BOTANY LETTERS
https://doi.org/10.1080/23818107.2019.1613262

Taylor & Francis
Taylor & Francis Group

Genome size variation and polyploidy in the geographical range of *Juniperus sabina* L. (Cupressaceae)

Perla Farhat[a,b], Sonja Siljak-Yakovlev[b], Robert P. Adams[c], Magda Bou Dagher Kharrat[a] and Thierry Robert[b,d]

[a]Laboratoire Biodiversité et Génomique Fonctionnelle, Faculté des Sciences, Université Saint-Joseph, Campus Sciences et Technologies, Beirut, Lebanon; [b]Ecologie Systématique Evolution, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, Orsay, France; [c]Biology Department, Baylor University, Waco, TX, USA; [d]Biology Department, Sorbonne Université, Paris, France

ABSTRACT

Polyploidy and natural hybridization are considered as two major evolutionary processes involved in plant speciation and diversification. In conifers, natural hybridization has been noticed to be more frequent than polyploidy. Nevertheless, a few cases of polyploidy have been reported in the genus *Juniperus*. In this genus, a new variety *Juniperus sabina* var. *balkanensis* has been postulated to have arisen from an ancient hybridization between the tetraploid species *Juniperus thurifera* and the diploid species *Juniperus sabina* var. *sabina*. The genome size variation and the ploidy level of two *J. sabina* taxa were estimated by flow cytometry in a panel of 29 populations. All 13 populations of *J. sabina* var. *sabina* were diploid, with genome sizes ranging from 22.09 to 25.03 pg/2C, while the 16 populations of *J. sabina* var. *balkanensis* were tetraploid, with genome sizes ranging from 41.99 pg to 51.33 pg/2C. These findings open new venues towards the discovering of the polyploidization pathway of *J. sabina* var. *balkanensis* and to understand historical and ecological factors that explain its current geographical distribution.

ARTICLE HISTORY
Received 21 December 2018
Accepted 25 March 2019

KEYWORDS
Cytogeography; genome size; interspecific hybridization; *Juniperus sabina* var. *balkanensis*; *Juniperus sabina* var. *sabina*; monoploid genome size; polyploidy

Introduction

Natural hybridization and polyploidy are two major evolutionary processes in plant speciation and diversification (Otto and Whitton 2000; Mable 2004; Ranney 2006; Abbott et al. 2013; Goulet, Roda, and Hopkins 2017). The frequency of both phenomena differs greatly between and within plant families (Ranney 2006; Wood et al. 2009; Marques et al. 2018). In conifers, polyploidy was reported to be rare, in contrast to natural hybridization that was found to be more frequent (Critchfield 1975; Ahuja 2005; Opgenoorth et al. 2010; Worth et al. 2016). Nevertheless, a few cases of polyploidy have been reported in the genus *Juniperus* (Hall, Mukherjee, and Crowley 1973; Siljak-Yakovlev et al. 2010; Romo et al. 2013; Vallès et al. 2015).

The genus *Juniperus* L. belongs to the family Cupressaceae. Species of this genus have been placed into three sections: *Caryocedrus, Juniperus* and *Sabina*. All *Juniperus* species occur in the Northern Hemisphere, except *J. procera* Hochst. ex. Endl. (sect. *Sabina*) which grows at high elevation in Western Saudi Arabia, and thence it spread to Ethiopia into the Southern Hemisphere along the east Africa mountains (Adams 2014).

Until recently, *Juniperus* species were believed to be mostly diploid, with the exception of the exclusively tetraploid *Juniperus thurifera* L. (section *Sabina*) demonstrated by genome size measurements (vary from 40.81 pg to 43.2 pg/2C) and by chromosome count ($2n= 4x= 44$) (Romo et al. 2013; Vallès et al. 2015). Intra-specific ploidy variation has been reported in *Juniperus chinensis* L. (section *Sabina*), for which some individuals were found to be diploid and others tetraploid (Sax and Sax 1933; Hall, Mukherjee, and Crowley 1973). Another noticeable case of polyploidy has also been reported in *Juniperus sabina* L. (section *Sabina*) in which one population from the Dinaric Alps of the Balkans region has been found to be tetraploid according to genome size of 39.62 ± 1.72 pg/2C (Siljak-Yakovlev et al. 2010), whereas Spanish populations were found to be diploid ($2n= 2x= 22$, Vallès et al. 2015), with a genome size of 21.41 ± 0.62 pg/2C (Romo et al. 2013). *Juniperus sabina* is a juniper with smooth leaf margins. It is a monoecious or dioecious multi-seeded shrub of 1 m in height and 2 m wide (Adams 2014). It is widely distributed in the Eastern Hemisphere from Spain throughout Europe to Kazakhstan, western China and Mongolia (Adams 2014) (Figure 1). Recently, a new variety named *J. sabina* var. *balkanensis* R. P. Adams and A. Tashev. was described based on molecular data (Adams, Schwarzbach, and Tashev 2016). This variety combines *J. sabina* var. *sabina* nuclear alleles at the ITS region,

CONTACT Perla Farhat perla.farhat@u-psud.fr 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

Published online 14 May 2019

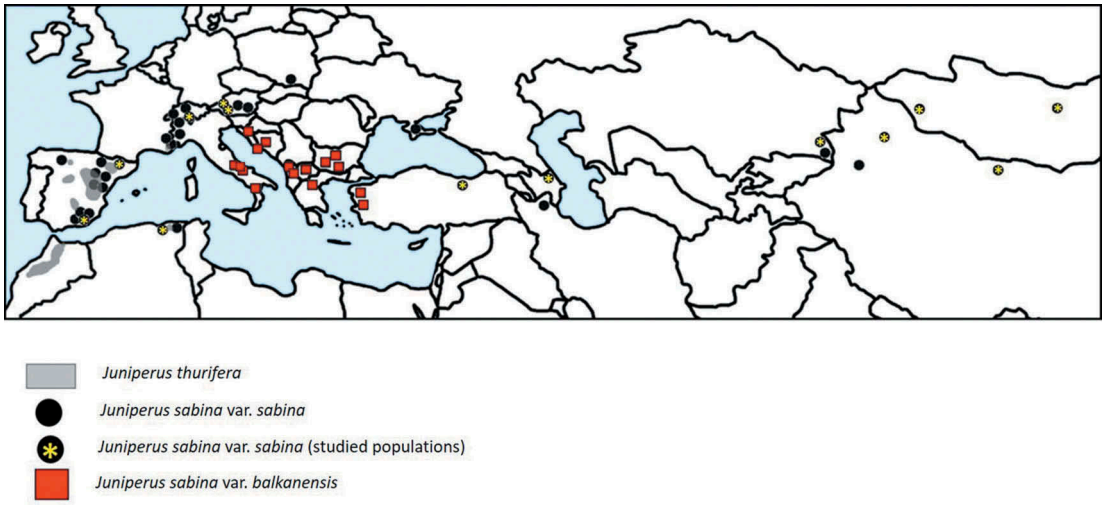

Figure 1. Geographic distribution of *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis*, with indication of studied populations. The actual distribution of *J. thurifera* according to Adams (2014).

