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**Nuclear and chloroplast DNAs reveal diverse origins and mis-identifications of
Juniperus cultivars from Windsor Gardens, UK, Part 3 of 3.**

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ABSTRACT

Ploidy was determined for 15 plants labeled as *Juniperus squamata* at the Windsor Gardens, UK and revealed 12 were tetraploids ($2n=4x=44$) and 3 were diploids ($2n=2x=22$). nrDNA (ITS) and cp DNA sequencing the tetraploids found: 4 *J. squamata* (4x); 4 *J. tibetica* (4x) x *J. squamata* (4x); 2 *J. sabina* var. *balkanensis* (4x) x *J. squamata* (4x); and one *J. chinensis* var. *sargentii* (4x) x *J. squamata* (4x). Sequencing the 3 diploids revealed: 2 *J. pingii* (2x) x *J. pingii* (2x); and 1 *J. pingii* (2x)? x *J. komarovii*(2x)? Ploidy analyses of 18 additional cultivars, putatively from *Juniperus davurica*, *J. recurva*, *J. rushforthiana*, *J. sabina*, and *J. virginiana* revealed 6 diploids, 5 triploids and 7 tetraploids. Cultivar 'Musgrave' (4x), by DNA, was identical to *J. xpfitzeriana* 'Wilhelm Pfitzer' (4x). The DNA of the 5 triploids were all nearly identical to *J. xpfitzeriana* 'Wilhelm Pfitzer' (4x). 'Tamariscifolia' and 'Variegata' both had *J. sabina* var. *sabina* as their maternal parent, but the first had *J. sabina* var. *balkanensis* as the male parent and the second had *J. sabina* var. *sabina* as the male parent. Thus, 'Tamariscifolia' is the first discovery of a *J. sabina* var. *balkanensis* x *J. s. var. sabina* hybrid in cultivation. None of the 3 'davurica' cultivars proved to be *J. davurica*, but rather *J. chinensis* var. *procumbens* x *J. chinensis* var. *sargentii*. Cultivars *J. indica* and *recurva* 'densa' were shown to be *J. indica* var. *caespitosa*. *recurva* 'Embley Park' appears to be *J. coxii* x *J. squamata* var. *wilsonii*. *J. wallichiana* (= *J. indica*) 15460 was found to be *J. rushforthiana*, whereas *J. wallichiana* (15487) was discovered to be *J. indica* x *J. rushforthiana*. Cultivar *virginiana* 'cannaertii' was shown to be *J. virginiana*. Botanic gardens provide a great opportunity for species to hybridize with other species that are not in contact in nature. The species care and suitable habitat provided in a garden setting, as well as vegetative propagation methods have allowed the preservation of those rare hybrids). Identification of juniper hybrids and variants is quite imprecise. DNA barcoding of cultivated plants in botanic gardens would greatly facilitate the recognition, study and utilization of rare hybrids and somatic mutations.

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KEY WORDS: *Juniperus davurica*, *J. recurva*, *J. rushforthiana*, *J. sabina*, *J. squamata*, *J. virginiana*, cultivars, origin, nrDNA, ITS, cp DNA, DNA barcoding.

This is the third report in an on-going study (Adams et al. 2019, Adams et al. 2020) on DNA barcoding of *Juniperus* at Windsor Gardens. Our initial study of *Juniperus x pfitzeriana* cultivars at Windsor Gardens (Adams, et al. 2019) discovered all of the 14 cultivars were identical in their chloroplast DNA, which was identical to that of *J. sabina* var. *balkanensis* (Table 1). In addition, 13 *J. xpfitzeriana* cultivars were allo-tetraploids with heterozygous bases at 5 to 7 sites that distinguish *J. chinensis* and *J. sabina* var. *balkanensis*. These cultivars had identical nrDNA. Two 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. xpfitzeriana* 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. xpfitzeriana* (Spath) Schmidt.

Table 1. nrDNA (ITS) variable sites in *J. chinensis* cultivars. (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*. Modified from Adams et al. (2019). Site numbers modified to correspond with site numbers in Table 3 of this report.

taxa: <i>J. xpfitzeriana</i> (=xmedia) unless noted otherwise	ploidy	212 ^a K	410 S	665 Y	986 Y	996 M	1034 K	1073 W	1137 R	ITS classification hybrid?	chloroplast, ex. pollen from:
Probable male (pollen) parent	4x	G	C	T	T	A	T	T	G	<i>J. sabina</i> var. <i>balkanensis</i>	<i>J. sabina</i> var. <i>balkanensis</i>
Probable female parent genotype	4x	T	G	C	C	C	G	A	A	<i>J. chinensis</i>	<i>J. chinensis</i>
15442 Arctic	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15454 Armstrongii	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15418 Aurea, Paris-sud	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15474 Aurea	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15423 Saybrook Gold	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15425 Carberry Gold	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15463 Carberry Gold	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15443 Gold Star	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15462 Golden Saucer	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15482 Goldenkissen	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15430 pfitzeriana prostate	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15435 Wilhelm Pfitzer	4x	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	<i>balkanensis</i>
15453 Old Gold	4x	G/T	C	C/T	C/T	A/C	G/T	A/T	G	chin x sab*	<i>balkanensis</i>
15436 Sea Green, Windsor	3x	G/T	C	T	C/T	A/C	G/T	A/T	G	chin x sab*	<i>balkanensis</i>
15604 Sea Green Home Depot	3x	G/T	C	T	C/T	A/C	G/T	A/T	G	chin x sab*	<i>balkanensis</i> ?

