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Molecular and Phytochemical Variability of Endemic *Juniperus sabina* var. *balkanensis* from Its Natural Range

Nemanja Rajčević [1,*], Tanja Dodoš [1], Smiljana Janković [1], Pedja Janaćković [1], Valtcho D. Zheljazkov [2] and Petar D. Marin [1]

[1] Faculty of Biology, University of Belgrade, 11000 Belgrade, Serbia
[2] Crop and Soil Science Department, Oregon State University, Corvallis, OR 97331, USA
* Correspondence: nemanja@bio.bg.ac.rs

Abstract: *Juniperus sabina* L. var. *balkanensis* R.P. Adams & Tashev is a recently described endemic variety from the Balkan Peninsula. Its strong sprouting ability and fast vegetative propagation, on one hand, and fragmented distribution, on the other, can lead to lower genetic diversity in local populations and to the differentiation of populations. As there has been no detailed investigation of this variety, we studied Balkan natural populations using phytochemical and molecular markers. Leaf essential oils (EOs) were chosen based on their proven usability in the population studies of *Juniperus* taxa, while ISSRs (Inter Simple Sequence Repeats) have been used due to their high resolution. In addition, since this variety is best described using molecular markers, the chloroplast *trn*S-*trn*G region was amplified from individuals from different populations having different chemotypes. Based on the essential oil profile, three chemotypes could be identified with a difference in their distribution. The analysis of molecular variance showed moderate differentiation of populations and regions, attesting to the start of the separation of three regions in the Balkans: west, east and south. The bioclimatic and environmental parameters and sex of the individual did not influence the EO profile, although some of the compounds present in low-to-medium concentrations showed strong correlation with several bioclimatic parameters.

Keywords: savin juniper; essential oils; ISSR; cpDNA; environmental factors

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1. Introduction

Juniperus sabina L. (Cupressaceae) is a dioecious coniferous evergreen shrub with both sexual and asexual reproduction strategies. *J. sabina* (savin juniper) covers a vast area of the northern hemisphere, but its distribution is fragmented across Eurasia, from Spain in the west to Mongolia and Siberia in the east. The World Flora (WFO) database recognises five varieties within this species: var. *sabina*, var. *mongoliensis* R.P. Adams, var. *davurica* (Pall.) Farjon, var. *arenaria* (E.H.Wilson) Farjon and var. *balkanesis* R.P. Adams & Tashev [1]. Due to its ability to spontaneously produce adventitious roots on soil covered branches and its resistance to pruning, it plays a significant role in the prevention of land desertification and the improvement of the urban landscape [2].

Balkan savin juniper is a just recently described variety [3]. It is an extremely rare variety that grows only in the Balkans. It can only be found in very inaccessible terrains, growing mostly on screes and vertical cliffs. The most recent research shows that it is common in the Balkans and Anatolia region [4–9]; however, there is no data for Serbia, where its central part of distribution should be found. While Adams found slight morphological differences (e.g., in the colour and texture of leaves, position of leaf glands and shape of seed cones), the most distinct characteristics are found in their genome, reflecting an ancient hybridisation event with *J. thurifera* [3]. This was also confirmed by Farhat et al. [4,10,11], who discovered that all of the var. *balkanensis* individuals had twice the amount of DNA than other varieties of this species, suggesting that the variety is tetraploid, rather than diploid.

In addition to its significance in natural and urban environments, savin juniper produces large amounts of bioactive compounds, mainly terpenoids; therefore, their essential oil composition has largely been studied from this aspect [8,12–17]. However, there are not many reports dealing with the intra- and inter-population variability of their essential oil. Essential oils (EOs) are mixtures of volatile organic compounds, mostly commonly terpenoids. Monoterpens and sesquiterpenes dominate juniper essential oils, in which up to 100 different terpene and non-terpene compounds could be found. In addition to their bioactivity, conifer essential oils, and particularly juniper essential oils, have been studied for their chemophenetic significance as chemophenetic markers that have proven useful in describing and assessing the genetic diversity of populations, as their composition is mostly genetically determined [18–22]. Present literature data on savin juniper shows the domination of two biosynthetically linked monoterpenes—sabinene and *trans*-sabinyl acetate (Figure 1); however, not all enzymes involved in the biosynthesis are known [23–26]. Nevertheless, it appears that sabinene is the starting compound for several highly abundant savin juniper terpenes.