and *J. thurifera* chloroplast sequences (cpDNA) (Adams, Schwarzbach, and Tashev 2016). It is morphologically very similar to *J. sabina* var. *sabina*, with a few differences in foliage and seed cone morphology (Adams, Schwarzbach, and Tashev 2016). This variety is distributed in the Balkans, in Italy and in the western edge of Turkey (Adams et al. 2018b). The current geographical distribution of the studied populations of *J. sabina* var. *balkanensis* is well distinct from that of *J. thurifera* (Adams et al. 2018a, 2018b) (Figure 1). These authors hypothesized that an ancient interspecific hybridization happened between *J. sabina* and the ancestor of *J. thurifera* lineage leading to the formation of *J. sabina* var. *balkanenis* when those taxa distributions overlapped (Adams, Schwarzbach, and Tashev 2016). Indeed, *Juniperus thurifera* has been considered as relict species, originated from Tertiary and it is supposed to have a wider geographic distribution area during Pleistocene's cold periods in comparison to its current one (Terrab et al. 2008). Moreover, the reconstruction of the ancestral geographic distribution area of *Juniperus* genus, has shown that the ancestral lineage of *J. thurifera* was more likely distributed across Eurasia (Mao et al. 2010).

These findings emphasize the importance of determining the ploidy level of those two taxa throughout their geographical distribution. This study aimed to establish the cytogeography of the two *Juniperus sabina* cytotypes and to discuss polyploidization pathways that could have been involved in the genesis of *J. sabina* var. *balkanensis*.

Material and methods

Plant material

Leaf samples from 13 populations of *J. sabina* var. *sabina* and 16 populations of *J. sabina* var. *balkanensis* covering their entire distribution area were collected (Table 1 and Figure 1). In all cases, leaves were immediately dried and preserved in silica gel until use.

Voucher specimens for all samples are deposited at Baylor University Herbarium (BAYLU) and at The University of Barcelona Herbarium (BCN).

Sample preparation

Nuclear DNA amount was assessed by flow cytometry (FCM) according to Bourge, Brown, and Siljak-Yakovlev (2018) on silica dried leaves of *Juniperus* samples and fresh *Hordeum vulgare* L. "Sultan" (2C = 9.81 pg in Garnatje et al. (2004)) used as a standard. The leaves (approx. 30 mg) of both the internal standard and *Juniperus* were simultaneously chopped using a razor blade in a plastic Petri dish with 600 µl of cold Gif nuclear-isolation buffer-GNB (Bourge, Brown, and Siljak-Yakovlev 2018): 45 mM MgCl2, 30 mM sodium citrate, 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 5 mM sodium metabisulphite and RNase (2.5 U/ml). The nuclei suspension was filtered through 30 µm nylon mesh. The nuclei were stained with 100 µg/ml propidium iodide (PI), a specific DNA fluorochrome intercalating dye, and kept 5 min at 4°C.

Flow cytometric analyses

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). In most cases, each population was represented by three individuals, measured separately and repeated twice. The software CytExpert was used for histogram analyses. The total

Table 1. Summary of data concerning the studied populations of *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis*.

Taxon	Locality	Collector and collection year	Altitude	GPS coordinates	2C (pg)	1Cx (Mbp)
J. sabina var. *balkanensis*	Above village Ceren, on the path to Sorokol, Albania	L. Shuka 2018	1430	41° 49′ 37.01"N, 20° 28′ 28.13"E	44.11 ± 0.58	10,785
J. sabina var. *balkanensis*	Above the read from Ceren to Radomira Village, Albania	L. Shuka 2018	1150	41° 49′ 43.86"N, 20° 27′ 43.05"E	45.74 ± 0.53	11,183
J. sabina var. *balkanensis*	Mts Cvrsnica and Cabulja, Bosnia-Herzegovina	F. Bogunic and S. Siljak Yakovlev 2017	1460	43° 34′ 18.09"N, 17° 30′ 39.88"E	45.57 ± 0.78	11,142
J. sabina var. *balkanensis*	Rila Moutains, Bulgaria	R. Adams 2018	1242	42° 14′ 26.5"N, 23° 32′ 33.8"E	48.53 ± 2.35	11,864
J. sabina var. *balkanensis*	Mt Rhodopes, Bulgaria	R. Adams 2018	1270	41° 14′ 44.7"N, 25° 15′ 31.9"E	47.02 ± 0.24	11,496
J. sabina var. *balkanensis*	Central Stara Planina, National Park "Central Balkan", Bulgaria	S. Stoyanov 2018	1500	42° 42′ 25.38"N, 25° 8′ 7.76"E	48.52 ± 0.44	11,862
J. sabina var. *balkanensis*	Mt Velebit, Croatia	K. Marcysiak 2017	1080	44° 32′ 36"N, 15° 10 09"E	49.66 ± 0.48	12,143
J. sabina var. *balkanensis*	Mt Tsena, Greece	A. Tashev 2015	1630	41° 08′ 29.4"N, 22° 14′ 42.2"E	45.46 ± 1.63	11,115
J. sabina var. *balkanensis*	Calabria, Italy	F. Roma-Marzio and L. Peruzzi 2017	1436	39° 54′ 48.56"N, 16° 17′ 8.81"E	51.26 ± 1.57	12,534
J. sabina var. *balkanensis*	Colle dell Angelo, Italy	F. Bartolucci, F. Conti, L. Di Martino 2018	1002	42° 11′ 37.39"N, 14° 7′ 15.1"E	44.43 ± 0.00	10,862
J. sabina var. *balkanensis*	Colle le Macchie, Italy	F. Bartolucci, F. Conti, L. Di Martino 2018	1030	42° 6′ 30.31"N, 14° 11′ 45.02"E	44,72 ± 0,00	10,934
J. sabina var. *balkanensis*	Colle Bandiera, Italy	F. Bartolucci, F. Conti, L. Di Martino 2018	1200	42° 6′ 18.68"N, 14° 11′ 32.82"E	42.72 ± 0.00	10,444
J. sabina var. *balkanensis*	San Domenico, Italy	F. Bartolucci, F. Conti, L. Di Martino 2018	1484	41° 55′ 42.74"N, 14° 12′ 40.86"E	45.70 ± 0.00	11,173
J. sabina var. *balkanensis*	Mavrovo area, Macedonia	K. Marcysiak 2017	1377	41° 39′ 18.16"N, 20° 44′ 01.21"E	45.08 ± 1.23	11,021
J. sabina var. *balkanensis*	Spil Dağı, south west Turkey	A. Boratyński, K. Boratyńska 2016	1250	38° 33′N, 27° 25′12"E	51.33 ± 0.00	12,549
J. sabina var. *balkanensis*	Ballıca Mahallesi, Akhisar/Manisa, south west Turkey	T. Mataraci 2016	1200	38° 57′N, 27° 41′E	41.99 ± 0.00	10,267
J. sabina var. *sabina*	Assouel, National Parc of Djurdjura, Algeria	A. Adjaoud 2011	1840	36° 27′ 36"N, 04° 04′ 15"E	22.09 ± 0.17	10,801
J. sabina var. *sabina*	Zwieselstein, Alps, Austria	A. Boratyński 2015	1440	46° 55.8′N, 11° 02.4′E	25.03 ± 0.62	12,241
J. sabina var. *sabina*	Tirol, Nordtirol, Stubaier Alpen, Otztal, Austria	P. Schonswetter and P. C. Campmany 2018	1100	47° 9′ 23"N, 10° 55′ 33"E	24.19 ± 0.18	11,831
J. sabina var. *sabina*	Caucasus Mtns. 1.4 km (by air) east of Jek village, Azerbaijan	V. Farzliyev 2014	1649	41° 11.79′N 48° 15.31′E	24.65 ± 0.00	12,056
J. sabina var. *sabina*	Gansu, China	J. Q. Liu 2004	3200	38° 26.63′N 101° 20.35′E	22.35 ± 0.99	10,927
J. sabina var. *sabina*	Tian Shan Mts, Xinjiang, China	R. P. Adams 1996	2008	43° 53.60′N 88° 06.06′E	24.26 ± 0.60	11,865
J. sabina var. *sabina*	Paniflor, Kazakhestan	R. P. Adams 1996	2000	44° 29.88′N 80° 04.14′E	24.33 ± 0.52	11,899
J. sabina var. *sabina*	Central Mongolia, Mongolia	R. P. Adams 1994	2010	47° 49.93′N 106° 54.73′E	24.17 ± 0.29	11,818
J. sabina var. *sabina*	Mts Altai, Mongolia	R. P. Adams 1995	1740	46° 36.49′N 91° 17.73′E	23.17 ± 0.34	11,332
J. sabina var. *sabina*	Mts Pyrenees, Spain	R. P. Adams 1995	1290	42° 46.47′N 0° 19.71′W	23.74 ± 0.35	11,608
J. sabina var. *sabina*	Sierra Nevada, Spain	R. P. Adams 1993	2100	37° 06.17′N 3° 24.52′W	22.41 ± 0.48	10,960
J. sabina var. *sabina*	South St. Niklaus, Baltschieder, Switzerland	R. P. Adams 1995	1300	46° 09.24′N 7° 47.40′E	23.74 ± 0.15	11,608
J. sabina var. *sabina*	Gumushane, N Cent., Turkey	A. Kandemir 2016	2376	40° 36′ 03"N, 38° 53′ 21"E	24.38 ± 0.00	11,923

