The origin of *Juniperus* x*pfitzeriana,* an allo-tetraploid hybrid of *J. chinensis* x *J. sabina*

Robert P. Adams and **Sam T. Johnson**
Baylor University, Biology Department, Utah Lab, Baylor University,
201 N 5500 W, Hurricane, UT, 84737, USA
robert_Adams@baylor.edu

John Anderson
Keeper of The Gardens, Crown Estate Office, Windsor Great Park, Windsor, *SL4 2HT* UK

Keith Rushforth
The Shippen, Ashill, Cullompton, Devon, *EX15 3NL*, UK

Perla Farhat
Laboratoire Biodiversité et Génomique Fonctionnelle, Faculté des Sciences, Université Saint-Joseph, Campus Sciences et Technologies, Mar Roukos, Mkalles, BP: 1514 Riad el Solh, Beirut 1107 2050, Lebanon and Ecologie Systématique Evolution, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, 91400 Orsay, France

Nicolas Valentin
Institute of Integrative Biology of the Cell (I2BC), Plateforme de Cytométrie, CEA, CNRS, Université Paris-Saclay, 91198, Gif-sur-Yvette, France

and

Sonja Siljak-Yakovlev
Ecologie Systématique Evolution, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, 91400 Orsay, France

ABSTRACT

Juniperus x*pfitzeriana* is one of the most commonly cultivated junipers in the world. The origin of *J.* x*pfitzeriana* has remained speculative, but it is thought to be a hybrid of *J. chinensis* x *J. sabina*. nrDNA (ITS) and 4 chloroplast gene regions were sequenced from 14 *J.* x*pfitzeriana* cultivars from Windsor Gardens, UK, and compared with all *Juniperus*, sect. *sabina*, smooth leaf margin species. All of the 14 cultivars were identical in their chloroplast DNA and their cp DNA was identical to that of *J. sabina* var. *balkanensis*. In addition, 13 *J.* x*pfitzeriana* cultivars were allo-tetraploids with heterozygous bases at 5 to7 sites that distinguish *J. chinensis* and *J. sabina* var. *balkanensis*. These cultivars had identical nrDNA. Two of the 14 cultivars, 'Old Gold' and 'Sea Green', showed a slightly different nrDNA pattern, being homozygous at sites 410 and 1139, as found in *J. s.* var. *balkanensis*. The origin of *J.* x*pfitzeriana* is from a cross of a male, tetraploid *J. sabina* var. *balkanensis* and a female, tetraploid, *J. chinensis*, resulting in an allo-tetraploid, dioecious, *J.* x*pfitzeriana* (Spath) Schmidt. Published on-line www.phytologia.org *Phytologia 101(2): 164-174 (June 21, 2019).* ISSN 030319430.

KEY WORDS: *Juniperus* x*pfitzeriana*, x*media*, *J. chinensis*, *J. sabina* var. *balkanensis*, tetraploid, origin.

Juniperus x*pfitzeriana* is one of the most commonly cultivated junipers in the world (Krussmann, 1991, listed 28 cultivars). The origin of the group of cultivars treated as 'Pfitzers' is thought to have been from a cross of *J. chinensis* x *J. sabina* (Le Duc, et al. 1999; Krussmann 1991; van Melle 1947). Van Melle (1947) proposed the name *Juniperus xmedia* for *J. chinensis* '*Pfitzeriana'*, having concluded that it was a hybrid. The missionary Armand David collected the seed from the Ho Lan (Helan) Shan, Inner Mongolia and sent seed back to France in the 1860s (van Melle 1947). Van Melle (1947) notes that, by the 1870s, the plants, obtained by growing the seeds, were cultivated 'extensively' in France and Belgium by nurserymen. The Spath nursery selected a male plant and named the cultivar 'Pfitzeriana' after W. Pfitzer, a nurseryman at Stuttgart (Den Oden and Boom, 1965). It is interesting that van Melle (1947) recognized several male and female cultivars as varieties, thus pfitzers are dioecious as are the putative parents, *J. chinensis* and *J. sabina* (Adams 2018). Van Melle (1947) recognized two male varieties: *J. xmedia* var. *pfitzeriana*, and var. *globosa*; and two female: var. *arbuscula* and var. *plumosa*. The seedlings grown in France and Belgium produced several 'sports' or somatic mutations, and these were further propagated by cloning to conserve the somatic mutation, to obtain commercially valuable cultivars. These seedlings matured to become male or female reproducing plants. This provides evidence that the natural hybrids in Ho Lan Shan, produced viable seed, yielding seedlings that later displayed the 'Pfitzer' hybrid phenotype.

Juniperus x*pfitzeriana* (Spath) Schmidt (Schmidt 1983) is widely accepted as the name of the 'Pfitzers', but *Juniperus xmedia* Van Melle is still in use, although the name has been rendered illegitimate because of prior usage of *J. media* V. D. Dmitriev (Le Duc et al. 1999, Czerepanov 1973). Lewis (1995) attempted to get the name *J. xmedia* conserved because of historical usage, but his proposal was rejected (Le Duc et al. 1999).

The volatile leaf oil of *J. chinensi*s contains bornyl acetate and the oil of *J. sabina* includes sabinyl acetate. Fournier et al. (1991) reported that the volatile leaf oils of pfitzeriana cultivars contained both bornyl acetate and sabinyl acetate. Thus, the oils supported (but not proving) the origin from *J. chinensis* x *J. sabina*.