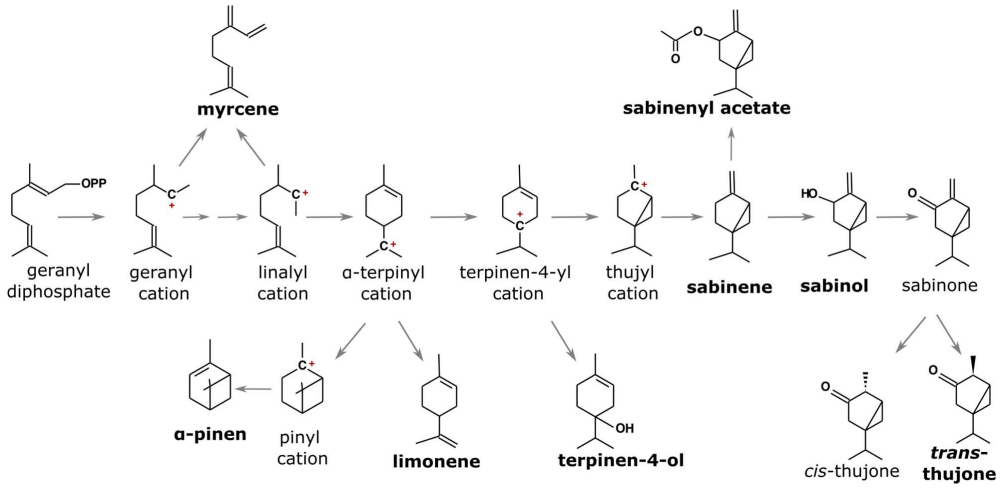


Figure 1. Biosynthetic pathway for the most common savin juniper terpenoids, adapted from [26].

While the species has been proven as hardy and is widely grown for ornamental purposes, it cannot withstand competition with other shrubs or trees in its natural habitat. As these remaining fragments of natural populations are important gene pools, it is important to preserve their genetic resources and reconstruct or find new habitats for this species. Thus, the starting point in these efforts is determining the genetic diversity and structure of natural populations of *J. sabina* var. *balkanensis*. However, previous investigations of the population variability using molecular markers have only been conducted in Asia [2], where two genetic pools were discovered. Recently, different authors have suggested using inter-simple sequence repeat (ISSR) markers as an alternative system with reliability and advantages over other molecular marker systems [27–30]. This technique involves the amplification of genomic segments flanked by inversely oriented microsatellite sequences. These regions are amplified using primers based on SSRs anchored with a couple of nucleotides. The resulting PCR products are multiple bands of different lengths that could be coded into a presence/absence matrix. ISSR markers are dominant markers that show Mendelian inheritance.

Protecting this narrowly distributed taxon and its genetic potential is of paramount significance in studies of changing climate on its growth and distribution. The aim of this study was to establish intra- and inter-population variability using leaf essential oil composition and their possible correlation with environmental factors. Individuals with different chemotypes were chosen for molecular analyses. ISSR were used to assess intra- and inter-population variability and correspondence with phytochemical markers, while

chloroplast *trn*G-*trn*S sequences were used to further confirm that the samples are *J. sabina* var. *balkanensis*.