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)

The mean 2C-value as well as the standard deviation of the mean values were calculated from measurements of samples comprising at least three individuals. The monoploid genome size (1Cx) which is the DNA content of genome with chromosome base number *x*, was calculated by dividing the 2C value by ploidy level (Greilhuber et al. 2005). The

value of 1Cx was given in Mbp (1 pg~978 Mbp according to Doležel et al. (2003)).

Statistical analyses

Differences in genome sizes between populations of the same variety and between the two varieties, *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis*, were tested using the non-parametric Kruskal–Wallis test. Pairwise comparisons using Dunn's all-pairs test with Holm's correction for multiple test (Holm 1979) were performed to test the difference in genome size between populations of the same variety. Populations represented by less than 3 individuals were discarded from the statistical analyses. *Juniperus sabina* var. *sabina* discarded populations were from Turkey and Azerbaijan. For *J. sabina* var. *balkanensis*, the two populations of Turkey were discarded. The four populations of *J. sabina* var. *balkanensis* from central Italy (Colle dell Angelo, Colle le Macchie, Colle Bandiera and San Domenico) were not discarded even if one individual was available for each locality, instead they were statistically treated as one population due to their relatively close locations.

The non-parametric Kruskal–Wallis test was used to test the differences in mean monoploid genome sizes between *J. sabina* var. *sabina*/*J. sabina* var. *balkanensis, J. sabina* var. *sabina*/*J. thurifera* (measurements of *J. thurifera* used are those published by Romo et al. (2013)) and *J. sabina* var. *balkanensis*/*J. thurifera*.

All statistical tests were performed with R software version 3.5.1.

Results

The genome size of the 29 populations of *Juniperus sabina* was successfully measured using flow cytometry. Results clearly showed the existence of two categories of genome size, corresponding to each of the varieties examined (Table 1). The 2C DNA values ranged from 22.09 pg to 25.03 pg for the 13 populations of *Juniperus sabina* var. *sabina*. Difference among *J. sabina* var. *sabina* populations was statistically significant (chi-squared = 26.91; df = 10; p-value = 0.00269). Pairwise population comparisons showed that the difference in 2C DNA value was significant just between two populations (Algeria and Zwieselstein, Austrian Alps (p-value = 0.032)). The genome size of the 16 populations of *J. sabina* var. *balkanensis* ranged from 41.99 pg to 51.33 pg (Table 1). Differences among *J. sabina* var. *balkanensis* populations were also statistically significant (chi-squared = 34.74; df = 10; p-value = 0.00014). Pairwise population comparisons showed that the 2C DNA value was significantly different only between two populations (Calabia, Italy and Albania (p-value = 0.014)).

The difference between mean genome size of *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis* was highly significant (chi-squared = 55.902; df = 1, p-value = 7.619×10^{-14}).

The difference in mean genome size value between populations of *Juniperus sabina* var. *balkanensis* (46.36 pg/2C) and populations of *J. sabina* var. *sabina* (23.73 pg/2C) is approximately two-fold. Because genome size is positively correlated with chromosome number and ploidy level within species, we can safely consider that this two-fold difference is mainly the result of a difference in ploidy level between both varieties. This study reveals that all studied populations of *J. sabina* var. *balkanensis* are tetraploid (4*x*) and all studied populations of *J. sabina* var. *sabina* are diploid (2*x*). On this basis, the inferred mean monoploid genome sizes (1C*x*) of the tetraploid *J. sabina* var. *balkanensis* was 11,336 Mbp (11.59 pg) and the one of *J. sabina* var. *sabina* was 11,605 Mbp (11.87 pg). The difference between the mean 1C*x* of *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis* was not statistically significant (chi-squared = 2.6255, df = 1, p-value = 0.1052). When comparing our data to the estimated monoploid genome size of the assumed parent *J. thurifera* (mean 1C*x* = 10,073 Mbp) estimated by Romo et al. (2013), a significant genome downsizing is observed between this species and both *J. sabina* var. *sabina* (chi-squared = 14.468, df = 1, p-value = 0.0001426) and *J. sabina* var. *balkanensis* (chi-squared = 17.359, df = 1, p-value = 3.094×10^{-5}).

Discussion

Inter-variety and inter-population genome size variation

Intra-specific variability in ploidy level is a well-documented phenomenon in the plant kingdom (Duchoslav, Šafářová, and Krahulec 2010; Husband, Baldwin, and Suda 2013; Krejčíková et al. 2013). During the last decade, the detection of this variability has dramatically increased due to the development of high throughput analyses such as FCM (Trávníček et al. 2010; Krejčíková et al. 2013).

