with entire (smooth) leaf margins. The serrate-leaf margined junipers are confined to the western hemisphere, except for *J. phoenicea*, which may have a greater affinity to the smooth-leaf margined junipers (Adams and Schwarzbach, 2013b).

The *Juniperus* of section *Sabina*, of the eastern hemisphere, can be further divided into two groups based on the number of seeds per female cone (often called berries) and female cone shape. The single seed/cone (single-seeded) *Juniperus* of the eastern hemisphere have cones that are ovoid with a noticeable pointed tip, whereas the multi-seeded *Juniperus* are generally globose and often have an irregular surface. *Juniperus sabina* L. is a smooth leaf-margined, multi-seeded juniper of the eastern hemisphere. It is very widely distributed from Spain through Europe to Kazakhstan, western China, Mongolia and Siberia (Fig. 1). *Juniperus sabina* has a range that is discontinuous between Europe and central Asia; the species is generally a shrub less than 1 m tall and ranges up to 1-2 m wide. But in the Sierra Nevada of Spain, it forms a horizontal shrub.



Fig. 1. Distribution of *J. sabina*. x = outlying populations of *J. sabina*.

DNA sequencing (Adams and Schwarzbach, 2013a), based on new collections of *J. sabina* var. *arenaria* from Lake Qinghai and a river bank in Gansu, as well as additional samples from Mongolia, has led to a different picture of the relationships in the *chinensis-erectopatens-davurica-sabina* complex (Fig. 2). Notice that *J. erectopatens* was 100% (posterior probabilities) supported as a distinct clade, as previously shown in both essential oils and RAPD data (Adams, 1999). There was no support for treating *J. erectopatens* as a synonym of *J. chinensis* (Farjon, 2005). *Juniperus erectopatens* is a cryptic species in its morphology, but it is quite distinct as an evolutionary unit in its terpenes, RAPD markers and DNA sequence data. *Juniperus chinensis* (and *J. procumbens*) were also well supported (100%) as being

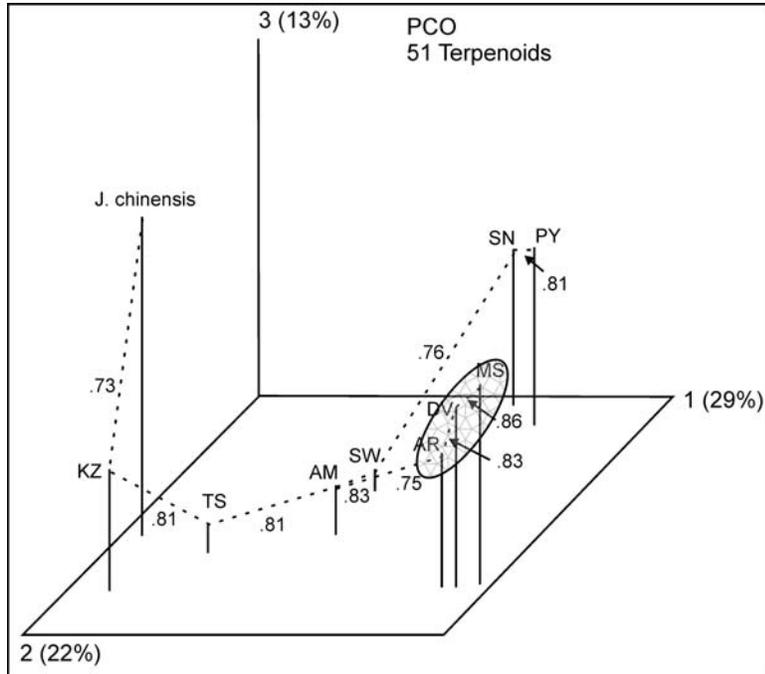
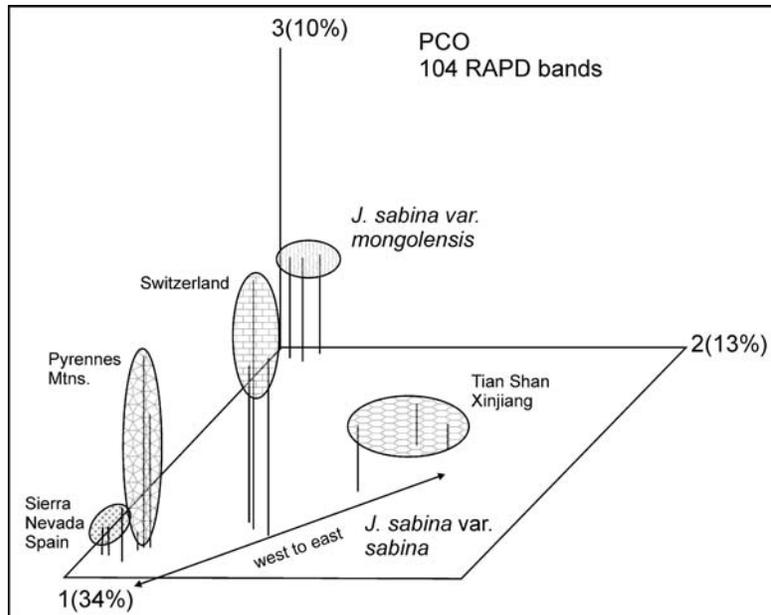