Le Duc et al. (1999) used RAPDs (Random Amplified Polymorphic DNAs) to ordinate *J. chinensis* (*Adams 6764-6766*, cv 'Kaizuka', cultivated at Northwest Normal University, Lanzhou, China), *J. sabina* var. *sabina* (*Adams 7611-7614*, Switzerland) and eight *J.* x*pfitzeriana* cultivars ('Fruitlandii', 'Gold Coast', 'Hetezii', 'Kallay's Compact', 'pfitzeriana Aurea', 'pfitzeriana Glauca' and 'Wilhelm Pfitzer', the cultivar of the type for *J.* x*pfitzeriana* by Schmidt, 1983. Using 122 RAPD bands, they found the x*pfitzeriana* samples ordinated intermediate between the putative parental species (*J. chinensis*, *J. sabina* var. *sabina*), as one would expect in hybrids (Adams1982). Again, these data supported (but did not prove) the origin from *J. chinensis* x *J. sabina*.

With the advances in DNA sequencing technology it is now possible to deduce the parents in conifer hybrids, and the inheritance of chloroplast (cp) in the Cupressaceae has been shown (Table 1) to be from the male, pollen parent (Adams 2019).

Scion Ltd., New Zealand recently made available materials from controlled crossings in *Hesperocyparis*, a genus closely related to *Juniperus*. Adams et al. (2016) analyzed 18 hybrids from a single, controlled cross, *H. arizonica* (male) x *H. macrocarpa* (female), and all 18 had perfect *H. arizonica* (paternal) chloroplast DNAs, confirming paternal inheritance of chloroplasts in *Hesperocyparis* (Table 2), and by inference, in the closely related genus, *Juniperus*.

Recently, it has been proved that genome size using flow cytometry (FC) was successfully used as a proxy for ploidy level in *Juniperus* (Farhat et al. 2019a, b). Therefore, the ploidy of Juniper hybrids can now be determined by FC. This is very important because it is known that several *J. chinensis* pfitzers are

tetraploid (Hall, et al. 1979). With the confluence of both DNA methodology and FC ploidy determination, this present us with a great opportunity to examine the origin of *J.* x*pfitzeriana*.

The purpose of the present research is to present new DNA sequencing utilizing both chloroplast and nuclear DNA in the determination of the origin of *J.* x*pfitzeriana*. We also present ploidy for *J.* x*pfitzeriana* cultivars and the putative parental species, *J. chinensis* and *J. sabina*.

Table 1. Inheritance of cp (chloroplasts) and mt (mitochondria) in conifers. ns = not studied. From Adams 2019, in part).

Cupressaceae	cp	mt	reference (see Adams 2019 for ref.)
Cunninghamioideae			
Cunninghamia konshii	mat	ns	Lu, et al. 2001
Sequoioideae			
Sequoia sempervirens	pat	pat	Neale, Marshall and Sederoff, 1989
Taxodioideae			
Cryptomeria japonica	pat, some mat leakage	ns	Ohba et al. 1971
Callitroideae			
Callitris (4 species)	pat	ns	Sakaguchi, et al. 2014
Cupressoideae			
Leyland cypress - *Callitropsis nootkatensis* x *Hesperocyparis macrocarpa*	4 plants: pat	pat	Kou, et al. 2014
	2 plants: mat	mat	
Calocedrus decurrens	pat	pat	Neale, Marshall and Harry, 1991
Chamaecyparis obtusa	pat, ~2.5% mat leakage	ns	Shirashi et al. 2001
Chamaecyparis obtusa x pisifera	pat	pat	Kondo, et al., 1998
Chamaecyparis lawsonia	pat	pat	Chesnoy, 1973
Platycladus orientalis	pat	pat	Chesnoy, 1969
Hesperocyparis arizonica x *Hesperocyparis macrocarpa*	pat	ns	Adams et al. 2018
Juniperus ashei, J. pinchotii, J. virginiana	pollen	pollen	Mohanty et al. 2016, ultrastructural presence of cp and mt in pollen was confirmed by TEM and DNA.

METHODS

Plant materials:

x***pfitzeriana*** samples: Leaf samples were collected in Windsor Gardens, Windsor Great Park, Windsor, *SL4 2HT* UK from 14 *J.* x*pfitzeriana* cultivar accessions and immediately placed in activated silica gel for DNA sequencing and Flow Cytometry - ploidy determination (see Table 2).

Reference Species: *Juniperus chinensis*, *J. sabina* var. *sabina*, *J. s*. var. *balkanensis* see Adams et al. (2018a) for collection details.

DNA extraction and sequencing

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 $MgCl_2$ 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. 2.31 (Technelysium Pty Ltd.).