^aVariable sites located at: 212, xGGCCAAGC; 410, xGTTGAGAT; 665, xTCTTCGTC; 986, xGCCCTCCC; 996, xGCGAGGAG; 1034, xGCCGTCCG; 1073, xCGCGACGA; 1137, xGAACCTTG.

In our second study of 24 *J. chinensis* cultivars at Windsor Gardens, we reported (Adams et al. 2020, this issue) that two cultivars were found to be mis-identified, and were actually *Cupressus gigantea* and *J. virginiana*. Interestingly, of the remaining 22 ‘*chinensis* cultivars’ only 3 plants were ‘pure, autotetraploid *J. chinensis*’ by DNA sequencing and Flow Cytometry (FC) ploidy determination. The parentage of the remaining 19 samples had mixed parents from several related species.

The purpose of the present research is to present new DNA sequencing utilizing both chloroplast and nuclear DNA to determine variation in *Juniperus davurica*, *J. recurva*, *J. rushforthiana*, *J. sabina*, *J. squamata*, and *J. virginiana*, cultivars at Windsor Gardens.

METHODS

Plant materials:

Samples: Leaf samples were collected in Windsor Gardens, Windsor Great Park, Windsor, *SL4 2HT* UK from 33 *Juniperus* cultivar accessions (see Table 2) and immediately placed in activated silica gel for DNA sequencing and Flow Cytometry - ploidy determination (Table 2).

Table 2. Windsor 33 *Juniperus* cultivars collected with cultivar origin (< is earlier than).

taxon (as labeled at Windsor Garden)	Adams. Coll. #	Windsor acc. #	ploidy (this study)	Chrom. number, 2n, litr.	Origin: based on Den Oden and Boom 1965; Krussmann 1991; Welch 2012, Lewis 1998, Auders & Spicer 2012
davurica 'Expansa'	15431	2001-448	4x		Netherlands 1940
davurica 'Expansa Variegata'	15475	1999-5915	4x		Netherlands 1938
davurica 'Expansa Aureo-spicata'	15444	1999-5914	4x		Netherlands 1940
indica'	15437	1999-6150	2x		unknown
pingii var. wilsonii	15459	2000-1308	2x		China 1910
recurva 'densa'	15419	1999-5967	2x	44	UK 1862
recurva 'Embley Park'	15420	1999-2968	2x	44	UK 1961
sabina 'Variegata'	15434	2001-405	2x		UK 1822
sabina 'Musgrave'	15479	1999-5986	4x		UK 1930
sabina 'Tamariscifolia'	15489	1999-5991	4x	22,44	UK 1789
squamata	15483	1999-6162	4x	44	unknown
squamata	15456	1999-6161	4x	44?	unknown
squamata	15485	1999-6163	2x	44	unknown
squamata 'Blue Alps'	15447	1999-6022	4x	44?	UK/Austria 1968
squamata 'Blue Spider'	15481	1999-6024	4x	44?	Netherlands <1980
squamata 'Chinese Silver'	15455	1999-6027	4x	44?	UK 1964. TTYu 7881/TTYu 15614
squamata 'Filborna'	15476	1999-6028	4x	44?	Sweden 1946
squamata 'Glassell'	15445	1999-6029	4x	44?	UK 1958
squamata 'Holger'	15486	1999-6031	4x	44?	Sweden 1946
squamata 'Prostrata'	15421	1999-6036	2x	(44)	UK ?
squamata 'Pygmaea'	15424	2001-778	2x	(44)	UK <1964
squamata var. fargesii	15480	1999-6167	4x	44?	China Rehder & Wilson 1914
squamata 'Wilsonii'	15449	1999-4563	4x	44?	China 1910
squamata 'Wilsonii'	15450	1999-6038	4x	44?	China 1910
squamata 'Yellow Tip'	15457	1999-6039	4x	44?	Netherlands <1991
virginiana Pfitzer Group 'Hetzii'	15422	2000-521	3x	33	USA 1920
virginiana 'Glauca' x Pfitzer Group) '= Grey Owl'	15429	2000-266	3x	33	Netherlands 1938
virginiana Pfitzer Group 'Sulphur Spray'	15438	1999-6114	3x	33	Netherlands 1962
virginiana 'cannaertii'	15440	1999-6045	2x	22	Belgium 1868
virginiana 'Glauca' x Pfitzer Group) '= 'Grey Owl'	15448	1999-6149	3x	33	Netherlands 1938
virginiana 'Blue Cloud'	15468	1999-6042	3x	33	Netherlands 1955
wallichiana	15460	2000-571	4x		unknown
wallichiana	15487	1999-6144	4x		unknown

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 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. Chromas 2.31 (Technelysium Pty Ltd.) was used viewing sequence chromatograms and Mafft used for alignment.