Fig. 3. Principal Coordinate Ordination (PCO) (from Adams, Nguyen and Liu, 2006) based on 51 terpenoids. Notice the distinct ordination of *J. chinensis* and the prostrate junipers of n China and Mongolia (AR = *J. d. var. arenaria*; DV = *J. davurica*; MS = *J. d. var. mongolensis*).

J. sabina:
KZ = Kazakhstan;
TS = Tian Shan Mtns., Xinjiang, China;
AM = Altai Mtns., w Mongolia;
SW = Switzerland;
PY = Pyrenees;
SN = Sierra Nevada, Spain

Fig. 4. PCO showing west to east clinal variation using RAPD bands, within *J. s. var. sabina* (from Adams et al. 2007).



Recently, in routine analyses of *J. sabina* from Bulgaria and northern Greece, we found plants that appeared to have non-*J. sabina* chloroplasts (based on petN-psbM, trnSG, trnDT and trnLF sequences). The purpose of the present paper is to report on these unusual plants and the taxonomy and evolution of this taxon.

MATERIAL AND METHODS

Specimens used in this study (species, popn. id., location, collection numbers): *J. chinensis*, CH, Lanzhou, Gansu, China, Adams 6765-6767; *J. sabina*: (SN), Sierra Nevada, Spain, Adams 7197, 7199, 7200; (PY), Pyrenees Mtns., Spain/ France border, Adams 7573-7577; (SW), Switzerland, Adams 7611, 7612, 7614, 7615; TS, Tian Shan Mtns., Xinjiang, China, Adams 7836-7838; Mongolia, Altai Mtns., Adams 7585-7587; Kazakhstan, Paniflor, Adams 7811-7812; Azerbaijan: Adams 14316-14320; *J. davurica* (DV), 15 km se Ulan Bator, Mongolia, Adams 7252, 7253, 7601; *J. davurica* var. *arenaria* (AR) sand dunes, Lake Qinghai, Qinghai, China, Adams 10347-10352; river bank, Gansu, J-Q. Liu and Adams 10354-10356; *J. davurica* var. *mongolensis* (MS) sand dunes, 80 km sw Ulan Bator, Mongolia, Adams 7254-7256;

Collections of new taxon with non-*J. sabina* cpDNA: (acronyms used in Fig. 7)

B1-B5 Eastern Rhodopes, Bulgaria, Adams 13725-13729 (A. Tashev 2012-1-5);

B6 Central Stara Planina, Sokolna reserve, Bulgaria, Adams 14721 (A. Tashev 2015 Balkan 1);

B7-B9, Ba, Bb Rila Mountain, Bulgaria, Adams 14722-14726 (A. Tashev 2015 Rila 1.1-1.3, 2.1-2.2);

G1-G5 Mt. Tsena, Greece, Adams 14727-14731 (A. Tashev 2015 So. 1-5 Tsena);

Voucher specimens for all collections are deposited at Baylor University Herbarium (BAYLU) and Herbarium (University of Forestry, Sofia, Bulgaria).