Flow cytometric analyses for ploidy level determination

Nuclear DNA amount was assessed by flow cytometry (FC) based on the technique of Bourge et al. (2018) on silica dried leaves of *Juniperus* samples and fresh leaves of *Hordeum vulgare* L. 'Sultan' (2C= 9.81 pg in Garnatje et al. (2004)) used as an internal standard. Approximately, 30 mg of leaves of both the internal standard and *Juniperus* were simultaneously chopped using a razor blade in a plastic Petri dish with 500 µl of cold Gif nuclear-isolation buffer-GNB (Bourge et al. 2018): 30 mM sodium citrate, 45 mM $MgCl_2$, 60 mM MOPS (4-morpholine propane sulphonate, pH 7), and 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2 containing 0.1% (w/v) Triton X–100, supplemented with 10 mM sodium metabisulphite and RNase (2.5 U/ml). The nuclei suspension was filtered through 50 µm. The nuclei were stained with 100 µg/ml propidium iodide (PI); a specific DNA fluorochrome intercalating dye, and kept at 4°C for 5 min. DNA content of about 3,000 stained nuclei was determined for each sample using the cytometer CytoFLEX S (Beckman Coulter- Life Science United States. Excitation 561 nm, 26 mW; emission through a 610/20 nm band-pass filter). Measurements of each sample were repeated twice. The software CytExpert was used for histogram analyses. The total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the species and the internal standard, according to the following formula:

2C DNA content/nucleus (pg) = (Sample 2C peak mean / Standard 2C peak mean) x Standard 2C DNA (pg).

RESULTS

Thirteen of *J.* x*pfitzeriana* accessions were tetraploids (4*x*, except Sea Green that was found to be a triploid (3*x*, Table 3). Analysis of three chloroplast regions: petN-psbM, trnS-trnG, and trnL-trnF sequences of the 14 cultivars and all the *Juniperus* taxa with smooth leaf margins in section *Sabina*, revealed that the sequences of all 14 accessions were identical (Fig. 1). Furthermore, the 14 *J.* x*pfitzeriana* accessions cp sequences were identical to that of *J. sabina* var. *balkanensis* (Table 3), and differed by three indels and one SNP from *J. thurifera* and *J. t*. var. *africana* (Fig. 1). Thus, revealing that the tetraploid male (paternal, pollen) parent of *J. xpfitzeriana* was *J. sabina* var. *balkanensis* (or an ancestor with the same chloroplast sequence for petN-psbM, trnS-trnG, and trnL-trnF).

Juniperus chinensis was found to be unacceptable as the male (cp) parent (Table 3) as each of the three cp gene regions were specific for *J. chinensis*. Likewise, *J. chinensis* var. *tsukusiensis* and var. *taiwanensis* (now recognized as *J. tsukusiensis* and *J. tsukusiensis var. taiwanensis,* Adams 2014) are unacceptable, because both contain the *chinensis* type cp, and, interestingly, are diploids (Table 3). *Juniperus chinensis* var. *sargentii* was found to be 4*x*, and contained a different type chloroplast (noted as *sargentii,* Table 3).

Table 2. Collection information for *J.* x*pfitzeriana* at Windsor Gardens, UK.

cultivar name and Adams collection number, All *Juniperus* x*pfitzeriana*	Windsor acc. #	Location in Windsor Gardens	Origin: [1]Krussmann, 1991, [2]The Conifer Manual, Welch, 2012, [3]Conifertreasury.org
'Aurea' *Adams 15474*	1999-6099	HG57 Grayswood 2 (435) 1 1	[1]Mutation of 'Pfitzeriana', similar to type. (= *J. media* var. *pfitzeriana* f. *aurea* van Melle) ex D. Hill Nursery, IL, 1923[1]
'Aurea' *Adams 15418*	na	University of Paris-sud campus	[1]Mutation of 'Pfitzeriana', similar to type. (= *J. media* var. *pfitzeriana* f. *aurea* van Melle) 1923, D. Hill Nursery, IL, Dundee, USA
'Arctic' *Adams 15442*	1999-6077	HG28 Bomer 2 (425) 62 1	[3]1972?, Mitsch Nursery, Aurora, OR, USA D. Hill Nursery, IL, Dundee, USA
'Armstrongii' *Adams 15454*	1999-6075	HG41 Hillier 1 (102) 31 1	[1]low, slow growing 'Pfitzeriana', Dev. 1932, Armstrong Nurseries, Ontario, CA, USA
'Carberry Gold' *Adams 15425*	2001-774	HG16 (423) 15 1	[3]Carbery Nurs., Bournemouth, UK [1] = Old Gold Carberry
'Carberry Gold' *Adams 15463*	1999-6081	HG49 Esveld 1 (97) 2 1	[3]Carbery Nurs., Bournemouth, UK [1] = Old Gold Carberry
'Gold Star' *Adams 15443*	1999-6088	HG28 Bomer 2 (425) 83 2	[1]discovered David Bakker (1961), introduced 1971, Bakker & Sons Nursery, St. Catherine, Ontario, CA
'Golden Saucer' *Adams 15462*	1999-6084	HG44 Bedgebury (88) 34 2	[1]sport of 'Pfitzeriana Aurea', more yellow in winter. 1976, MW Van Nierop, Boskoop, Holland
'Goldenkissen' *Adams 15482*	1999-6086	HG64 Mason (110) 21 5	[3]D M van Delderen, 1983, G. Oltsman Nursery., Ekern, Germany
'Old Gold' *Adams 15453*	1999-6097	HG41 Hillier 1 (102) 21 1	[1]mutation of 'Pfitzeriana Aurea', ex FJ Grootendorst, Holland, 1958.
'Pfitzeriana Prostrate' *Adams 15430*	1999-6102	HG20 (333) 31 1	prostrate sport of *J. Xpfitzeriana* propagated from plant at Windsor
'Saybrook Gold' *Adams 15423*	2001-2555	HG14 (337) 24 4	[2,3]1980, Girard Nursery, Geneva, OH, USA
'Sea Green' *Adams 15436*	1999-6110	HG27 Bomer 1 (91) 30 2	commercial plant, locally available.
'Sea Green' Adams 15604	na	na	Home Depot Inc. nursery, St. George, UT, USA
'Wilhelm Pfitzer' *Adams 15435*	2000-179	HG25 (317) 1 1	[1]male, putative natural hybrid (*J. chinensis x J. sabina*), seeds ex Ho Lan Shan, inner Mongolia, purchased as *J. chinensis pendula,* 1876 by Simon Louis Nursery, Metz, France, and plants sold to the public by Spath Nursery in 1899.