2. Materials and Methods

2.1. Plant Material

Twigs with leaves of 100 individuals from seven natural populations of *J. sabina* var. *balkanensis* were collected from 2009 to 2015, along an altitudinal gradient from 700 to 1450 m in the Balkan Peninsula. One population was sampled in two different years to account for seasonable variability. All samples were placed in individual PP zip-lock bags and placed in a portable freezer on the location site. Within a day, the bags were packed in the freezer, and kept at a temperature of −18 °C until extraction. Plant material for DNA isolation was desiccated in the field using silica gel and stored at room temperature until DNA extraction. In total, 21 individuals were used in ISSR and 12 in *trn*G-*trn*S analyses. Population details, geographic information, terrain inclination, exposure, altitudes and voucher numbers are given in Table 1 and Figure 2. Taxonomic identification of the species was conducted by the authors in the field (NR, PJ and PM) and confirmed in the taxonomical lab using keys for the identification of *Junipers* [31,32]. The voucher specimens (accession No. given in Table 1) were deposited at the Herbarium of the University of Belgrade, Faculty of Biology (BEOU).

Table 1. Geographic distribution of studied *Juniperus sabina* L. var. *balkanensis* R.P.Adams & Tachev populations from the Balkans.

Location	Country	Longitude	Latitude	Altitude	Incl. [1]	Exp. [2]	S [3]	BEOU
Mt. Velebit	Croatia	44.245	15.810	1350	35	SW	Sa	17213
Mt. Biokovo	Croatia	43.299	17.077	700	30	E	Li	17094
Mt. Prokletije	Montenegro	42.510	19.834	1300	15	S	Li	17409
Mt. Korab	Albania	41.786	20.546	1250	45	W	Li	17837
Mt. Bistra	North Macedonia	41.594	20.665	1450	60	S	Si	17838
Resava gorge	Serbia	44.085	21.681	720	65	SW	Li	17209
Central Balkan	Bulgaria	42.688	25.123	1350	25	SE	Sa	17403

[1] Inclination presented as difference in m.a.s.l on a 100 m line; [2] Exposition; [3] Geological substratum, Sa—sandstone, Li—limestone, Si—silicate.

Figure 2. Spatial distribution of analysed populations of *Juniperus sabina* var. *balkanensis;* for population details, cf. Table 1.

2.2. Essential Oil Isolation

The leaves from the collected frozen material were ground in a laboratory mill prior to essential oil extraction. Distilled water (150 mL) was added to the homogenised material of each sample (ca. 3 g) and subjected to 2 h simultaneous hydrodistillation and extraction (SDE) in a Likens-Nickerson type apparatus [33]. Dichloromethane (CH_2Cl_2) 5 mL was used as primary solvent. The obtained extracts (0.5 mL) were stored in amber vials at 4 °C until further analyses.

2.3. GC-FID and GC/MS Analysis

The GC analysis was carried out using an Agilent 7890A apparatus equipped with a 5975C mass-selective detector, flame ionisation detector (FID) and DB-5 MS fused-silica gel cap. The conditions and analysis procedures for GC-FID and GC/MS are described in Rajčević et al. [19]. The relative amounts of volatile components were expressed as percentages of the peak area of total ion chromatograms. Values under 0.05% were not considered during compound identification. A library search and mass spectral deconvolution and extraction were performed using the software NIST AMDIS version 2.64.113.71, with the retention index (RI) calibration data analysis parameters set to "strong" level and 10% penalty for compounds without RI. The search was performed against our homemade library, containing 4972 spectra. The relative contents of the identified compounds were computed from the GC peak areas. The linear RI was calculated for all compounds using the following formula: $LRI = 100 \times (t_{rs} - t_{rn})/(t_{rn} + 1 - t_{rn}) + 100 \times n$.

2.4. DNA Extraction

The DNA was isolated from the young leaves of seven populations (Table 1) using a modified CTAB protocol, previously described in Rajčević et al. [18]. DNA purity and quantity were determined using the Perkin Elmer spectrophotometer (LambdaBio, Beaconsfield, UK). The sample concentration was adjusted to 100 ng/µL and stored at −80 °C until further analyses.