Our results support the conclusion that difference in genome size between *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis* reflects a difference in ploidy level between them (diploidy for *J. sabina* var. *sabina* versus tetraploidy for *J. sabina* var. *balkanensis*). It is also noticed that the mean genome size of studied populations of *J. sabina* var. *sabina* measured in this work is very close to mean genome size previously reported for the diploid Spanish populations (2*n*= 2*x*= 22, Vallès et al. 2015) (21.41 ± 0.62 pg/2C, Romo et al. 2013).

In addition, we found a significant variation in 2C value between only two populations within each of the two varieties. Bennett (1976) found that genome size variation was highly correlated with the geographical distribution of populations and environmental factors. Interpopulation variation of genome size has been observed in many angiosperm species such as *Armeria maritima* (Mill.) Willd. (Vekemans et al. 1996),*Berberis sp*. (Bottini et al. 2000),*Retama sp*. (Benmiloud-Mahieddine et al. 2011), and also in gymnosperms as *Pinus banksiana* Lamb.,*Pinus sylvestris* L., *Picea glauca* (Moench) Voss. and *Picea sitchensis* (Bong) Carr. (Mergen and Thielges 1967).

Hypothetical polyploidy pathways of J. sabina *var.* balkanensis

Polyploidy pathways have been mainly studied in angiosperms. The main mechanisms leading to the formation of a polyploid are: somatic doubling, unreduced gametes and more rarely the polyspermy (Ramsey and Schemske 1998; Tayalé and Parisod 2013).

For conifers, the only well-studied case of polyploidy concerns the hexaploid coast redwood (*Sequoia sempervirens* (D. Don) Endl.). Current research suggests that coast redwood is likely an autopolyploid. However, the polyploidy pathway has not been yet well defined (Scott et al. 2016).

For *Juniperus*, cytological data concerning tetraploid *J. thurifera* and *J. chinensis* were not sufficient to answer the question of the origin of polyploidy in these species, i.e., whether they are auto- or allopolyploids, due to the high homogeneity of their karyotypes (Teixeira, Rodríguez-Echeverría, and Nabais 2014; Vallès et al. 2015). Nevertheless, for *J. thurifera*, although no definite conclusion on its auto- or allopolyploidy origin has been achieved, at least the palaeopolyploid condition has been mentioned (Vallès et al. 2015).

Regarding the tetraploidy pathway of *J. sabina* var. *balkanensis*, the autopolyploidy has been discarded in the first step of this variety formation, based on the fact that this variety holds in one part, the chloroplast sequences of *J. thurifera* which are very different from those of *J. sabina* var. *sabina* with a minimum of 36 mutations, including indels within 3114 bp sequenced and on the other part it holds the nuclear patterns (ITS region) of *J. sabina* var. *sabina* (Adams, Schwarzbach, and Tashev 2016). This polymorphism pattern clearly supports the fact that *J. sabina* var. *balkanensis* is the result of an interspecific hybridization between *J. sabina* var. *sabina* and *J. thurifera*. This last hypothesis is not supported by the fact that the current geographical distributions of the three taxa do not overlap. However, it has been proposed that *J. thurifera* ancestral lineage had previously a wider distribution (Terrab et al. 2008; Mao et al. 2010). It is therefore possible that the geographical distributions of three taxa overlapped in the past. The high similarity between ITS sequences of the two *J. sabina* varieties suggests that the interspecific hybridization event was followed by several backcross generations to *J. sabina* var. *sabina* parent. Homogenization of ITS sequences within *J. sabina* var. *balkanensis* could also have been accelerated by concerted evolution of these sequences, a mechanism frequently invoked to explain the lack of intragenomic polymorphism between ITS sequences (Kovarik et al. 2005; Tang et al. 2015).

Therefore, we assumed that *J. sabina* var. *balkanensis* is an allopolyploid that arose from an ancient interspecific hybridization followed by several backcrosses to *J. sabina* var. *sabina* parent leading to the morphological and the ITS region similarities to this parent.

Given that *J. sabina* var. *balkanensis* possesses chloroplast DNA sequences that are the most similar to *J. thurifera* than any other studied *Junieprus* species, this implies that *J. thurifera* is the most likely paternal parent, because in conifers in general, and in Cupressaceae in particular, chloroplasts are predominantly paternally inherited (Neale and Sederoff 1988; Hipkins, Krutovskii, and Strauss 1994; Kondo et al. 1998).

Figure 2 presents four of the most parsimonious hypothetical pathways that could have led to the polyploidization of *J. sabina* var. *balkanensis*. However, further research is needed in order to determine the most plausible pathway.

In the first proposed pathway (Figure 2(a)), polyploidization would have happened in one step, in which haploid pollen of the tetraploid *J. thurifera* (TT) ($n= 2x$ in this case) would have fertilized an unreduced female gamete of *J. sabina* var. *sabina* (SS) giving rise to a tetraploid (TTSS). The possible gametes produced by this interspecific tetraploid hybrid (TS, SS and TT) could be fertilized by other unreduced gametes of *J. sabina* var. *sabina* (SS) giving rise to a tetraploid interspecific hybrid (SSSS; TTSS; TSSS). Thus, a minimum of one backcross with the maternal parent is needed to produce the tetraploid interspecific hybrid having a *J. sabina*-like genome composition.

Unreduced gametes (with the somatic chromosome number) have been extensively studied in angiosperms, and have been considered as the most frequent mechanism leading to polyploidy (Ramsey and Schemske 1998; Soltis, Soltis, and Tate 2004). However, studies on the production of unreduced gametes were mainly concentrated on male gametes, since pollen (male gametophyte) studies are easier than female gamete studies. Until now, a single conifer species from the same family as *Juniperus,*