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 MgCl₂, 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 nylon mesh. 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 620/10 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:

$$2C \text{ DNA sample (pg)} = (\text{Sample } 2C \text{ peak mean} / \text{Standard } 2C \text{ peak mean}) \times \text{Standard } 2C \text{ DNA (pg)}.$$

RESULTS AND DISCUSSION

Analyses of *Juniperus 'squamata'* cultivars (15)

Ploidy levels determined for 15 *Juniperus 'squamata'* cultivars revealed that 12 are tetraploids (4x) and 3 are diploids (2x, Table 3). It should be noted that all the '*squamata*' cultivars were shrubs, not trees.

Analyses of trnSG (SG hereafter) found the paternal (male, pollen) parent varied considerably with 4 cultivars having SG cp DNA about equally similar to *J. pingii* or *J. squamata* (green, Table 3). *Juniperus tibetica* (or a shrub form in cultivation) appears to be the paternal parent of 5 cultivars, 15450 'Wilsonii', 15479 'Filborna', 15480 'var. fargesii', 15481 'Blue Spider', and 15449 'Wilsonii' (Table 3). Two cultivars, 'Yellow Tip' and 'Holger', have the cp of *J. thurifera* or *J. sabina* var. *balkanensis*. Because *J. thurifera* is a tree and *J. s.* var. *balkanensis* is a shrub, it seems more likely the paternal parent is *J. s.* var. *balkanensis*. 'Blue Alps' is interesting as its male parent is *J. chinensis* var. *sargentii* (Table 3).

nrDNA (ITS) indicates only 4 cultivars appear to be from *J. squamata* x *J. squamata* parents (in green, Table 3). Most of the 15 cultivars have a maternal parent of *J. squamata* or a closely related taxon (Table 3). The nrDNA for 15450 *J. squamata* 'Wilsonii' was equal to *J. pingii* and *J. squamata*, but as *J. pingii* a diploid, that favors *J. squamata* as the maternal parent. Both 15450 *J. squamata* 'Wilsonii' and

15450 *J. squamata* 'Wilsonii' appear to be of hybrid origin (*J. tibetica* x *J. squamata*), but as *J. tibetica* is a tree (in the wild), there may be a shrub form in cultivation that is the male parent. The parents of 15480 'var. fargesii' and 15481 'Blue Spider' appear to be *J. tibetica* (or a shrub form in cultivation) x *J. squamata*. Plant 15449 'Wilsonii', has ITS DNA most similar to *J. squamata* var. *wilsonii*, but not definitive. It is likely that its ITS sequences are not yet in GenBank.

Both 'Yellow Tip' and 'Holger' seem derived from a *J. sabina* var. *balkanensis* x *J. squamata* cross. This is of some interest because *J. s.* var. *balkanensis* and *J. squamata* do not grow near each other in nature (Adams 2014), so the *J. s.* var. *balkanensis* male parent must be (or have been) in cultivation in a garden where the cross occurred. So far as known, var. *balkanensis* has not been found in cultivation. The origin of 'Holger' (Sweden, 1946) is older than that of 'Yellow Tip' (Netherlands, 1991, Table 1), so it is possible that 'Holger' was acquired later in the Netherlands (1991) and a yellow, somatic mutation occurred, thence the new cultivar 'Yellow Tip'.

Two of the diploids 15421 'Prostrata' (UK ?) and 15424 *J. squamata* 'Pygmaea' (UK before 1964) have identical male and female parent matches (Table 3) and their ITS differs by only 1 bp. The third diploid, 15485 *squamata* (origin unknown) differs by 3 bp in ITS and has quite different putative parents (pingii x komarovii). As both *J. pingii* and *J. komarovii* are trees, this seems unlikely.

Table 3. ITS (13 informative ITS SNPs) and trnS-trnG (cp) analyses of *J. squamata* cultivars at Windsor Gardens.

accession name at Windsor Gardens, with ploidy (this study)	paternal (male) parent by trnSG cp sequence, with ploidy from Farhat et al. 2019	maternal parent based on ITS, with ploidy from Farhat et al. 2019	putative origin of Windsor Gardens accession, with ploidy (this study) (paternal x maternal)
15445 <i>J. squamata</i> 'Glassell' 4x	pingii 2x/ squamata 4x	squamata? 4x	squamata x squamata 4x
15455 <i>J. squamata</i> 'Chinese Silver' 4x	pingii 2x/ squamata 4x	squamata 4x	squamata x squamata 4x
15483 <i>J. squamata</i> 4x	pingii 2x/ squamata 4x	squamata? 4x	squamata x squamata 4x
15456 <i>J. squamata</i> 4x	pingii 2x/ squamata 4x	indica 4x x squamata 4x?	squamata x squamata 4x
15480 <i>J. squamata</i> var. fargesii 4x	tibetica 4x	tibetica 4x/squamata 4x	tibetica x squamata 4x
15481 <i>J. squamata</i> 'Blue Spider' 4x	tibetica 4x	tibetica 4x/ squamata 4x	tibetica x squamata 4x
15449 <i>J. squamata</i> 'Wilsonii' 4x	tibetica 4x	wilsonii 4x/ squamata 4x	tibetica x sq. var. wilsonii? 4x
15450 <i>J. squamata</i> 'Wilsonii' 4x	tibetica 4x	pingii 2x/ squamata 4x	tibetica x squamata 4x
15476 <i>J. squamata</i> 'Filborna' 4x	tibetica 4x	squamata 4x	tibetica x squamata 4x
15457 <i>J. squamata</i> 'Yellow Tip' 4x	thurifera 4x /sabina var. balkanensis 4x	squamata 4x	sabina var. balkanensis x squamata 4x
15486 <i>J. squamata</i> 'Holger' 4x	thurifera 4x /sabina var. balkanensis 4x	squamata 4x	sabina var. balkanensis x squamata 4x
15447 <i>J. squamata</i> 'Blue Alps' 4x	sargentii 4x	squamata 4x	sargentii x squamata 4x
15421 <i>J. squamata</i> 'Prostrata' 2x	pingii 2x/ squamata 4x	pingii 2x	pingii/squamata x pingii 2x
15424 <i>J. squamata</i> 'Pygmaea' 2x	pingii 2x/ squamata 4x	pingii 2x	pingii/squamata x pingii 2x
15485 <i>J. squamata</i> 2x	pingii 2x/ squamata 4x	komarovii 2x	pingii? x komarovii? 2x