2.5. Chloroplast Sequence Analysis

The DNA isolates were assessed by PCR amplification of the one cpDNA region, the *trn*G-*trn*S spacer region. The *trn*G-*trn*S spacer was PCR amplified using *trn*S^{GCU} 5′-GCCGCTTTAG TCCACTCAGC-3′ as the forward, and *trn*G^{UCC} 5′-GAACGAATCACA CTTTTACCAC -3′ as the reverse primer [34]. PCR amplification was performed in 25 µL volumes, containing: 50 ng template DNA, 2.5 µL 1 × Taq Buffer with $(NH_4)_2SO_4$ (Fermentas UAB, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each forward and reverse primer, 0.80% BSA (Bovine Serum Albumin, Fermentas UAB, Vilnius, Lithuania), and 0.025 U/µL of Platinum taqDNA polymerase (Fermentas UAB, Vilnius, Lithuania). PCR amplifications were performed using Eppendorf Mastercycler Nexus GSX1 with the following program: initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. Macrogen Europe B.V. performed sequencing using ABI 3730XLs capillary DNA analyser. The resulting sequences were checked (Chromas, 2.6.6) and manually aligned (Mega X) [35].

2.6. ISSR Analysis

PCRs were performed in 25 µL volume consisting of 1 × KCl PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs mix, 0.8 µM primer, 0.5 U of Taq DNA polymerase (Thermo Fisher Scientific, Chesire, UK), 5% DMSO (Sigma Aldrich, St. Louis, MO, USA), and 50 ng of template DNA. Five ISSR primers were tested in this research (Table 4). Amplification was performed in an Eppendorf Mastercycler Nexus GSX1 using touchdown protocol of the following conditions: 10 min at 94 °C for 1 cycle, followed by 30 s at 94 °C, 60 s at T_m for specific primer (cf. Table 4), and 2 min at 72 °C for, next 30 s at 94 °C, 60 s at T_m −5 °C for specific primer, and 2 min at 72 °C for 37 cycles and 10 min at 72 °C for a final extension.

Amplification products were separated on 2% agarose gels run for 45 min at 4.5 V/cm in 1 × TAE, visualized by staining with green safe (NZY tech, Lisboa, Portugal), and 6X Orange DNA Loading Dye (Thermo Scientific). Gels were photographed under ultraviolet light using a gel documentation and image analysis system (Vilber Loumat Doc-Print VX2). Fragment lengths were estimated using a GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific). Polymorphic bands were scored as present (1) or absent (0) and assembled in a data matrix. Only reproducible and clear bands in the replications were considered potential polymorphic markers.

2.7. Environmental Data

The bioclimatic data (temperature, precipitation, solar radiation) of all seven studied localities were taken from the WorldClim 2.0 set of global climate layers, with the precision of ~1 sq km [36]. The data were analysed using QGIS software (QGIS Development Team 2015). Geological substratum information was obtained from the International Geological Map of Europe and Adjacent Areas [37].

2.8. Statistical Analyses

The mean, standard deviation and distribution were tested before performing univariate (Analysis of variance, ANOVA) and multivariate analyses (principal components analysis, PCA; discriminant analysis, DA; hierarchical cluster analysis, HCA; multivariate analysis of variance, MANOVA). Simple linear correlation and multivariate correlation (Mantel and partial Mantel tests) were used to analyse the correlation of EO components with the environmental data. All statistical analyses were performed using PAST 4.11 [38].

For the ISSRs, the efficiency of each marker in giving polymorphic DNA bands was expressed with the total number of bands, number of polymorphic bands, number of monomorphic bands, percentage of polymorphic bands and polymorphism information contents (PICs) [39]. AMOVA (Analysis of Molecular Variance) was calculated using GenoDive v.3.0 [40], with 9999 permutations. The population structure, based on a squared Euclidean distance matrix using Fst-analogue independent of ploidy level and breeding system [41,42], was analysed. Bayesian model-based estimation of population structure was performed using Structure version 2.3.4 [43]. The analyses were performed under the admixture model assuming independent allele frequencies and using a burn-in period of 10,000, followed by 5000 Markov Chain Monte Carlo, and the most likely value of K was determined according to [44]. Principal coordinate analysis (PCoA) was performed to illustrate the overall similarity among the individuals using GenAlEx 6.5 [45]. The PCoA was inferred from the Jaccard's distance between all pairs of ISSR phenotypes. The Mantel test was carried out to assess the correlation between genetic (Nei's genetic distances of populations) and geographic distances.

