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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Chloroplast capture by a new variety, *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev, from the Balkan peninsula: A putative stabilized relictual hybrid between *J. sabina* and ancestral *J. thurifera*

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**ABSTRACT**

An example of chloroplast capture has been found in *Juniperus sabina* from Bulgaria and Greece in the Balkan peninsula. The cpDNA from these populations is very uniform and is nearly identical to that of *J. thurifera* (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, the variety, with the *thurifera* cpDNA, is known only from Bulgaria and Greece. Published on-line www.phytologia.org *Phytologia* 98(2): 100-111 (Apr 1, 2016). ISSN 030319430.

**KEY WORDS:** *Juniperus sabina* balkanensis sp 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 cp markers has dominated phylogenetic research. However, even two decades ago, Rieseberg and Soltis (1991) warned of chloroplast capture (both recent via hybridization and ancient) 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* (Apiaceae, 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 *Juniperus osteosperma* in western North America. More recently, Adams (2015a, b) found widespread hybridization an 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 berry) 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 small 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.

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% 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 distinct from *J.
sabina and J. davurica (Fig. 2), again as has been shown by their essential oils and RAPD data (Adams, 1999). Among the J. sabina, J. davurica, J. d. var. arenaria and J. d. var. mongolensis samples, there was some support (61-68%) for infraspecific taxa. The J. sabina plants from Mongolian sand dunes are distinctly separated (Fig. 2) and have recently been recognized as a new variety, J. sabina var. mongolensis R. P. Adams (2006).

It is noteworthy that Adams and Schwarzbach (2013a) included only one OTUs of J. sabina (Fig. 2) and that was from Europe (Switzerland). It grouped in a clade with J. davurica (Fig. 2).

Adams, Nguyen and Liu (2006) used Principal coordinate analyses (PCO) of the leaf essential oils of these taxa to confirm (Fig. 3) that J. chinensis was distinct from the J. sabina - davurica complex (oval, Fig. 3). Adams, Nguyen and Liu (2006) also found that J. davurica var. davurica (DV, Fig. 3) had a terpenoid composition very similar to that of J. d. var. arenaria (AR, Qinghai sand dunes) and J. d. var. mongolensis plants growing on Mongolian sand dunes southwest of Ulan Batar (MS).

The Iberian J. sabina (SN, PY) plants' terpenoids were quite different (Fig. 3) from nearby Switzerland (SW) and central Asia (AM, TS, KZ).

In addition, Adams, Nguyen and Liu (2006) found the essential oils of J. sabina (sensu stricto) ranged (Fig. 3) from Kazakhstan (KZ) and neighboring Xinjiang (TS) and the Altai Mtns., Mongolia (AM) to Switzerland (SW) to the Iberian Peninsula [Pyrenees (PY), Sierra Nevada, Spain (SN)].

Adams et al. (2007) examined RAPDs in the same populations studied for leaf volatile oils (Adams, Nguyen and Liu, 2006). They found the RAPDs data separated J. sabina (Europe, Tian Shan) from the more easterly, J. s. var. davurica, J. s. var. arenaria and J. s. var. mongolensis. There also appeared to be a trend from J. sabina in Spain, to Pyrenees to Switzerland thence to the Tian Shan, Xinjiang (Fig. 4).
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


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 Plania, 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 Epicentre 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 Epicentre 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. in is in a clade with *J. sabina* from the Pyrenees. The use of nrDNA 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.

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 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!

![Minimum spanning network](image)

**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 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*. But that is beyond the scope of the present report.

If *J. sabina* 'balkanensis' is a product of chloroplast capture, then its nrDNA (and other nuclear genes) should be essentially the same as that of *J. sabina* (*sensu stricto*). And we do see that in the nrDNA analysis (Fig. 5) where 'balkanensis' is 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*).

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

There are many reasons to recognize a plant variety (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 as a new variety to aid in the conservation of this unusual evolutionary product 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. 41º08'29.4" N; 22º14'42.2" 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 the *J. thurifera* 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 Plania, 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 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).
Fig. 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.

**ACKNOWLEDGEMENTS**

Thanks to G-L Chu, China; A. D. Dembitsky, Kazakhstan; A. Lara, Spain; S. Shatar, Mongolia; Y. Turuspekov, Kazakhstan; J-P. Zhong, and J. Liu, China for assistance on field trips. This research was supported with funds Baylor University.

**LITERATURE CITED**


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