Four of the 'squamata' cultivars were clearly hybrids in their nrDNA (Table 4) with 13 heterozygous sites in 957 bp sequenced and a region with slipped sequences (427-750) with flanking indels that prevented sequencing. No differences were found among the 4 hybrids (in the 957 bp sequence), except at sites 802 and 995 (Table 4), yet, their putative male parents were all *squamata* (Table 3).

Table 4. Thirteen (13) heterozygous nrDNA sites in 4 hybrids, identical, except at sites 802 and 995.

hybrids	site# 179	212	351	363	365	366	389	no seq 427- 750	802	985	995	1071	1243	1169
15476 Filborna 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	A/G	C/T	A/C	A/T	A/G	C/T
15457 Yellow Tip 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	A/G	C/T	A/C	A/T	A/G	C/T
15486 Holger 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	A	C/T	C	A/T	A/G	C/T
15447 Blue Alps 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	G	C/T	C	A/T	A/G	C/T

Analyses of the other 18 *Juniperus davurica*, *J. recurva*, *J. rushforthiana*, *J. sabina*, *J. virginiana* cultivars

This group contained 6 diploids, 5 triploids, and 7 tetraploids (Table 5). The nrDNA of 15479 sabina 'Musgrave' (4x) was found to be identical to 15435 xpfitzeriana 'Wilhelm Pfitzer', considered the 'mother' of all pfitzer cultivars (Adams et al. 2019). All of the triploids were similar or somewhat similar to 'xpfitzeriana' in their ITS (Table 5). Both 15489 sabina 'Tamariscifolia' (4x) and 15434 sabina 'Variegata' (2x) had identical ITS DNA, which was 100% identical to *Adams 14317*, *J. sabina*, Type 2 ITS from Azerbaijan. This is suggestive that 'Tamariscifolia' might be an auto-tetraploid from 'Variegata'.

All three *davurica* cultivars were tetraploids that had NCBI matches of 99.31 to 99.74% to *J. chinensis* var. *sargentii* (no origin listed in NCBI). No heterozygous sites were found suggesting the tetraploid cultivars are auto-tetraploids. Two cultivars, 15437 *indica* and 15437 *recurva* 'densa', both diploids, had ITS sequences nearly identical (99.82, 99.85%) to *J. indica* var. *caespitosa*, a shrub, in contrast to *J. indica* (var. *indica*), a tree.

Accessions 15420 *recurva* 'Embley Park' 15459 'pingii var. wilsonii' had 100% matches to *J. squamata* var. *wilsonii* (*Adams 5521*, Arnold Arboretum).

Both accessions named 'wallichiana' (treated as *J. indica* in Adams 2014), were 99.83 and 99.49% similar to *Adams 8140*, ex Bhutan (from a field collection by K. Rushforth). 15460 differed at site 167, being heterozygous (Table 5). Finally, 15440 *virginiana* 'cannaertii' (2x) had a 99.37% match to *J. virginiana*, *Adams 10231*, Knoxville, TN

Analysis of trnSG (cp DNA) confirmed the paternal parent (by pollen) of 15479 sabina 'Musgrave' was *J. sabina* var. *balkanensis*, being the same as found in the Wilhelm Pfitzer (Table 6). All of the triploids plus the tetraploid 'Tamariscifolia' were also found to have *J. sabina* var. *balkanensis* as the paternal parent.

Surprisingly, 15434 sabina 'Variegata', which had identical ITS DNA with 'Tamariscifolia' (Table 5), had cp DNA of *J. sabina* var. *sabina* (100% to *Adams 14317*, Azerbaijan). In contrast, Tamariscifolia had *J. sabina* var. *balkanensis* cp DNA.

Table 5. ITS classification of the 18 cultivars. 15435 xpfitzeriana 'Wilhelm Pfitzer' from Adams et al. (2019) is included as a pfitzer exemplar. MAFFT and NCBI (BLASTn) search reported at % pairwise similarity (i.e. 100% = identical sequences, etc.)