3. Results

3.1. Essential Oil Composition

In the essential oils (EOs) obtained from 100 individuals, 114 compounds were detected and identified, representing, on average, 98.1–99.4% of the total oil composition (Supplementary Table S1). The number of compounds per EO varied between 62 and 89. The chemical composition was dominated by monoterpenes (46.4–73.8%), followed by either sesquiterpenes or diterpenes, depending on the population. Oxygenated compounds were dominant in most of the studied populations, ranging between 50.7% in the population from Central Balkan and 79.3% in the Resava gorge population, with the exception of the population of Mt. Korab, where oxygenated compounds were present below 48%. The two most abundant compounds were sabinene and *trans*-sabinyl acetate, accounting for approximately 31.5–53.2% of the total oil content on average. Four compounds were also present in medium-to-high concentrations: (>5.0)—abieta-7,13-dien-3-one, 4-*epi*-abietal, *trans*-sabinol, and germacrene D-4-ol. Twelve other compounds were present, on average, in low-to-medium concentrations—α-pinene, myrcene, limonene, γ-terpinene, bornyl ac-

etate, germacrene D, γ-cadinene, δ-cadinene, τ-muurolol, 1,10-di-*epi*-cubenol, α-cadinol, and abietadiene. All other compounds were present under 1%, primarily only in traces (<0.1%). While the concentration of sabinene and *trans*-sabinyl acetate reached high values in individual samples (e.g., 54.5% and 59.2%, respectively, in individual samples), most of the populations showed relatively similar concentrations of these two compounds or slight domination of one of the compounds. In populations from Mt. Velebit and Resava gorge, *trans*-sabinyl acetate was four and ten times more abundant than sabinene, respectively. On the other hand, in Mt. Korab and Central Balkan populations, sabinene was approximately three times more abundant than *trans*-sabinyl acetate.

3.2. Essential Oil Variability

Twenty-one compounds not-correlated ($-0.7 > R < 0.7$) and present above 0.1%, on average, were used for further statistical analyses, with the exception of the two most abundant compounds ($R = -0.81$, $p < 0.01$). The first two eigenvectors of the PCA explained 93.1% of the total variability. Three compounds were responsible for most of the separation: sabinene, *trans*-sabinyl acetate, and abieta-7,13-dien-3-one, separating individuals into three clusters (Supplementary Figure S1).