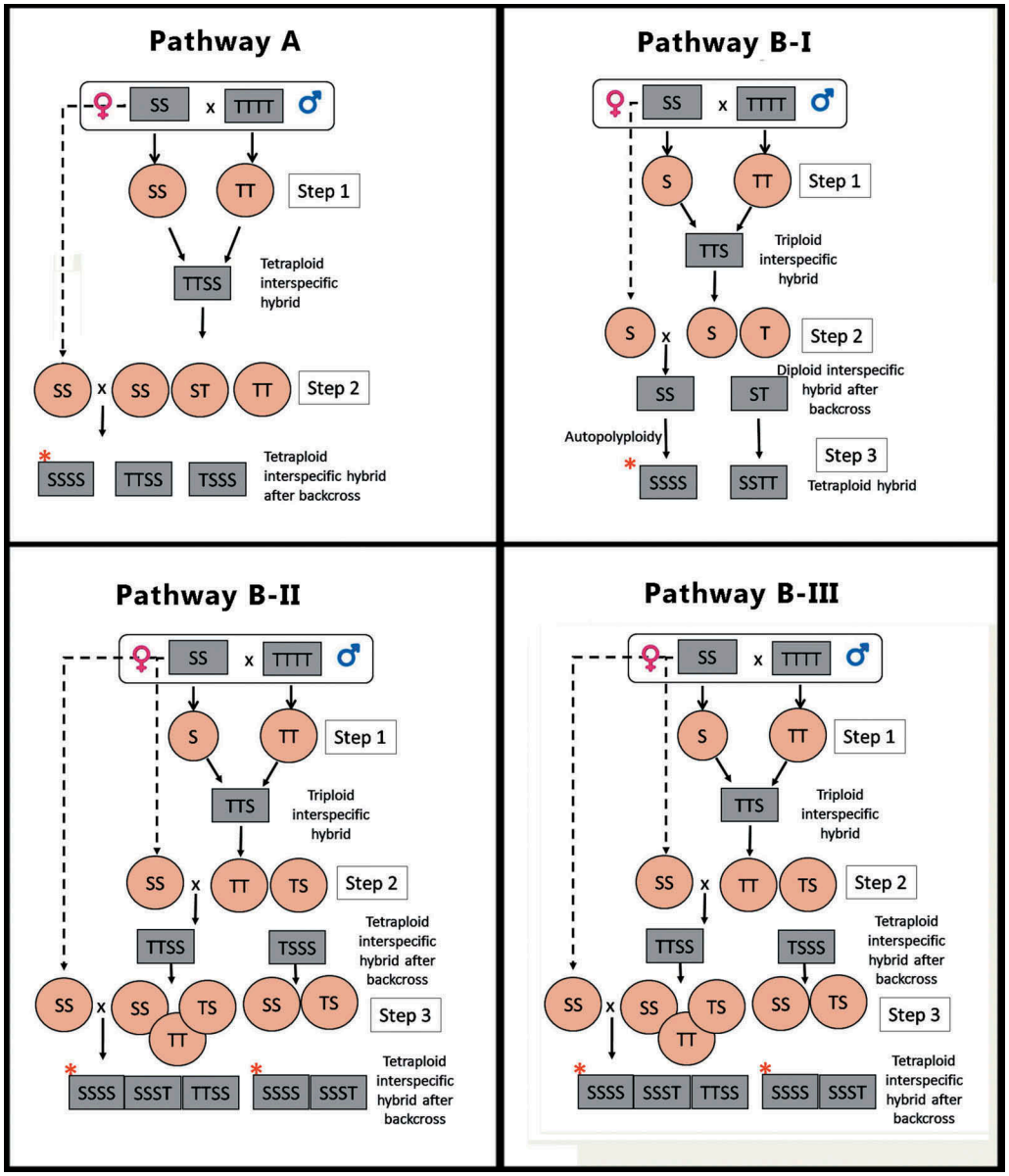

Figure 2. Parsimonious hypothetical polyploidization pathways leading to *J. sabina* var. *balkanensis*. Rectangles present taxa and circles gametes. T: *J. thurifera*; tetraploid *J. thurifera* genotype (TTTT), reduced *J. thurifera* gamete (TT). S: *J. sabina*; diploid *J. sabina* genotype (SS), unreduced *J. sabina* gamete (SS), reduced *J. sabina* gamete (S). Pathway (a) Step 1. 2*n* gamete of *J. sabina* var. *sabina* with *n* gamete of the tetraploid *J. thurifera*. Step 2. 2*n* gamete of *J. sabina* var. *sabina* with *n* gamete of the tetraploid hybrid. Three pathways with reduced gametes in the first step involving triploid bridge: Pathway B-I: Step 2. *n* gamete of the triploid hybrid with *n* gamete of *J. sabina* var. *sabina* parent. Step 3. Autopolyploidization. Pathway (b)-II: Step 2. Partially reduced gamete of the triploid hybrid with 2*n* gamete of *J. sabina* var. *sabina* parent. Step 3. *n* gamete of the tetraploid hybrid with 2*n* gamete of *J. sabina* var. *sabina* parent. Pathway B–III: Step 2. *n* gamete of *J. sabina* var. *sabina* with 3*n* gamete of the triploid hybrid. Step 3. 2*n* gamete of *J. sabina* var. *sabina* with *n* gamete of the tetraploid hybrid.

Cupressus dupreziana A.Camus. has been reported to produce unreduced pollen (Pichot and El Maâtaoui 2000). Such studies are very rare in conifers, and therefore this process may be more widespread than currently admitted in this group of species. Thus, the uncertainty of this pathway is mainly related to the possibility of an unreduced female gamete being produced by *J. sabina*.

The second pathway to polyploidization (Figure 2 (b)) involves a triploid bridge. This pathway is very frequent in angiosperms (Ramsey and Schemske 1998). In the present case, a normal hybridization between a reduced pollen ($n= 2x$) of the tetraploid *J. thurifera* (TT) and a reduced female gamete ($n= 1x$) of the diploid *J. sabina* (S) would produce a triploid (TTS). However, natural triploids are usually unfertile and unstable, due to meiotic irregularities (Ramsey and Schemske 1998). Therefore, triploid individuals that survive in nature are those that have the ability for vegetative propagation (Leitch et al. 2008). Interestingly, it has been shown that *J. sabina* has the ability for vegetative propagation (Bedell et al. 1993; Thomas, El-Barghathi, and Polwart 2007). This capacity could allow triploid individuals to

persist. Furthermore, recent investigations in angiosperms have shown that natural triploids can produce fertile *x*, 2*x* and 3*x* gametes (Ramsey and Schemske 1998; Schinkel et al. 2017). Thence, a fertilization between a reduced gamete (S) (*n*= 1*x*) produced by the triploid interspecific hybrid (TTS) with a reduced gamete (S) (*n*= 1*x*) of *J. sabina* would produce a diploid with (SS) nuclear *J. sabina*-like genome and the chloroplast of *J. thurifera*. Its subsequent autopolyploidization will produce a tetraploid (SSSS) holding the nuclear genome of *J. sabina* and the chloroplast of *J. thurifera* (Figure 2- Pathway B-I).

Another pathway (Figure 2- Pathway B-II) involves a triploid bridge, as in the pathway B-I. This step is followed by a cross between a partially reduced gamete (*n*= 2*x*) (TS; TT) produced by an interspecific triploid (TTS) and an unreduced gamete (*n*= 2*x*) of *J. sabina* var. sabina (SS). Such an event would produce a tetraploid interspecific hybrid with two possible nuclear genomic combinations (TTSS or TSSS). After at minimum one backcross to *J. sabina* var. *sabina* involving an unreduced gamete (SS; *n*= 2*x*), progeny having a *J. sabina*-like (SSSS) genome composition and the chloroplast of *J. thurifera* will appear among other possibilities.

A third pathway of triploid bridge involving unreduced gamete of the triploid interspecific hybrid would be suggested (Figure 2- Pathway B-III). A fertilization between a triploid gamete (TTS) (*n*= 3*x*) produced by a triploid interspecific hybrid with a reduced gamete (S) (*n*= 1*x*) of *J. sabina* would produce a tetraploid (TTSS). One backcross with an unreduced gamete (SS) (*n*= 2*x*) of the female parent *J. sabina* will give a tetraploid interspecific hybrid having a *J. sabina*-like genome composition (SSSS) and the chloroplast of *J. thurifera* among other possibilities.