Adams coll. #, Windsor accession name, ploidy(this study)	ITS classification, ploidy from Farhat et al. 2019.	168 C/T	212 G/T	350 A/G	410 C/G	663 C/T	985 C/T	995 A/C	1033 G/T	1071 A/T	1135 A/G	1147 A/T
15435 xpfitzeriana 'Wilhelm Pfitzer 4x, ex Adams et al. 2019.	J. xpfitzeriana (hybrid sabina v. balkanensis 4x X chinensis 4x¹)	C	G/T	A	C/G	C/T	C/T	A/C	G/T	A/T	A/G	T
15479 sabina 'Musgrave' 4x	J. xpfitzeriana 4x	C	G/T	A	C/G	C/T	C/T	A/C	G/T	A/T	A/G	T
15438 virginiana Pfitzer Group 'Sulphur Spray' 3x	~ J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x ¹	C/T	G/T	A/G	C/G	C/T	C/T	A/C	G/T	A/T	A/G	A/T
15422 virginiana Pfitzer Group 'Hetzii' 3x	~ J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x ¹	C/T	G/T	A/G	C/G	T	C/T	A/C	G	A/T	A/G	A/T
15429 virginiana 'Glaucua' = 'Grey Owl' 3x	~ J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x ¹	C/T	G/T	A/G	C/G	T	C/T	A/C	G	A/T	A/G	A/T
15448 virginiana 'Glaucua' = 'Grey Owl' 3x	~ J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x ¹	C/T	G/T	A/G	C	T	C/T	A/C	G	A/T	G	A/T
15468 virginiana 'Blue Cloud' 3x	~ J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x ¹	C/T	G/T	A/G	C	T	C/T	A/C	G	A/T	G	A/T
15489 sabina 'Tamariscifolia' 4x	J. sabina var. sabina, Type 2, ITS 2x	MAFFT 100%, to Adams 14317, Azerbaijan, Type 2 ITS Note Identical to 15434 Variegata										
15434 sabina 'Variegata' 2x	J. sabina var. sabina, Type 2, ITS 2x	MAFFT 100%, to Adams 14317, Azerbaijan, Type 2 ITS Note Identical to 15489 Tamariscifolia										
15444 davurica 'Expansa Aureo-spicata' 4x	J. chin. var. sargentii 4x	NCBI 99.31%, origin of J. c. var. sargentii, not listed in NCBI										
15475 davurica 'Expansa Variegata' 4x	J. chin. var. sargentii 4x	NCBI 99.74%, origin of J. c. var. sargentii, not listed in NCBI										
15431 davurica 'Expansa' 4x	J. chin. var. sargentii 4x	NCBI 99.74%, origin of J. c. var. sargentii, not listed in NCBI										
15437 indica 2x	J. indica v. caespitosa 2x?	NCBI 99.82% to Adams 7625, Nepal										
15419 recurva 'densa' 2x	J. indica v. caespitosa 2x?	NCBI 99.85% to Adams 7625, Nepal										
15420 recurva 'Embley Park' 2x	J. squamata var. wilsonii 2x	MAFFT 100% to Adams, 5521, Arnold Arbor., #1010-64A										
15459 pingii var. wilsonii 2x	J. squamata var. wilsonii 2x	MAFFT 100% to Adams, 5521, Arnold Arbor., #1010-64A										
15460 wallichiana 4x	J. rushforthiana 4x	NCBI 99.83% to Adams 8140, Bhutan, site 167 A/G										
15487 wallichiana 4x	J. rushforthiana 4x	NCBI 99.49% to Adams 8140, Bhutan										
15440 virginiana 'cannaertii' 2x	J. virginiana 2x	NCBI 99.37% to Adams 10231, Knoxville, TN										

All three 'davurica' accessions had *J. chinensis* var. *procumbens* (4x) as the paternal parent. The diploid accessions, 15437 indica, and 15419 recurva 'densa' had *J. indica* var. *caespitosa* as the paternal parent (Table 6).

Accession 15419 recurva 'densa' had a 100% match to *J. coxii* in GenBank (origin not given), but as *J. coxii* is a tetraploid, this should be viewed some caution. 15459 pingii var. wilsonii (2x), had matches of 99.92% to *J. pingii* (Adams 8506, tree, Yunnan) and *J. carinata* (Adams 8498, shrub, Yunnan), because *J. carinata* is a shrub (as is acc. 15459), this favors *J. carinata* as the pollen parent.

Interestingly the 2 'wallichiana' tetraploids had different paternal parents: *J. rushforthiana* (4x) for 15460, and *J. indica* (2x), for 15487 (Table 6). 15440, virginiana 'cannaertii' (2x), had the cp of *J. virginiana* (2x).

Table 6. Analyses of putative paternal (pollen) parents by trnSG cp DNA.