All four samples of *J. sabina* var. *sabina* are diploids (2*x*, Table 3) and contain the var. *sabina* chloroplast, eliminating *J. s*. var. *sabina* as a possible male parent for *J.* x*pfitzeriana.* Having established that the paternal parent of *J.* x*pfitzeriana* is *Juniperus sabina* var. *balkanensis* (4*x*) or an ancestor, it seemed fruitful to investigate the maternal parent of *J.* x*pfitzeriana* by use of the nuclear gene region, nrDNA. Analysis of the 14 cultivars vs. 44 taxa in sect. *Sabina*, smooth leaf junipers revealed that the *J.* x*pfitzeriana* cultivars grouped with *J. chinensis* and *J. s*. var. *balkanensis.* (Fig. 2). Further analysis of nrDNA (1270 bp) revealed 8 variable sites, with 7 of them indicative of hybridization (Table 4). Site 410 was heterozygous in 12 of 14 cultivars, and homozygous in 'Old Gold' and 'Sea Green'. Thus, the nrDNA (ITS) region clearly supports that *J.* x*pfitzeriana* is of hybrid origin. All the 12 *J.* x*pfitzeriana* cultivars had identical nrDNA, except 'Old Gold' and 'Sea Green', that have C and G at 410 and 1075 (Table 4). Interestingly, 'Sea Green' also has a T at site 663, and is a triploid. Sea Green may have been derived from a tetraploid xpfitzer, backcrossed to a diploid *J. chinensis*, giving the triploid Sea Green, based on their having C and G at 410 and 1075.

Table 3. Classification of the 14 *J.* x*pfitzeriana* (=x*media*) accessions by cp markers. chloroplast types: *balkanensis* = *J. sabina* var. *balkanensis*; *sabina* = *J. sabina var. sabina*; and *chinensis* = *J. chinensis*; *sargentii* = *J. chinensis var. sargentii.*

J. xpfitzeriana (=x*media*), unless noted otherwise	ploidy	petN	trnSG	trnLF	chloroplast, ex pollen
15442 Arctic	4*x*	balk	balk	balk	*balkanensis*
15454 Armstrongii	4*x*	balk	balk	balk	*balkanensis*
15418 Aurea, Paris-sud	4*x*	balk	balk	balk	*balkanensis*
15474 Aurea	4*x*	balk	balk	balk	*balkanensis*
15423 Saybrook Gold	4*x*	balk	balk	balk	*balkanensis*
15425 Carberry Gold	4*x*	balk	balk	balk	*balkanensis*
15463 Carberry Gold	4*x*	balk	balk	balk	*balkanensis*
15443 Gold Star	4*x*	balk	balk	balk	*balkanensis*
15462 Golden Saucer	4*x*	balk	balk	balk	*balkanensis*
15482 Goldenkissen	4*x*	balk	balk	balk	*balkanensis*
15430 pfitzeriana prostate	4*x*	balk	balk	balk	*balkanensis*
15435 Wilhelm Pfitzer	4*x*	balk	balk	balk	*balkanensis*
15453 Old Gold	4*x*	balk	balk	balk	*balkanensis*
15436 Sea Green, Windsor	3x	balk	balk	balk	*balkanensis*
15604 Sea Green, Home Depot nursery	3x	balk	balk	balk	*balkanensis?*
Most likely male parent from cp data					
14723 sabina v. balkanensis, Bulg.	4*x*	balk	balk	balk	*balkanensis*
14728 sabina v. balkanensis, Greece	4*x*	balk	balk	balk	*balkanensis*
Unacceptable as male (pollen) parent					
8535 chinensis, Japan, Kaizuka?	4*x*	chin	chin	chin	*chinensis*
8536 chinensis, Japan, Kaizuka?	4*x*	chin	chin	chin	*chinensis*
9061 chin. v. taiwanensis, Taiwan (=tsukusiensis var. taiwanensis)	2*x*	chin	chin	chin	*chinensis*
8805 chin, v. tsukusiensis, Japan (= tsukusiensis v. tsukusiensis)	2*x*	chin	chin	chin	*chinensis*
8688 chinensis v. sargentii, Japan	4*x*	sarg	sarg	sarg	*sargentii*
14316 sabina v. sabina, Azerbaijan	2*x*	sab	sab	sab	*sabina*
7614 sabina v. sabina, Switzerland	2*x*	sab	sab	sab	*sabina*
7573 sabina v. sabina, Pyrenees	2*x*	sab	sab	sab	*sabina*
7811 sabina v. sabina, Kazakhstan	2*x*	sab	sab	sab	*sabina*

Examining the variable sites (i.e., hybrid indicating sites, or hybrid sites) of *J. chinensis, J. chinensis* var. *tsukusiensis* (now *J. tsukusiensis* var. *tsukusiensis*), *J. c*. var. *taiwanensis (now J. tsukusiensis* var. *taiwanensis*), and *J. sargentii*, revealed that all of these taxa (except *J. c*. var. *sargentii*) have the correct sequences at the hybrid sites to be the maternal parent of *J.* x*pfitzeriana* (Table 4). *Juniperus sargentii* is not likely the maternal parent because it has 3 non-matching bases at sites 663, 985, 1075 (Table 4).