Genome size evolution of J. sabina *var.* balkanensis

It has been frequently stated that a genome downsizing generally occurs after polyploidization, (Leitch and Bennett 2004; Dodsworth, Chase, and Leitch 2015). In our study, the mean monoploid genome size (1C*x*) of the tetraploid *J. sabina* var. *balkanensis* (1C*x* = 11,336 Mbp) was shown to be slightly but not significantly smaller than the one estimated for the diploid *J. sabina* var. *sabina* (1C*x* = 11,605 Mbp). In contrast, the mean monoploid genome size (1C*x*) of the tetraploid *J. thurifera* (mean 1C*x* = 10,073 Mbp (Romo et al. 2013)) showed a significant downsizing relatively to both *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis*. This result suggests that the polyploidization event that has produced *J. sabina* var. *balkanensis* might have been recent, because it has been shown that, in general, the amount of genome size changes increases with the age of the polyploidization (Leitch et al. 2008). This was the case, for example, in the genus *Nicotiana*, for which minimal genome downsizing was observed in young polyploids (ca. 200,000 years old) in contrast to older polyploids (ca. 4.5 million years old) (Leitch et al. 2008). Adams, Schwarzbach, and Tashev (2016) hypothesized that the interspecific hybridization between the ancestor lineage of *J. thurifera* and *J. sabina* leading to *J. sabina* var. *balkanensis* was ancient, at a time when their probable distribution overlapped. Differences of monoploid genomes sizes between *J. thurifera* and *J. sabina* var. *balkanensis* suggest that *J. thurifera* has undergone a significant genome downsizing since the hybridization event with *J. sabina* var. *sabina* whereas the genome size of *J. sabina* var. *balkanensis* remained stable. This stability may indicate that *J. sabina* var. *balkanensis* remained at a triploid level for a long time period if the pathway B-II and B-III were involved. Otherwise, if the pathway B-I was involved, more probably this variety remained for long time at the diploid level (Pathway B-I- step 2) due to the fact that diploid hybrids are more stable in nature than triploids. It is possible that the genome of this taxon reached its current tetraploid state more recently, because a more significant downsizing would have also been expected if the tetraploidization was ancient. This interpretation is therefore in favor of the "triploid bridge" pathways described above. Alternatively, the absence of a significant downsizing of *J. sabina* var. *balkanensis* relatively to *J. sabina* var. *sabina* could also be related to the slow growth and long biological cycle of junipers because of their woody habit (Bedell et al. 1993; Thomas, El-Barghathi, and Polwart 2007).

Conclusion

Despite the rarity of polyploidy in conifers, this study discovered a new allotetraploid in *Juniperus: J. sabina* var. *balkanensis* which has evolved from a hybridization between the tetraploid *J. thurifera* and the diploid *J. sabina*. Genome size of all 16 studied populations of *J. sabina* var. *balkanensis* demonstrated the tetraploid state of this variety, in contrast to 13 studied populations of *J. sabina* var. *sabina* which were all diploid. Four parsimonious possible pathways were hypothesized concerning the origin of *J. sabina* var. *balkanensis*. However, further investigations are needed to specify the most likely pathway.

This study should foster additional studies to provide insights on potential factors (environmental conditions, geographical barrier to dispersion, plant community…) responsible for the relatively limited distribution of the tetraploid *J. sabina* var. *balkanensis* compared to that of the diploid *J. sabina* var. *sabina*.

Acknowledgments

This study was supported with funds provided by National Council for Scientific Research grant number CNRS-FS 90 – Lebanon, the Saint Joseph University Research Counsel (CR-USJ) FS-111 and Baylor University, project 0324512 to RPA.

Also, authors would like to thank colleagues who provided samples of *Juniperus* and Dr Sophie Nadot for English revision of the paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Baylor University, project 0324512 to RPA [project 0324512];Saint Joseph University Research Council (CR-USJ) [FS-111];National Council for Scientific Research- Lebanon [CNRS-FS90].

Notes on contributors

Perla Farhat is a PhD student at Saint Joseph University (Lebanon) and University Paris Sud (France). Her research field is polyploidy and hybridization in conifers, and more broadly plant genome evolution. Contribution: She conceived and designed the study, performed most of the experiments, and wrote the manuscript.

Sonja Siljak-Yakovlev is a Research Director at CNRS (France). She is an expert in plant cytogenetics and genome evolution and plant systematics. Contribution: She conceived and designed the study, collected some plant material, performed experiments, contributed to the manuscript writing and approved the final version of manuscript.

Robert P. Adams is a Professor at Baylor University (USA). He is an expert in evolutionary and systematic studies of *Juniperus*. Contribution: He provided most of the plant material, and contributed to the writing.

Magda Bou Dagher Kharrat is a Professor at Saint Joseph University (Lebanon). She is specialized in plant conservation biology, with expertise in cytogenetics. Contribution: She insured the funding of this study, co-supervised the study and contributed to the writing.

Thierry Robert is an Associate Professor at Sorbonne University (France). He is a plant population geneticist. Contribution: He supervised the study and coordinated the writing.

ORCID

Perla Farhat http://orcid.org/0000-0002-6134-788X
Sonja Siljak-Yakovlev http://orcid.org/0000-0002-6247-3351
Robert P. Adams http://orcid.org/0000-0001-8408-1271
Magda Bou Dagher Kharrat http://orcid.org/0000-0001-7969-1673
Thierry Robert http://orcid.org/0000-0003-4718-5658

References

Abbott, R., D. Albach, S. Ansell, J. W. Arntzen, S. J. E. Baird, N. Bierne, J. Boughman, et al. 2013. "Hybridization and Speciation." *Journal of Evolutionary Biology* 26 (2): 229–246. doi:10.1111/j.1420-9101.2012.02599.x.

Adams, R. P. 2014. *Junipers of the World: The Genus Juniperus, 4th Edition*. Bloomington: Trafford Publishing.

Adams, R. P., A. Boratynski, K. Marcysiak, F. Roma-Marzio, L. Peruzzi, F. Bartolucci, F. Conti, et al. 2018a. "Discovery of *Juniperus sabina* var. *balkanensis* RP Adams and AN Tashev in Macedonia, Bosnia-Herzegovina, Croatia and Central and Southern Italy and Relictual Polymorphisms Found in nrDNA." *Phytologia* 100 (2): 117–127.

Adams, R. P., P. Farhat, L. Shuka, and S. Siljak-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., A. E. Schwarzbach, and A. N. Tashev. 2016. "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*." *Phytologia* 98 (2): 100–111.

Ahuja, M. R. 2005. "Polyploidy in Gymnosperms: Revisited." *Silvae Genetica* 54 (2): 59–69. doi:10.1515/sg-2005-0010.