Adams coll #, accession name at Windsor Gardens, and ploidy (this study)	paternal (male) parent by trnSG cp sequence, with ploidy from Farhat et. al. 2019	notes on identification
15435 xpfitzeriana 'Wilhelm Pfitzer' 4x	<i>J. sabina</i> var. <i>balkanensis</i> (Adams et al. 2019) 4x	<i>J. xpfitzeriana</i> (hybrid <i>sabina</i> v. <i>balkanensis</i> 4x X <i>chinensis</i> 4x (Adams et al. 2019)
15479 <i>sabina</i> 'Musgrave' 4x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15438 virginiana Pfitzer Group 'Sulphur Spray' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15422 virginiana Pfitzer Group 'Hetzii' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15429 virginiana 'Glaucula' = 'Grey Owl' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15448 virginiana 'Glaucula' = 'Grey Owl' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15468 virginiana 'Blue Cloud' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15489 <i>sabina</i> 'Tamariscifolia' 4x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	MAFFT 99.92%, Adams 13725, Bulgaria
15434 <i>sabina</i> 'Variegata' 2x	<i>J. sabina</i> var. <i>sabina</i> 2x	MAFFT 100.0%, Adams 14317, Azerbaijan
15444 <i>davurica</i> 'Expansa Aureo-spicata' 4x	<i>J. chinensis</i> var. <i>procumbens</i> 4x	NCBI 100.0%, no origin given
15475 <i>davurica</i> 'Expansa Variegata' 4x	<i>J. chinensis</i> var. <i>procumbens</i> 4x	NCBI 100.0%, no origin given
15431 <i>davurica</i> 'Expansa' 4x	<i>J. chinensis</i> var. <i>procumbens</i> 4x	NCBI 100.0%, no origin given
15437 <i>indica</i> 2x	<i>J. indica</i> var. <i>caespitosa</i> 2x?	MAFFT 99.92%, Adams 7625, Nepal
15419 <i>recurva</i> 'densa' 2x	<i>J. indica</i> var. <i>caespitosa</i> 2x?	NCBI 99.85%, Adams 7625, Nepal
15420 <i>recurva</i> 'Embley Park' 2x	<i>J. coxii</i> 4x	NCBI 100.0%, no origin given
15459 <i>pingii</i> var. <i>wilsonii</i> 2x	<i>J. pingii</i> (tree) 2x/ <i>J. carinata</i> (shrub) 2x	MAFFT 99.92%, Adams 8506, China MAFFT 99.92%, Adams 8498, China
15460 <i>wallichiana</i> 4x	<i>J. rushforthiana</i> 4x	MAFFT 100.0%, Adams 8140, Bhutan
15487 <i>wallichiana</i> 4x	<i>J. indica</i> 2x	NCBI 99.85%, no origin given
15440 virginiana 'cannaertii' 2x	<i>J. virginiana</i> 2x	MAFFT 100.0%, Adams 10231 TN, USA

A summary of the paternal and maternal parents and putative origin of these 18 cultivars is given in Table 7. Notice that *xpfitzeriana* 'Wilhelm Pfitzer', 'Musgrave' and all the triploids have the same parents: male, *J. sabina* var. *balkanensis*; female, *J. chinensis* (Table 7). However, the triploids, seem unlikely to have come from 2 tetraploid parents. Farhat et al. (2019) reported only tetraploids in *J. chinensis* accessions, but cited literature reports of *J. chinensis* diploids. So, perhaps a diploid *J. chinensis* is the maternal parent of all the triploids. Alternatively, perhaps a meiotic abnormality occurred in in *J. sabina* var. *balkanensis*, producing haploid pollen that led to the first triploid in this group. Then, subsequent selection for somatic mutation(s) in a vegetative character led to the cloning of that (those) 'sports', and thence to the other triploid cultivars. They are certainly very closely related in their DNAs and appear to be as similar as siblings.

Cultivar 15434 *sabina* 'Tamariscifolia' is interesting in that it has *J. sabina* var. *balkanensis* (4x) as the paternal parent, but *J. sabina* var. *sabina* Type 2 ITS (2x) as the maternal parent. ITS Types 1 and 2 DNAs differ by 8 sites (Adams et al. 2018a,b), and both var. *balkanensis* and var. *sabina* have Types 1 and 2 ITS DNA, as well as numerous occurrences of hybridization between Type 1 and Type 2 plants (heterozygous for some or all of the 8 sites, Adams et al. 2018a,b). The origin of 'Tamariscifolia' seems to be from a reduced male gamete (2x pollen) of var. *balkanensis* fertilizing a diploid (un-reduced gamete, 2x) of var. *sabina* (Table 7). It is interesting that Le Duc et al. (1999) found that 'Tamariscifolia' grouped with *J. sabina* in PCO ordination using RAPDs (Random Amplified Polymorphic DNAs).

Complementing the origin of 'Tamariscifolia' is the origin of 'Variegata', a diploid arising from pollen of *J. sabina* var. *sabina* (2x, Type 2 ITS), fertilizing *J. sabina* var. *sabina* (2x, Type 2 ITS). Type 2 ITS for both parents was deduced by the lack of heterozygous sites in the ITS DNA for accession 'Variegata'.

None of the Windsor 'davurica cultivars' were, in fact, related to *J. davurica*. *Juniperus davurica* grows in Mongolia and far eastern Russia. It seems unlikely that it is in cultivation in nurseries and Botanic gardens. All 3 of these 'davurica cultivars' have *J. chinensis* var. *procumbens* as the pollen (paternal) parent and *J. chinensis* var. *sargentii* as the maternal parent (Table 7) and both parents are tetraploids as well as cultivars *Expansa Aureo-spicata* and *Expansa Variegata*. However, Windsor *davurica* 'Expansa' was found to be a triploid, indicating that one of the parents might be a diploid *J. chinensis* taxon.