Another factor to consider in the potential maternal parent of *J. xpfitzeriana* is the ploidy level. Notice that *J. chinensis* (samples from Japan) is a tetraploid (4x), whereas *J. c*. var. *tsukusiensis* and *J. c.* var. *taiwanensis* are both diploids (2x, Table 4). Thus, *J. chinensis* (4x) seems more probable as the maternal parent of *J. xpfitzeriana.*

Table 4. nrDNA (ITS) variable sites in *J.* x*pfitzeriana* (=x*media*) (Windsor Gardens), *J. chinensis*, and *J. sabina*. K=G/T; S=C/G; Y=C/T; M=A/C; W=A/T; R=A/G. chloroplast types: *balkanensis* = *J. sabina* var. *balkanensis*/ *J. thurifera*; *sabina* = *J. sabina var. sabina*; and *chinensis* = *J. chinensis.*

taxa: *J.* x*pfitzeriana* (=x*media*), unless noted otherwise	ploidy	212 K	410 S	663 Y	985 Y	995 M	1033 K	1075 W	1139 R	ITS classif. hybrid?	chloroplast, ex. pollen
15442 Arctic	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15454 Armstrongii	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15418 Aurea, Paris-sud	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15474 Aurea	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15423 Saybrook Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15425 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15463 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15443 Gold Star	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15462 Golden Saucer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15482 Goldenkissen	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15430 pfitzeriana prostate	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15435 Wilhelm Pfitzer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15453 Old Gold	4*x*	G/T	**C**	C/T	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15436 Sea Green, Windsor	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15604 Sea Green Home Depot	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis?*
8535 chinensis, Japan	4*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
8536 chinensis, Japan	4*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
9061 chin. v. taiwanensis, Taiwan (=tsukusiensis var. taiwanensis)	2*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
8805 chin, v. tsukusiensis, Japan (= tsukusiensis v. tsukusiensis)	2*x*	T	C	C	C	C	G	A	G	*chinensis*	*chinensis*
8688 chinensis v. sargentii, Japan	4*x*	T	C	T	T	C	G	T	G	chin sarg.	*sargentii*
sabina Type 2 ITS											
14723 sabina v. balkanensis, Bulg.	4*x*	G	C	T	T	A	T	T	G	*sab.* v. *balk*	*balkanensis*
14316 sabina v. sabina, Azerbaijan	2*x*	G	C	T	T	A	T	T	G	*sabina*	*sabina*
7614 sabina v. sabina, Switzerland	2*x*	G	C	T	T	A	T	T	G	*sabina*	*sabina*
sabina Type 1 ITS:											
14728 sabina v. balkanensis, Greece	4*x*	G	C	T	T	A	G	T	G	*balkanensis*	*balkanensis*
7573 sabina v. sabina, Pyrenees	2*x*	G	C	T	T	C	G	T	G	*sabina*	*sabina*
7811 sabina v. sabina, Kazakhstan	2*x*	G	C	T	T	A	G	T	G	*sabina*	*sabina*
Most probable male (pollen) parent genotype	4*x*	G	C	T	T	A	T	T	G	*balkanensis* Type 2 ITS	*balkanensis*
male parent: pollen, with balk cp. 14723 sabina v. balkanensis, Bulg.	4*x*	G	C	T	T	A	T	T	G	*balkanensis* Type 2 ITS	*balkanensis*
typical x*pfitzeriana*, cf 15442, above	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x balk	*balkanensis*
Most probable female parent genotype	**4*x***	T	G	C	C	C	G	A	A	*chinensis*	*chinensis*
female parent: 8535 chinensis, Japan	4*x*	T	C	C	C	C	G	A	G	chinensis	*chinensis*

Variable sites located at: 212, xGGCCAAGC; 410, xGTTGAGAT; 663, xTCTTCGTC; 985, xGCCCTCCC; 995, xGCGAGGAG; 1033, xGCGGTCGG; 1075, xCGCGACGA; 1139, xGAACTTTG.

Although we have established that the paternal parent is *J. sabina* var. *balkanensis* (or an ancestor). There is an 8 site polymorphism in the nrDNA of *J. sabina*, which Adams et al. (2018a, b) referred to as Type 1 and Type 2. Examination of nrDNA (ITS) Type 1 and Type 2 variation (Table 5) shows that there is no variation in the 14 *J.* x*pfitzeriana* cultivars. *Juniperus chinensis* (8535, 8536) has a slightly different Type 2 pattern with a C in 995 and a G in 1036 that perfectly complements the paternal *balkanensis* ITS Type 2 pattern to make the observed A, C, G, C, T, A/C, G, G/T pattern of *J.* x*pfitzeriana.*

Table 5. nrDNA (ITS) Type 1 and Type 2 nrDNA at 8 variable sites in *J.* x*pfitzeriana* (=x*media*) (Windsor Gardens), *J. chinensis*, and *J. sabina*. [1]Eight polymorphic sites are 350(R), 391(S), 432(R), 604(M), 745(Y), 995(M), 1036(R), 1037(K).