Bedell, T. E., T. L. Deboodt, C. Jacks, and L. E. Eddleman. 1993. "Western Juniper: Its Impact and Management in Oregon Rangelands". Oregon State University Extension Service, Extension Circular EC 1417. 1–15.

Benmiloud-Mahieddine, R., M. Abirached-Darmency, S. C. Brown, M. Kaid-Harche, and S. Siljak-Yakovlev. 2011. "Genome Size and Cytogenetic Characterization of Three Algerian *Retama* Species." *Tree Genetics & Genomes* 7 (5): 987–998. doi:10.1007/s11295-011-0389-z.

Bennett, M. D. 1976. "DNA Amount, Latitude, and Crop Plant Distribution." *Environmental and Experimental Botany* 16 (2–3): 93–108. doi:10.1016/0098-8472(76)90001-0.

Bottini, M. C. J., E. J. Greizerstein, M. B. Aulicino, and L. Poggio. 2000. "Relationships among Genome Size, Environmental Conditions and Geographical Distribution in Natural Populations of NW Patagonian Species of *Berberis* L. (Berberidaceae)." *Annals of Botany* 86 (3): 565–573. doi:10.1006/anbo.2000.1218.

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. doi:10.31383/ga.vol2iss2pp1-12.

Critchfield, W. B. 1975. Interspecific hybridization in Pinus: a summary review." In Proceedings of the 14th Canadian Tree Improvement Association, Part 2, Symposium on interspecific and interprovenance hybridization in forest trees, Fredericton, New Brunswick, Canada, 28-30 August 1973, edited by D.P. Fowler and C.W. Yeatman, 99–105. Canadian Forestry Service, Ottawa, ON.

Dodsworth, S., M. W. Chase, and A. R. Leitch. 2015. "Is Post-Polyploidization Diploidization the Key to the Evolutionary Success of Angiosperms?." *Botanical Journal of the Linnean Society* 180 (1): 1–5. doi:10.1111/boj.12357.

Doležel, J., J. Bartos, H. Voglmayr, and J. Greilhuber. 2003. "Nuclear DNA Content and Genome Size of Trout and Human." *Cytometry. Part A: the Journal of the International Society for Analytical Cytology* 51 (2): 127–128. doi:10.1002/cyto.a.10013.

Duchoslav, M., L. Šafářová, and F. Krahulec. 2010. "Complex Distribution Patterns, Ecology and Coexistence of Ploidy Levels of *Allium oleraceum* (Alliaceae) in the Czech Republic." *Annals of Botany* 105 (5): 719–735. doi:10.1093/aob/mcq035.

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. doi:10.1016/j.biolcel.2003.11.005.

Goulet, B. E., F. Roda, and R. Hopkins. 2017. "Hybridization in Plants: Old Ideas, New Techniques." *Plant Physiology* 173 (1): 65–78. doi:10.1104/pp.16.01340.

Greilhuber, J., J. Doležel, M. A. Lysák, and M. D. Bennett. 2005. "The Origin, Evolution and Proposed Stabilization of the Terms 'Genome Size'and 'C-Value'to Describe Nuclear DNA Contents." *Annals of Botany* 95 (1): 255–260. doi:10.1093/aob/mci019.

Hall, M. T., A. Mukherjee, and W. R. Crowley. 1973. "Chromosome Counts in Cultivated Junipers." *Journal of the Arnold Arboretum* 54 (3): 369–376.

Hipkins, V. D., K. V. Krutovskii, and S. H. Strauss. 1994. "Organelle Genomes in Conifers: Structure, Evolution, and Diversity." *Forest Genetics* 1 (4): 179–189.

Holm, S. 1979. "A Simple Sequentially Rejective Multiple Test Procedure." *Scandinavian Journal of Statistics* 6: 65–70. http://www.jstor.org/stable/4615733.

Husband, B. C., S. J. Baldwin, and J. Suda. 2013. "The Incidence of Polyploidy in Natural Plant Populations: Major Patterns and Evolutionary Processes." In *Plant Genome Diversity*, edited by J. Greilhuber, J. Doležel, and J. Wendel, 255–276. Vol. 2. Vienna: Springer. doi:10.1007/978-3-7091-1160-4_16.

Kondo, T., Y. Tsumura, T. Kawahara, and M. Okamura. 1998. "Paternal Inheritance of Chloroplast and Mitochondrial DNA in Interspecific Hybrids of *Chamaecyparis* spp." *Japanese Journal of Breeding* 48 (2): 177–179. doi:10.1270/jsbbs1951.48.177.

Kovarik, A., J. C. Pires, A. R. Leitch, K. Y. Lim, A. M. Sherwood, R. Matyasek, J. Rocca, D. E. Soltis, and P. S. Soltis. 2005. "Rapid Concerted Evolution of Nuclear Ribosomal DNA in Two *Tragopogon* Allopolyploids of Recent and Recurrent Origin." *The Genetics Society of America* 169: 931–944. doi:10.1534/genetics.104.032839.

Krejčíková, J., R. Sudová, M. Lučanová, P. Trávníček, T. Urfus, P. Vít, H. Weiss-Schneeweiss, et al. 2013. "High Ploidy Diversity and Distinct Patterns of Cytotype Distribution in a Widespread Species of *Oxalis* in the Greater Cape Floristic Region." *Annals of Botany* 111 (4): 641–649. doi:10.1093/aob/mct030.

Leitch, I. J., and M. D. Bennett. 2004. "Genome Downsizing in Polyploid Plants." *Biological Journal of the Linnean Society* 82 (4): 651–663. doi:10.1111/j.1095-8312.2004.00349.x.

Leitch, I. J., L. Hanson, K. Y. Lim, A. Kovarik, M. W. Chase, J. J. Clarkson, and A. R. Leitch. 2008. "The Ups and Downs of Genome Size Evolution in Polyploid Species of *Nicotiana* (Solanaceae)." *Annals of Botany* 101 (6): 805–814. doi:10.1093/aob/mcm326.

Mable, B. K. 2004. "Why Polyploidy Is Rarer in Animals than in Plants: Myths and Mechanisms." *Biological Journal of the Linnean Society* 82 (4): 453–466. doi:10.1111/j.1095-8312.2004.00332.x.

Mao, K., G. Hao, J. Liu, R. P. Adams, and R. I. Milne. 2010. "Diversification and Biogeography of *Juniperus* (Cupressaceae): Variable Diversification Rates and Multiple Intercontinental Dispersals." *New Phytologist* 188 (1): 254–272. doi:10.1111/j.1469-8137.2010.03351.x.

Marques, I., J. Loureiro, D. Draper, M. Castro, and S. Castro. 2018. "How Much Do We Know about the Frequency of Hybridisation and Polyploidy in the Mediterranean Region?". *Plant Biology* 20: 21–37. doi:10.1111/plb.12639.

Mergen, F., and B. A. Thielges. 1967. "Intraspecific Variation in Nuclear Volume in Four Conifers." *Evolution* 21 (4): 720–724. doi:10.1111/j.1558-5646.1967.tb03429.x.