The parents of 15437 *indica* (2x) and 15419 *recurva* 'densa' appear to both be *J. indica* var. *caespitosa* (2x), the shrubby variety of *J. indica* var. *indica*, a tree. The diploid, *recurva* 'Embley Park' (15420) had cp of *J. coxii* (or perhaps a close relative that is 2x) and *J. squamata* (4x) (or a relative that is diploid) as parents (Table 7).

A *pingii* plant seems unusual in cultivation as *J. pingii* is a large tree in Kunming, China. It appears that 15459 *pingii* var. *wilsonii* (2x) male parent is likely *J. carinata* (a shrub, 2x) rather than *J. pingii* (a tree). The maternal parent is *J. squamata* var. *wilsonii* (2x) or a relative that is diploid (Table 7). The 15460 'wallichiana' cultivar seems to be a good *J. rushforthiana*. But 15487 'wallichiana' appears to have a *J. indica* pollen parent and *J. rushforthiana* maternal parent. Finally, 15440 *virginiana* 'cannaertii' (2x), is *J. virginiana* by both parents.

Table 7. Putative origin of 18 cultivars at Windsor Gardens.

Adams coll. #, acc. name at Windsor Gardens, and ploidy (this study)	paternal (male) parent by trnSG cp sequence, with ploidy from Farhat et al. 2019	maternal parent based on ITS, with ploidy from Farhat et. al. 2019	putative origin of Windsor Gardens accession, with ploidy (this study) (paternal x maternal)
15435 xpfitzeriana 'Wilhelm Pfitzer' 4x	<i>J. sabina</i> var. <i>balkanensis</i> (Adams et al. 2019) 4x	<i>J. chinensis</i> 4x	<i>J. xpfitzeriana</i> ; ie., <i>J. sabina</i> var. <i>balkanensis</i> 4x x <i>J. chinensis</i> 4x ¹
15479 <i>sabina</i> 'Musgrave' 4x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. chinensis</i> 4x	<i>J. xpfitzeriana</i> ; ie., <i>J. sabina</i> var. <i>balkanensis</i> 4x x <i>J. chinensis</i> 4x
15438 <i>virginiana</i> Pfitzer Group 'Sulphur Spray' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. chinensis</i> 4x	~= <i>J. xpfitzeriana</i> ; <i>J. sabina</i> var. <i>balkanensis</i> x <i>J. chinensis</i> 3x
15422 <i>virginiana</i> Pfitzer Group 'Hetzii' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. chinensis</i> 4x	~= <i>J. xpfitzeriana</i> ; <i>J. sabina</i> var. <i>balkanensis</i> x <i>J. chinensis</i> 3x
15429 <i>virginiana</i> 'Glauca' = 'Grey Owl' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. chinensis</i> 4x	~= <i>J. xpfitzeriana</i> ; <i>J. sabina</i> var. <i>balkanensis</i> x <i>J. chinensis</i> 3x
15448 <i>virginiana</i> 'Glauca' = 'Grey Owl' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. chinensis</i> 4x	~= <i>J. xpfitzeriana</i> ; <i>J. sabina</i> var. <i>balkanensis</i> x <i>J. chinensis</i> 3x
15468 <i>virginiana</i> 'Blue Cloud' 3x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. chinensis</i> 4x	~= <i>J. xpfitzeriana</i> ; <i>J. sabina</i> var. <i>balkanensis</i> x <i>J. chinensis</i> 4x
15489 <i>sabina</i> 'Tamariscifolia' 4x	<i>J. sabina</i> var. <i>balkanensis</i> 4x	<i>J. sab. var. sabina</i> Type 2 ITS 2x	<i>J. sabina</i> v. <i>balkanensis</i> Type 2 ITS x <i>J. sab. var. sabina</i> Type 2 ITS 4x
15434 <i>sabina</i> 'Variegata' 2x	<i>J. sabina</i> var. <i>sabina</i> 2x	<i>J. sab. var. sabina</i> Type 2 ITS 2x	<i>J. sab. var. sabina</i> Type 2 ITS x <i>J. sab. var. sabina</i> Type 2 ITS 2x
15444 <i>davurica</i> 'Expansa Aureo-spicata' 4x	<i>J. chinensis</i> var. <i>procumbens</i> 4x	<i>J. chinensis</i> var. <i>sargentii</i> 4x	<i>J. chinensis</i> var. <i>procumbens</i> x <i>J. chinensis</i> var. <i>sargentii</i> 4x
15475 <i>davurica</i> 'Expansa Variegata' 4x	<i>J. chinensis</i> var. <i>procumbens</i> 4x	<i>J. chinensis</i> var. <i>sargentii</i> 4x	<i>J. chinensis</i> var. <i>procumbens</i> x <i>J. chinensis</i> var. <i>sargentii</i> 4x
15431 <i>davurica</i> 'Expansa' 4x	<i>J. chinensis</i> var. <i>procumbens</i> 4x	<i>J. chinensis</i> var. <i>sargentii</i> 4x	<i>J. chinensis</i> var. <i>procumbens</i> x <i>J. chinensis</i> var. <i>sargentii</i> 4x
15437 <i>indica</i> 2x	<i>J. indica</i> var. <i>caespitosa</i> 2n?	<i>J. indica</i> var. <i>caespitosa</i> 2n?	<i>J. indica</i> var. <i>caespitosa</i> 2x
15419 <i>recurva</i> 'densa' 2x	<i>J. indica</i> var. <i>caespitosa</i> 2n?	<i>J. indica</i> var. <i>caespitosa</i> 2n?	<i>J. indica</i> var. <i>caespitosa</i> 2x
15420 <i>recurva</i> 'Embley Park' 2x	<i>J. coxii</i> 4x	<i>J. squamata</i> var. <i>wilsonii</i> 2x	<i>J. coxii</i> x <i>J. squamata</i> var. <i>wilsonii</i> 2x
15459 <i>pingii</i> var. <i>wilsonii</i> 2x	<i>J. pingii</i> (tree) 2x/ <i>J. carinata</i> (shrub) 2x	<i>J. squamata</i> var. <i>wilsonii</i> 2x	<i>J. carinata</i> x <i>J. squamata</i> var. <i>wilsonii</i> 2x
15460 <i>wallichiana</i> 4x	<i>J. rushforthiana</i> 4x	<i>J. rushforthiana</i> 4x	<i>J. rushforthiana</i> 4x
15487 <i>wallichiana</i> 4x	<i>J. indica</i> 2x	<i>J. rushforthiana</i> 4x	<i>J. indica</i> x <i>J. rushforthiana</i> 4x
15440 <i>virginiana</i> 'cannaertii' 2x	<i>J. virginiana</i> 2x	<i>J. virginiana</i> 2x	<i>J. virginiana</i> 2x