taxa: *J. xpfitzeriana* (=x*media*), unless noted otherwise	ploidy	[1]350	391	432	604	745	995	1036	1037	ITS Type	cp male parent
Type 1 nrDNA (ITS) pattern		G	G	A	C	C	C	A	G	1	
Type 2 nrDNA (ITS) pattern		A	C	G	A	T	A	G	T	2	
15442 Arctic	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15454 Armstrongii	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15418 Aurea, Paris-sud	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15474 Aurea	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15423 Saybrook Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15425 Carberry Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15463 Carberry Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15443 Gold Star	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15462 Golden Saucer	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15482 Goldenkissen	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15430 pfitzeriana prostate	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15435 Wilhelm Pfitzer	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15453 Old Gold	4*x*	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15436 Sea Green, Windsor	3x	A	C	G	A	T	A/C	G	G/T	2	balkanensis
15604 Sea Green, Windsor	3x	A	C	G	A	T	A/C	G	G/T	2	balkanensis
8535 chinensis, Japan	4*x*	A	C	G	A	T	C	G	G	2*	maternal chinensis
8536 chinensis, Japan	4*x*	A	C	G	A	T	C	G	G	2*	
9061 chin. v. taiwanensis, Taiwan (=tsukusiensis var. taiwanensis)	2*x*	A	C	G	A	T	C	G	G	2*	
8805 chin, v. tsukusiensis, Japan (= tsukusiensis v. tsukusiensis)	2*x*	A	C	G	A	T	C	G	G	2*	
8688 chinensis v. sargentii, Japan	4*x*	A	C	G	A	T	C	G	G	2*	
14723 sabina v. balkanensis, Bulg.	4*x*	A	C	G	A	T	A	G	T	2	paternal balkanensis
14316 sabina v. sabina, Azerbaijan	2*x*	A	C	G	A	T	A	G	T	2	
7614 sabina v. sabina, Switzerland	2*x*	A	C	G	A	T	A	G	T	2	
14728 sabina v. balkanensis, Greece	4*x*	G	G	A	C	C	C	A	G	1	
7573 sabina v. sabina, Pyrenees	2*x*	G	G	A	C	C	C	A	G	1	
7811 sabina v. sabina, Kazakhstan	2*x*	G	G	A	C	C	C	A	G	1	

[1]Eight polymorphic sites (1-8): polymorphic sites are 350(R), 391(S), 432(R), 604(M), 745(Y), 995(M), 1036(R), 1037(K). 350 xTGTCGGAG; 391 xGAGGTCCG; 432 xTCGTGTGC; 604 CGACAAGAx; 745(105) xCCAAAAGA; 995(333) xGCGAGGAG; 1036(392) xNGCGGTCGG;1037 xGCGGTCGG

A caveat to the aforementioned analysis is that the *J. chinensis* (8535, 8536) from Japan appear to be cv. Kaizuka, with spiral, twisted branches. Krussmann (1991) noted that 'Kaizuka' or 'Hollywood' juniper came from the Yokohama Nursey in the 1920s to the USA. Although I (RPA) collected from trees growing in a 'natural appearing' site; the site may have been planted in a 'randomly natural' manner. *Juniperus chinensis* is a very widely cultivated in China and Farjon notes in his contribution to the ICUN Red List (https://www.iucnredlist.org/species/42227/2962948#habitat-ecology):

"In a few localities this widespread species forms groves of tall trees (e.g. in S Gansu), or it is mixed with pines and deciduous angiosperms at canopy level. It is much more common, under conditions largely determined by man's agricultural practices, in secondary vegetation, on open, rocky slopes. The altitudinal range is (100-)1,400-2,400(-2,700) m a.s.l. Widespread planting and subsequent establishment in areas where it was not originally native have made it difficult to establish it original habitat and types of vegetation."

In a recent communication with Kangshan Mao (Chengdu), he wrote that there may be a few isolated trees in the mountains of southern Gansu, and that his students will undertake a survey/ collection trip in the summer of 2019. Collecting samples of *J.* x*pfitzeriana* plants, *J. chinensis* and *J. sabina* in the Ho Lan (Helan) Mountains (Shan) seems promising (research in progress).

ACKNOWLEDGEMENTS

This research was supported in part with funds from Baylor University. Thanks to the Issy Bean and Claire Sadler at Windsor Gardens for assistance in collection.

LITERATURE CITED

Adams, R. P. 1982. A comparison of multivariate methods for the detection of hybridization. Taxon 31: 646-661.

Adams, R. P. 2014. The Junipers of the World: The genus *Juniperus*. Trafford Publ., Bloomington, IN.

Adams, R. P., M. Miller and C. Low. 2016. Inheritance of nrDNA in artificial hybrids of *Hesperocyparis arizonica* x *H. macrocarpa*. Phytologia 98: 277-283.

Adams, R. P. 2018. Evolution of dioicous/ monecious taxa in *Juniperus*, contrasted with *Cupressus*, *Hesperocyparis*, *Callitropsis* and *Xanthocyparis* (Cupressaceae). Phytologia 100(4): 248-255.

Adams, R. P. 2019. Inheritance of chloroplasts and mitochondria in Conifers: A review of paternal, maternal, leakage and facultative inheritance. Phytologia 101(2): 134-138.

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., A. Boratynski, K. Marcysiak, F. Roma-Marzio, L. Peruzzi, F. Bartolucci, F. Conti, T. Mataraci, A. N. Tashev and S. Siljak-Yakovlev. 2018a. Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams & Tashev in Macedonia, Bosnia-Herzegovina, Croatia and southern Italy and relictual polymorphisms found in nrDNA. Phytologia 100(2): 117-127.