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, Bartel and Price, 2009; Adams, 1975; Veldman, 1967).

RESULTS

Sequencing nrDNA for the *J. sabina* from Bulgaria and Greece resulted in 1270 bp of data. Bayesian analysis (including all the smooth leaf, globose cone *Juniperus* of section *Sabina*), shows (Fig. 5) that the *J. sabina* 'balkanensis' plants are in a diverse clade with most of the other *J. sabina* accessions. They are not distinct. In addition, *J. sabina* from Azerbaijan and Switzerland, plus one plant from the Altai Mtns., Mongolia form a distinct, disjunct clade. The other sample from Altai Mtns. is in a clade with *J. sabina* from the Pyrenees. Unfortunately, the use of nrDNA alone is just not sufficient to resolve this group of smooth junipers. This group includes *J. chinensis* and all the smooth leaf junipers from the

western hemisphere. Compare Fig. 5 with Fig. 2, that, based on combined nrDNA plus 4 cp regions, clearly resolved the smooth leaf junipers of the western hemisphere).

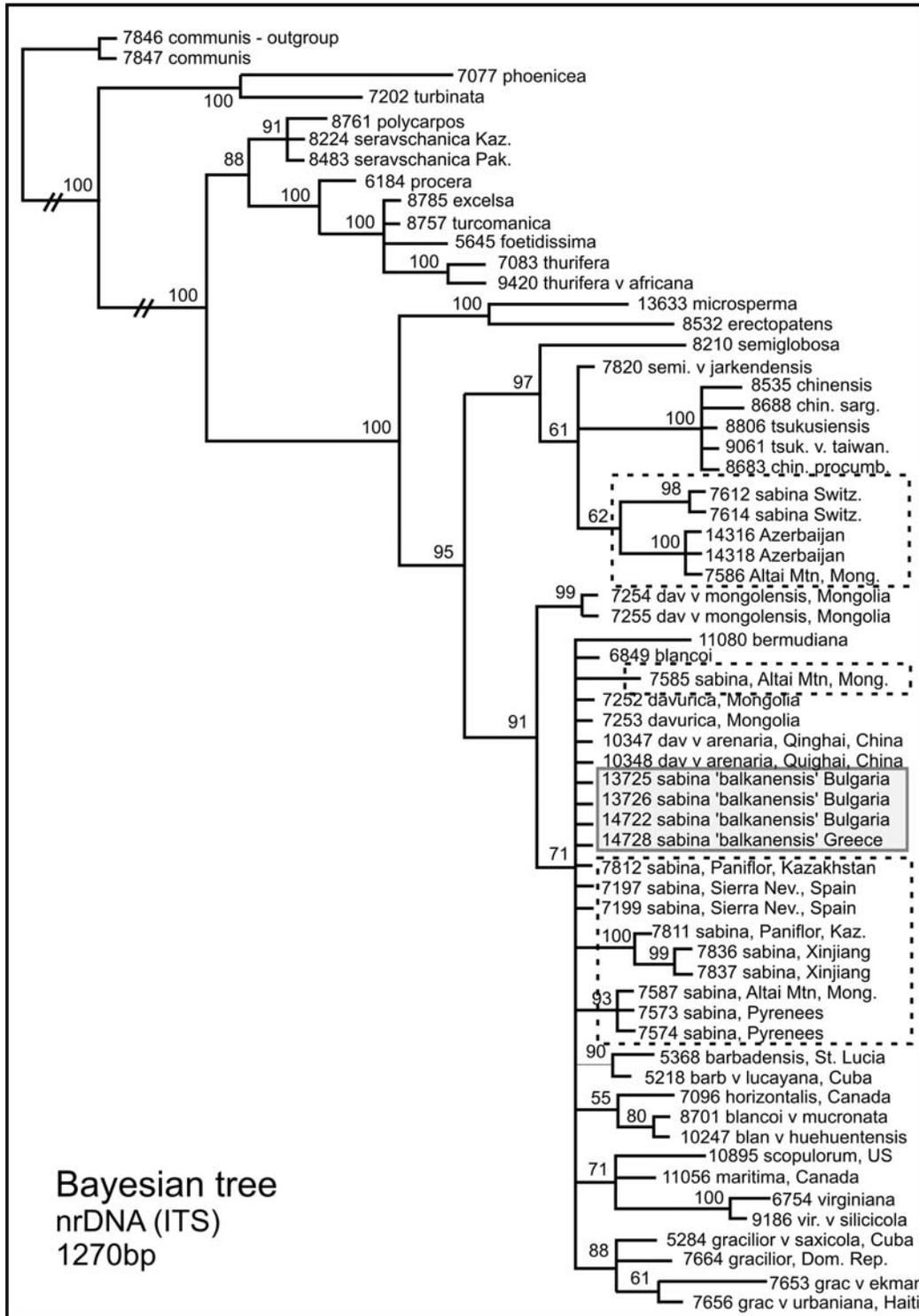


Figure 5. Bayesian analysis of the smooth leaf margined junipers of section Sabina using nrDNA (1270 bp). The numbers at the branch points are posterior probabilities (as percent). 'balkanensis' OTUs are in the shaded block. Other *J. sabina* OTUs are in the dashed line boxes.

Sequencing four cp regions (petN-psbM, trnSG, trnDT, and trnLF) resulted in 3114 bp of data. Bayesian analysis (including all the smooth leaf *Juniperus* of section *Sabina*), shows (Fig. 6) that the *J. sabina* 'balkanensis' plants are in a clade with *J. thurifera* (var. *thurifera* and var. *africana*). Clearly the chloroplast of 'balkanensis' is allied most closely with that of *J. thurifera*, not *J. sabina*. It seems likely that the 'balkanensis' chloroplast was captured from an ancestor of *J. thurifera* because *J. thurifera* (extant) is nested within 'balkanensis'. If 'balkanensis' captured its chloroplast from an extant *J. thurifera*, one would expect all of 'balkanensis' to be nested within *J. thurifera*. Further indicative of an ancient hybridization event is the measurable level of variation found in generally conserved chloroplast DNA among 'balkanensis' accessions.