Neale, D. B., and R. R. Sederoff. 1988. "Inheritance and Evolution of Conifer Organelle Genomes." In *Genetic Manipulation of Woody Plants*, edited by J. W. Hanover, D. E. Keathley, C. M. Wilson, and G. Kuny, 251–264. Boston, MA: Springer.

Opgenoorth, L., G. G. Vendramin, K. Mao, G. Miehe, S. Miehe, S. Liepelt, J. Liu, and B. Ziegenhagen. 2010. "Tree Endurance on the Tibetan Plateau Marks the World's Highest Known Tree Line of the Last Glacial Maximum." *New Phytologist* 185 (1): 332–342. doi:10.1111/j.1469-8137.2009.03007.x.

Otto, S. P., and J. Whitton. 2000. "Polyploid Incidence and Evolution." *Annual Review of Genetics* 34 (1): 401–437. doi:10.1146/annurev.genet.34.1.401.

Pichot, C., and M. El Maâtaoui. 2000. "Unreduced Diploid Nuclei in *Cupressus dupreziana* A. Camus Pollen." *Theoretical and Applied Genetics* 101 (4): 574–579. doi:10.1007/s001220051518.

Ramsey, J., and D. W. Schemske. 1998. "Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants." *Annual Review of Ecology and Systematics* 29 (1): 467–501. doi:10.1146/annurev.ecolsys.29.1.467.

Ranney, T. G. 2006. "Polyploidy: From Evolution to New Plant Development". *Combined Proceedings International Plant Propagators' Society* 56: 137–142.

Romo, A., O. Hidalgo, A. Boratyński, K. Sobierajska, A. K. Jasińska, J. Vallès, and T. Garnatje. 2013. "Genome Size and Ploidy Levels in Highly Fragmented Habitats: The Case of Western Mediterranean *Juniperus* (Cupressaceae) with Special Emphasis on *J. thurifera* L." *Tree Genetics & Genomes* 9 (2): 587–599. doi:10.1007/s11295-012-0581-9.

Sax, K., and H. J. Sax. 1933. "Chromosome Number and Morphology in the Conifers." *Journal of the Arnold Arboretum* 14 (4): 356–375. doi:10.5962/bhl.part.9959.

Schinkel, C. C. F., B. Kirchheimer, S. Dullinger, D. Geelen, N. D. Storme, and E. Hörandl. 2017. "Pathways to Polyploidy: Indications of a Female Triploid Bridge in the Alpine Species *Ranunculus kuepferi* (Ranunculaceae)." *Plant Systematics and Evolution* 303 (8): 1093–1108. doi:10.1007/s00606-017-1435-6.

Scott, A. D., N. W. M. Stenz, P. K. Ingvarsson, and D. A. Baum. 2016. "Whole Genome Duplication in Coast Redwood (*Sequoia sempervirens*) and Its Implications for Explaining the Rarity of Polyploidy in Conifers." *New Phytologist* 211 (1): 186–193. doi:10.1111/nph.13930.

Siljak-Yakovlev, S., F. Pustahija, E. M. Šolić, F. Bogunić, E. Muratović, N. Bašić, O. Catrice, and S. C. Brown. 2010. "Towards a Genome Size and Chromosome Number Database of Balkan Flora: C-Values in 343 Taxa with Novel Values for 242." *Advanced Science Letters* 3 (2): 190–213. doi:10.1166/asl.2010.1115.

Soltis, D. E., P. S. Soltis, and J. A. Tate. 2004. "Advances in the Study of Polyploidy since Plant Speciation." *New Phytologist* 161 (1): 173–191. doi:10.1046/j.1469-8137.2003.00948.x.

Tang, L., J. M. Tang, S. Tan, J. Li, X. Ma, and Z. Q. Zhou. 2015. ""ITS Sequence Variation and Concerted Evolution in the Natural Hybrid Species *Malus toringoides*." *Nordic Journal of Botany* 33: 109–119. doi:10.1111/j.1756-1051.2012.00108.x.

Tayalé, A., and C. Parisod. 2013. "Natural Pathways to Polyploidy in Plants and Consequences for Genome Reorganization." *Cytogenetic and Genome Research* 140 (2–4): 79–96. doi:10.1159/000351318.

Teixeira, H., S. Rodríguez-Echeverría, and C. Nabais. 2014. "Genetic Diversity and Differentiation of *Juniperus thurifera* in Spain and Morocco as Determined by SSR." *PLoS One* 9 (2): e88996. doi:10.1371/journal.pone.0088996.

Terrab, A., P. Schönswetter, S. Talavera, E. Vela, and T. F. Stuessy. 2008. "Rangewide Phylogeography of *Juniperus thurifera* L., A Presumptive Keystone Species of Western Mediterranean Vegetation during Cold Stages of the Pleistocene." *Molecular Phylogenetics and Evolution* 48: 94–102. doi:10.1016/j.ympev.2008.03.018.

Thomas, P. A., M. El-Barghathi, and A. Polwart. 2007. "Biological Flora of the British Isles: *Juniperus communis* L." *Journal of Ecology* 95 (6): 1404–1440. doi:10.1111/j.1365-2745.2007.01308.x.

Trávníček, P., B. Kubátová, V. Čurn, J. Rauchová, E. Krajníková, J. Jersáková, and J. Suda. 2010. "Remarkable Coexistence of Multiple Cytotypes of the *Gymnadenia conopsea* Aggregate (The Fragrant Orchid): Evidence from Flow Cytometry." *Annals of Botany* 107 (1): 77–87. doi:10.1093/aob/mcq217.

Vallès, J., T. Garnatje, O. Robin, and S. Siljak-Yakovlev. 2015. "Molecular Cytogenetic Studies in Western Mediterranean *Juniperus* (Cupressaceae): A Constant Model of GC-rich Chromosomal Regions and rDNA Loci with Evidences for Paleopolyploidy." *Tree Genetics & Genomes* 11 (3): 1–8. doi:10.1007/s11295-015-0860-3.

Vekemans, X., C. Lefebvre, J. Coulaud, S. Blaise, W. Gruber, S. Siljak-Yakovlev, and S. C. Brown. 1996. "Variation in Nuclear DNA Content at the Species Level in *Armeria maritima*." *Hereditas* 124 (3): 237–242. doi:10.1111/j.1601-5223.1996.00237.x.

Wood, T. E., N. Takebayashi, M. S. Barker, I. Mayrose, P. B. Greenspoon, and L. H. Rieseberg. 2009. "The Frequency of Polyploid Speciation in Vascular Plants." *Proceedings of the National Academy of Sciences* 106 (33): 13875–13879. doi:10.1073/pnas.0811575106.

Worth, J. R. P., M. J. Larcombe, S. Sakaguchi, J. R. Marthick, D. M. J. S. Bowman, M. Ito, and G. J. Jordan. 2016. "Transient Hybridization, Not Homoploid Hybrid Speciation, between Ancient and Deeply Divergent Conifers." *American Journal of Botany* 103 (2): 246–259. doi:10.3732/ajb.1500433.