Adams, R. P., P. Farhat, L. Shuka and S. Silak-Yakovlev. 2018b. Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev in Albania and relictual polymorphisms found in nrDNA. Phytologia 100(3): 187-194.

Adams, R. P. and M. E. Kauffmann. 2010. Geographic variation in nrDNA and cp DNA of *Juniperus californica, J. grandis, J. occidentalis* and *J. osteosperma* (Cupressaceae). Phytologia 92: 266-276.

Bourge, M., S. C. Brown and S. Siljak-Yakovlev. 2018. Flow cytometry as tool in plant sciences, with emphasis on genome size and ploidy level assessment. Genetics & Application 2 (2): 1-12.

Czerepanov, S. K. 1973. Addidamenta et corrigenta and Floram URSS (Russ.). Leningrad, Nauka.

Den Oden, P. and B. K. Boom. 1965. Manual of cultivated conifers: Hardy in the cold- and warm-temperate zone. Martinus Nijhoff Publ., The Hague, Belgium

Farhat, P., O. Hidalgo, T. Robert, S. Siljak-Yakovlev, I. J. Leitch, R. P. Adams, and M. B. Dagher-Kharrat. 2019a. Polyploidy in the conifer genus *Juniperus*: an unexpectedly high rate. Frontiers in Plant Science (in press).

Farhat, P., S. Siljak-Yakovlev, R. P. Adams, T. Robert and M. B. Dagher-Kharrat. 2019b. Genome size variation and polyploidy in the geographical range of *Juniperus sabina* L. (Cupressaceae). Botany Letters (in press).

Garnatje, T., J. Vallès, S. Garcia, O. Hidalgo, M. Sanz, M. Á. Canela and S. Siljak-Yakovlev. 2004. Genome size in *Echinops* L. and related genera (Asteraceae, Cardueae): karyological, ecological and phylogenetic implications. Biology of the Cell 96 (2): 117-124.

Hall, M. T., A. Mukherjee and W. R. Crowley. 1979. Chromosome numbers of cultivated Junipers. Bot. Gaz. 140: 364-370.

Krussmann, G. 1991. Manual of cultivated conifers. Timber Press, Portland, OR

Le Duc, A., R. P. Adams and M. Zhong. 1999. Using random amplification of polymorphic DNA for a taxonomic reevaluation of Pfitzer junipers. HortScience 36: 1123-1125.

Lewis, J. 1995. Proposal to conserve the name *Juniperus xmedia* Melle (Cupressaceae). Taxon 44: 229-231.

Schmidt, R. 1983. *Juniperus* X*media* Van Melle, Invalid name for pfitzeriana and plumosa group of *Juniperus chinensis*. Folia Dendrologica 10: 291-296.

Van Melle, P. J. 1947. Review of *Juniperus chinensis* et al. 108 pp. New York Botanical Garden Press, NY.