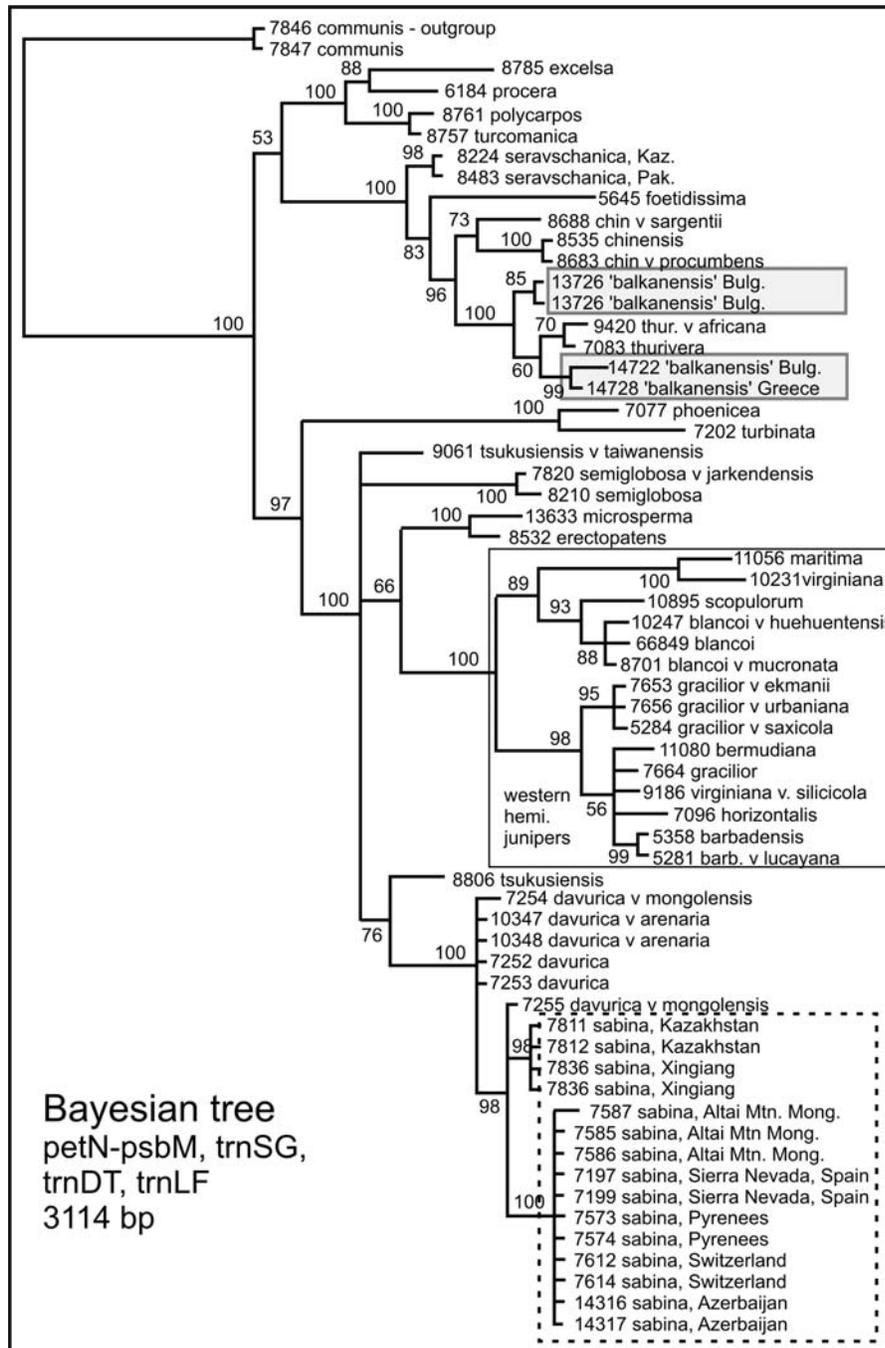


Figure 6. Bayesian analysis based on four cp regions.

It might be noted that *J. sabina* from Kazakhstan and Xinjiang form a clade (Fig. 6). The use of four cp regions resulted in a clade of the junipers from the western hemisphere (box, Fig. 6).

In order to more closely investigate the amount of divergence of the 'balkanensis' chloroplast from that of present day *J. thurifera*, a minimum spanning network was computed using both SNPs and indels, herein called mutations. This analysis found 52 mutations within the set: *J. sabina* (sensu stricto), *J. sabina* 'balkanensis' and *J. thurifera*. The minimum spanning network (Fig. 7) shows that all the 'balkanensis' plants differ by only 6-8 mutations from *J. thurifera* chloroplast. However, the nearest link connecting 'balkanensis' to *J. sabina* (sensu stricto) is 36 mutations!