In this study, we found tremendous variation in the origin of cultivars as evidenced by highly diverse nrDNA and cp parentage. Botanic gardens provide unusual laboratories for the production of hybrids whose parents are seldom if ever sympatric in nature. *Juniperus* species from very diverse regions and habitats are grown in close proximity, under favorable conditions, such that opportunities for cross pollination are favorable. A hybrid seedling that grows under the maternal plant may be protected from weeding, and later discovered and rescued into a greenhouse. Survival in Botanic Gardens and private estates is common and has led to commercialization of many 'sports' (mutations) and hybrids that are now sold as cultivars. One has only to peruse books on cultivated conifers (Den Oden and Boom 1965; Krussmann 1991; Welch 2012) to see the number of bizarre shapes that have been cloned and propagated in the past two centuries. So, it is not surprising that this study revealed great variation in parentage and ploidy of the cultivars. The development and implementation of a DNA barcode system would greatly aid botanic gardens to screen current and incoming accessions to assign taxonomic names to junipers and other conifers.

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Table 3. ITS (13 informative ITS SNPs) and petN-psbM (cp), trnS-trnG (cp) analyses of *J. squamata* cultivars at Windsor Gardens.

accession name at Windsor Gardens	Adams coll. #	ploidy this acc.	Paternal ID by trnSG cp data	Windsor acc. identity by ITS data.	179 ¹ C/T	212 G/T	351 C/T	363 C/G	365 C/G	366 C/T	389 C/G	802 A/G	985 C/T	995 A/C	1071 A/T	1143 A/G	1169 C/T
<i>J. squamata</i> 'Blue Alps'	15447	4x	sargentii	sargentii x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	C	A/T	A/G	C/T
<i>J. squamata</i> 'Yellow Tip'	15457	4x	thurifera	thurifera x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	A/C	A/T	A/G	C/T
<i>J. squamata</i> 'Holger'	15486	4x	thurifera	thurifera x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	A/C	A/T	A/G	C/T
<i>J. squamata</i> 'Filborna'	15476	4x	tibetica	tibetica x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	A/C	A/T	A/G	C/T
ITS complement of 15480, 15481, etc. below	na	na	na	sargentii (NCBI blast)	C	T	T	C	G	C	C	G	C	A	A	G	T
<i>J. squamata</i> var. fargesii	15480	4x	tibetica	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Blue Spider'	15481	4x	tibetica	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Glassell'	15445	4x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Chinese Silver'	15455	4x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i>	15483	4x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Wilsonii'	15449	4x	tibetica	wilsonii	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Wilsonii'	15450	4x	tibetica	pingii?	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i>	15456	4x	pingii/squamata	indica/squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Prostrata'	15421	2x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i> 'Pygmaea'	15424	2x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
<i>J. squamata</i>	15485	2x	pingii/squamata	pingii/squamata / komarovii	T	G	C	G	C	T	G	A	T	C	T	A	C

¹ 179-xGCGGACAC, 212- zGCCCAAGC, 351- xGTCGGAGC, 363,365,366- GAGCGAGxGyz, 389- xGAGGTCCG, 803-xAAACATAA, 985(492)-xGCCCTCCC, 995(502)-xGCGAGGAG, 1071(578)-xCGGACGA, 1143(650)- xTCTTTGGT, 1169(676)- xGCGGGCAT.