Welch, H. J. 2012. The conifer manual. Springer Publ., Netherlands

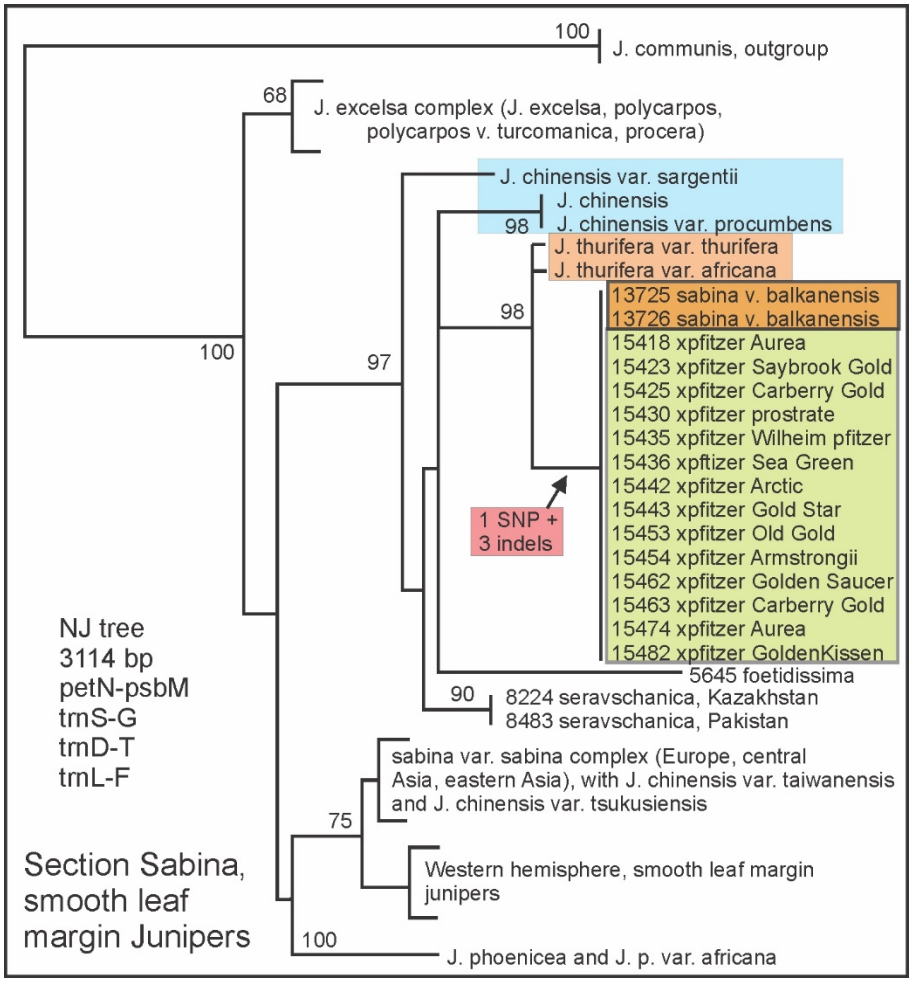

Figure 1. Chloroplast tree for *Juniperus*, sect. *Sabina*, smooth leaf margined junipers, based on four chloroplast gene regions, 3114 bp: petN-psbM, trnS-trnG, trnD-trnT, and trnL-trnF. Numbers at branch points are posterior probabilities as percent. Probabilities below 68 are not shown. Notice that *J. thurifera and J. thurifera var. africana* differ by only 1 SNP and 3 indels from xpfitzer (*J.* x*pfitzeriana*) cultivars. There are no sequence differences among the xpfitzer (*J.* x*pfitzeriana*) cultivars, nor with *J. sabina* v. *balkanensis* (inside yellow and orange boxes).

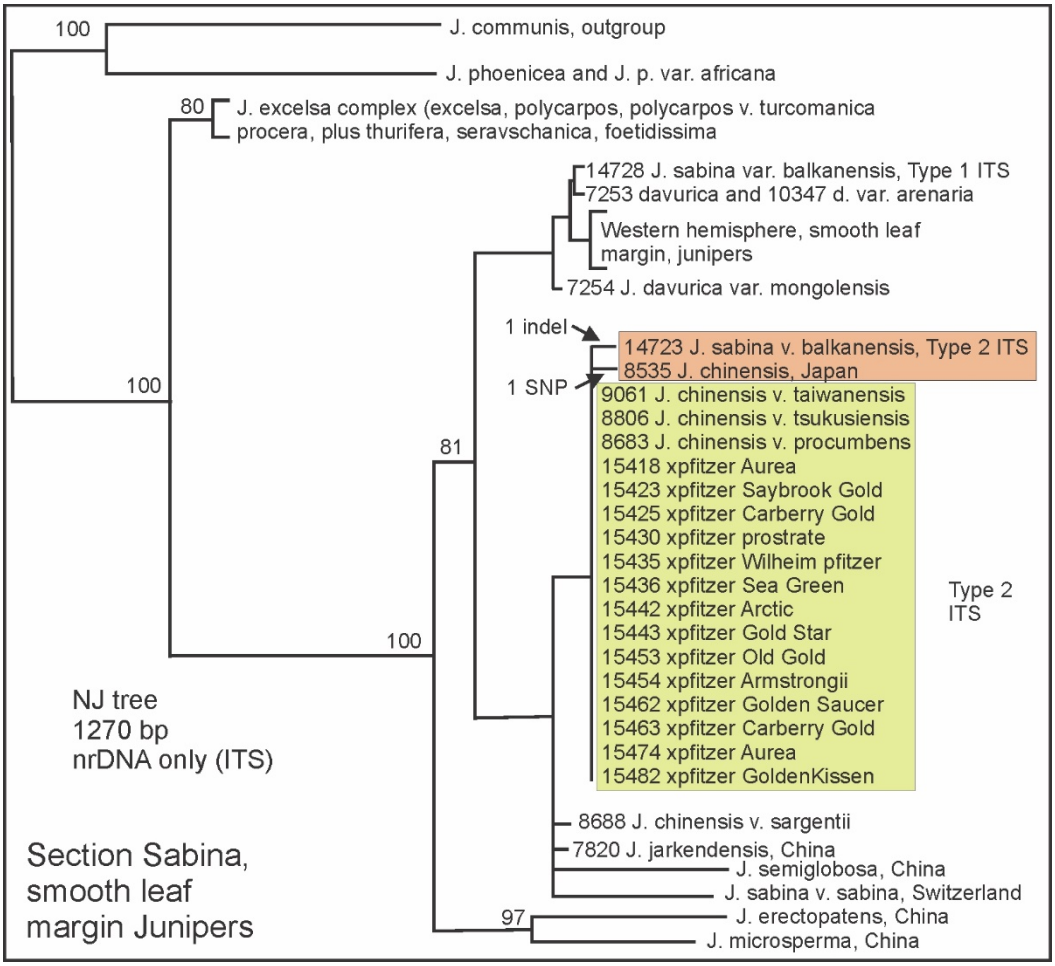

Figure 2. nrDNA (ITS) tree for *Juniperus*, sect. *Sabina*, smooth leaf margined junipers, based nrDNA (ITS), 1270 bp. Numbers at branch points are posterior probabilities as percent. Probabilities below 68 are not shown. Notice that 14723 *J. sabina* var. *balkanensis* differs by only 1 indel from xpfitzer cultivars and 8535 *J. chinensis*, Japan differs by only 1 SNP from the xpfitzer cultivars. There are no sequence differences among the xpfitzer (*J.* x*pfitzeriana*) cultivars, nor with *J. chinensis* v. *procumbens*, v. *taiwanensis* or v. tsukusiensis, except for the heterozygous sites in the xpfitzer cultivars (inside yellow box).