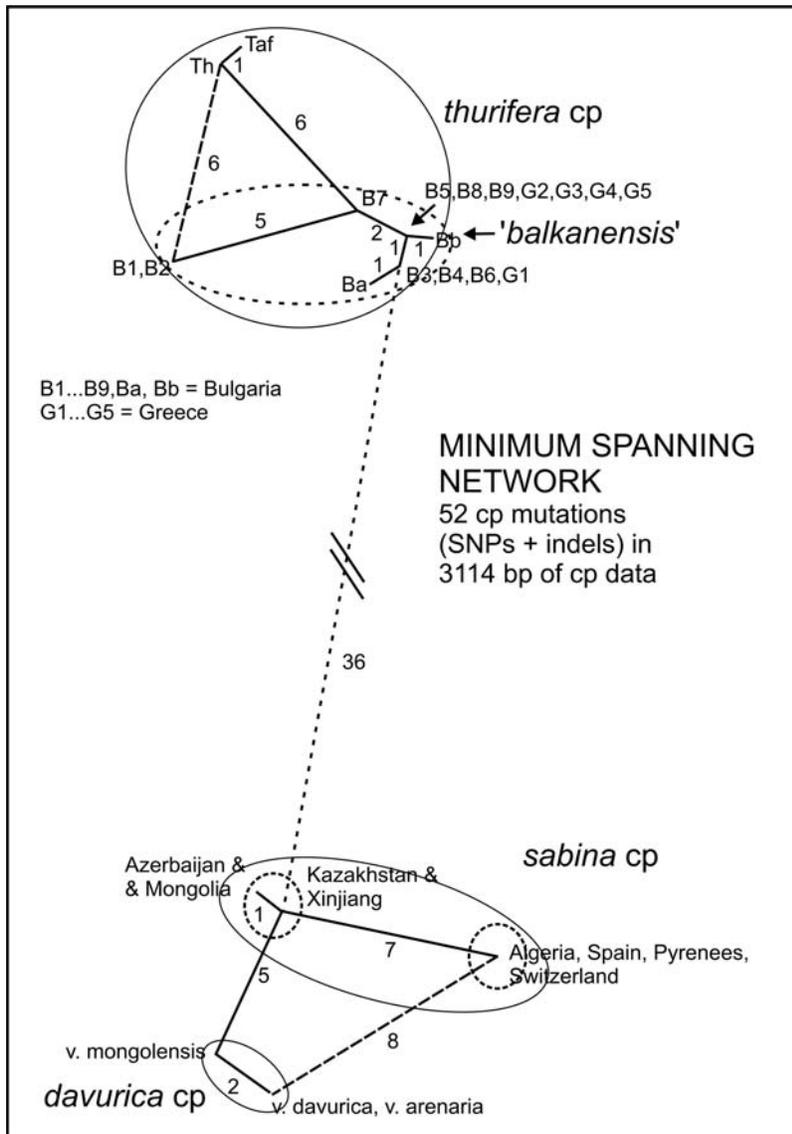


Figure 7. Minimum spanning network based on 52 mutations (SNPs + indels) in 4 cp markers (3114 bp). The numbers next to the lines are the number of mutations for that link. The dotted line connects the *thurifera* cp taxa to the *sabina* cp taxa by 36 mutations. The dashed line is the second nearest neighbor of *J. sabina* to *J. davurica* cp type. (8 mutations).

Notice (Fig. 7) that Azerbaijan/ Mongolia accessions group with Kazakhstan/ Xinjiang and this group differs by 7 mutations from the Europe/ Algeria group. This suggests that *J. sabina* in central Asia may be a different variety of *J. sabina*. That needs to be looked at in more details but this is beyond the scope of the present report.

The current data suggest that *J. sabina* 'balkanensis' captured the chloroplast of an ancestor of the *thurifera* lineage during an ancient hybridization event at a time when species distributions overlapped. Since it displays a general morphology similar of *J. sabina* this hybridization event was likely followed by successive backcrosses to *J. sabina* after the hybridization event, resulting largely in a nuclear genome as well as a morphology similar to *J. sabina* (*sensu stricto*). In fact, we do see that in the nrDNA analysis (Fig. 5), where 'balkanensis' is clearly interspersed in a clade with other *J. sabina*. So it is not surprising that a comparison of the morphology of 'balkanensis' and *J. sabina* has, to date, revealed only a few quantitative differences (Table 1). It may be that further morphological analysis will find additional differences but that seems unlikely, as no known genes for morphology reside in the chloroplast.

Table 1. Comparison of the morphology of *J. sabina* var. *balkanensis* and *J. sabina* (*sensu stricto*).

	<i>sabina</i> var. <i>balkanensis</i>	<i>sabina</i> var. <i>sabina</i>
foliage	fine, green	coarse, yellow-green
scale leaf tips	obtuse to acute	acute
scale leaf glands	not apparent	apparent
whip leaf glands	flat to sunken, most 3/4 length of leaf, elongate	level or above the surface mostly less than 3/4 length of leaf, oval to elongate
seed cones	mostly reniform (bi-lobed), some ovate; 1,2 seeded	mostly ovate, some reniform mostly ovate; 1,2,3 seeded

There are many reasons to recognize a given taxon (species, subspecies, etc.). Often it is just a logical way to organize morphological groups into named taxa. In this case, although 'balkanensis' is cryptic in its morphology, it seems worthy of recognition so as to call attention to this unusual evolutionary entity as:

***Juniperus sabina* var. *balkanensis* R.P.Adams and A. N. Tashev, var. nov.** Fig. 8.

Type: Greece, Northern Central Greece. Region Central Macedonia. Mount Tsena near the village of Notia. 41°08' 29.4" N; 22° 14'42.2" E., 1630 m, R. P. Adams 14730 (*Alexander Tashev Tsena 4*), 31 Aug. 2015 (HOLOTYPE: BAYLU, ISOTYPE University of Forestry, Dept. of Dendrology, Sofia, Bulgaria).

Prostrate shrubs similar to *J. sabina*, but differing in having a *J. thurifera*-like chloroplast, coarser foliage, leaf tips more acute, whip leaf glands flat to sunken, and more elongate.

Other specimens studied: TOPOTYPES: *Tashev Tsena 1,2,3,5* (*Adams 14727, 14728, 14729, 12731*) at BAYLU and Herbarium, University of Forestry, Sofia, Bulgaria.

Additional specimens studied: B1-B5 Eastern Rhodopes, *Adams 13725-13729* (*A. Tashev 2012-1-5*); Bulgaria. B6 Central Stara Planina, Sokolna reserve, *Adams 14721* (*A. Tashev 2015 Balkan 1*, Bulgaria; B7-B9, Ba, Bb Rila Mountain, *Adams 14722-14726* (*A. Tashev 2015 Rila 1.1-1.3, 2.1-2.2*) Bulgaria. G1-G5 Mt. Tsena, n Greece, *Adams 14727-14731* (*A. Tashev 2015 So. 1-5 Tsena*); Greece at Baylor University Herbarium (BAYLU) and Herbarium, University of Forestry, Sofia, Bulgaria.

At present, *J. sabina* var. *balkanensis* is known only from sloping rocky limestone, at 1240 - 1630m, in the mountains of Bulgaria and northern Greece (Fig. 9). It may occur northward into Romania,

westward into Macedonia and/ or eastward into northern Turkey. Additional research is in progress to more accurately determine its range (Fig. 10).



Figure 8. Holotype of *Juniperus sabina* var. *balkanensis* (Adams 14730)



Fig. 9. Habit and habitat of *J. s.* var. *balkanensis* in the eastern Rhodopes mountains, Bulgaria. *Juniperus communis*, columnar trees are in the background.

The distribution of *J. sabina* var. *balkanensis* and *J. sabina* is shown in Fig. 10. The distribution of *J. thurifera* is presented in the insert, lower left (Fig. 10). At present the distributions of *J. s.* var. *balkanensis* and *J. thurifera* do not appear to overlap, negating hybridization. However, there were large changes in plant distributions in the Pleistocene and earlier, that would have given opportunity for a *J. thurifera*-like ancestor to co-occur with *J. sabina*. This would have presented an opportunity for chloroplast capture by *J. sabina*.

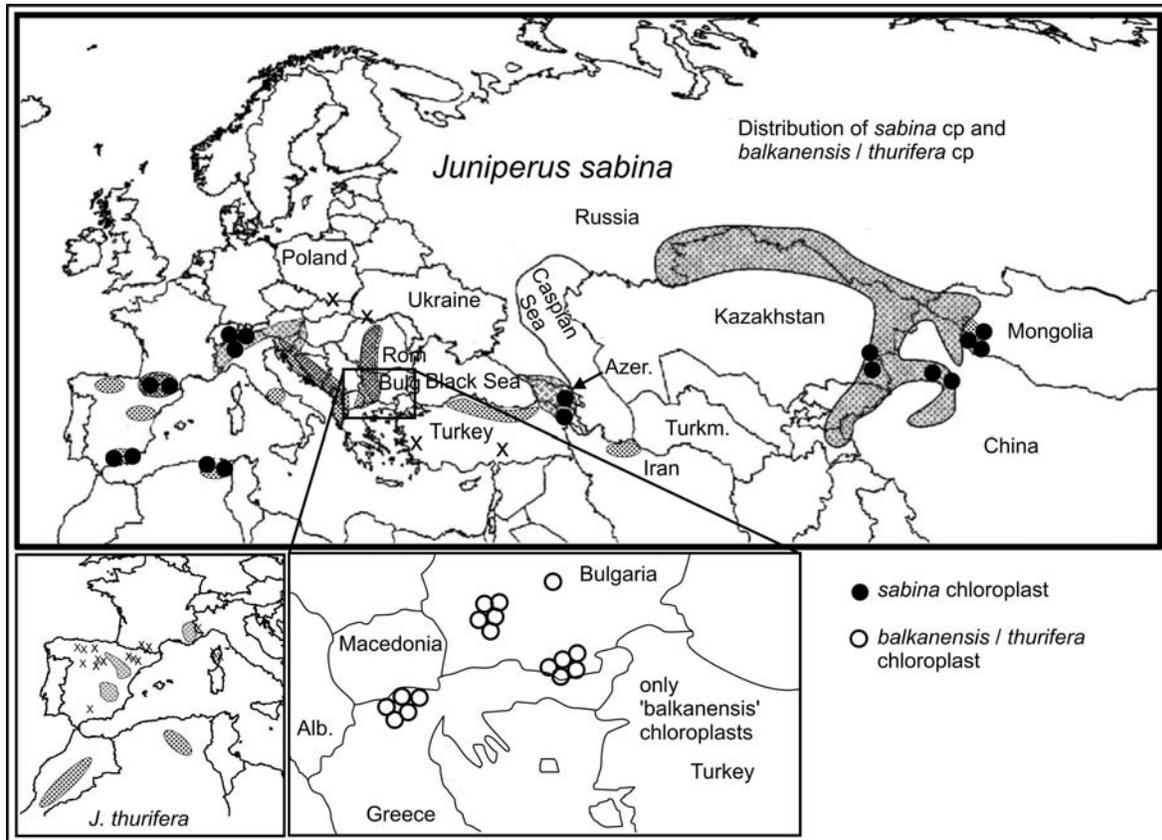


Figure 10. Distribution of *J. sabina* var. *balkanensis* and typical *J. sabina* chloroplast. The distributions of *J. thurifera* and var. *africana* (in north Africa) are shown in the insert on the lower left.

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