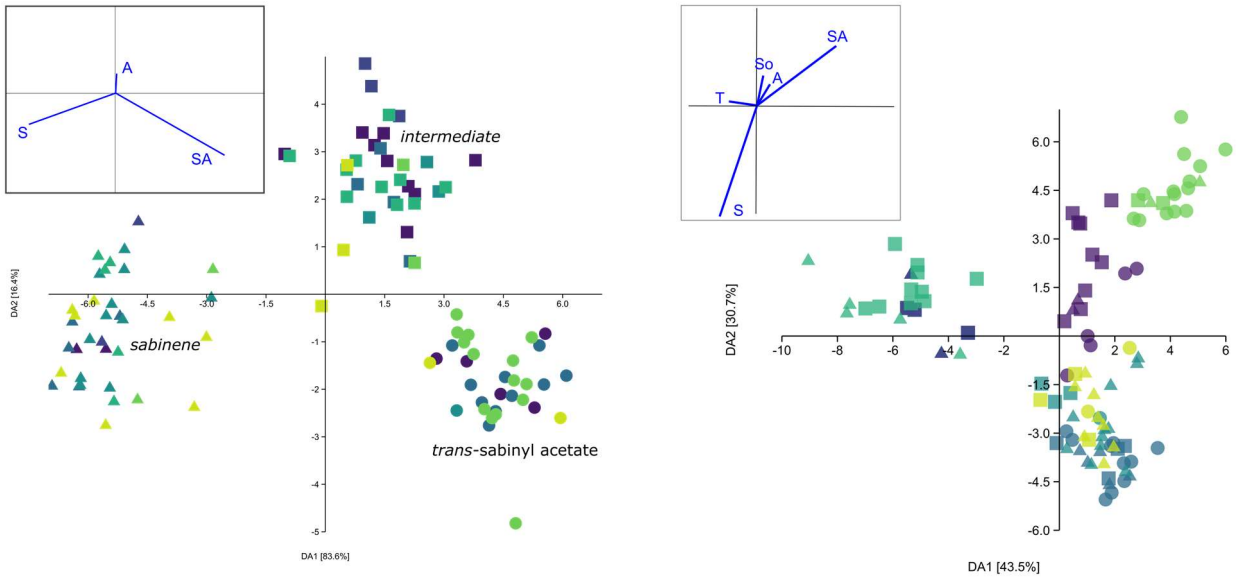
K-cluster analysis (K = 3) was performed, and the grouping of the individuals was checked against PCA scatter plot. Three obtained groups of similar size (cf. Table 2) correlated with the trimodal distributions of sabinene and *trans*-sabinyl acetate, while abieta-7,13-dien-3-one showed a slightly skewed normal (unimodal) distribution. These results suggested the existence of three possible chemotypes. To test the differences between these three putative chemotypes, a series of univariate and multivariate tests were performed. ANOVA showed separation in all 21 analysed compounds. The more conservative post hoc pairwise test (Tukey's) showed a strong separation in all three putative chemotypes and only in the concentration of *trans*-sabinyl acetate, sabinene and *cis*-thujone, while on all other components, different pairs were formed (Table 2).

Table 2. ANOVA of three detected chemotypes of leaf essential oil of *Juniperus sabina* var. *balkanensis* from the Balkans.

	Compound	F	*p*	SA *n* = 32	I *n* = 34	S *n* = 35
1	α-Pinene	10.38	***	1.2 ± 0.6 ^{a}	1.7 ± 1.0 ^{a}	2.1 ± 0.8
2	**Sabinene**	**180.6**	***	**5.6 ± 5.4**	**11.0 ± 7.0**	**37.4 ± 9.2**
3	Myrcene	19.6	***	1.2 ± 1.1 ^{a}	1.7 ± 1.2 ^{a}	2.8 ± 0.9
4	Limonene	8.8	***	0.6 ± 0.5	1.4 ± 1.2 ^{a}	1.3 ± 0.8 ^{a}
5	***cis*-Thujone**	**18.6**	***	**1.5 ± 1.6**	**0.7 ± 0.5**	**0.1 ± 0.1**
6	*trans*-Thujone	26.81	***	0.9 ± 0.5 ^{a}	1.1 ± 0.9 ^{a}	0.1 ± 0.2
7	*trans*-Sabinol	19.52	***	4.0 ± 3.7 ^{a}	3.1 ± 2.6 ^{a}	0.3 ± 0.4
8	Terpinen-4-ol	11.6	***	0.8 ± 0.3	2.6 ± 2.3 ^{a}	3.6 ± 3.4 ^{a}
9	Citronellol	6.5	***	0.5 ± 0.7 ^{a}	0.5 ± 0.6 ^{a}	1.0 ± 0.8
10	Methyl citronellate	3.1	***	1.5 ± 1.6 ^{a}	2.0 ± 1.7 ^{ab}	2.5 ± 1.4 ^{b}
11	Bornyl acetate	6.9	***	0.2 ± 0.9 ^{a}	2.4 ± 3.9	0.5 ± 1.9 ^{a}
12	***trans*-Sabinyl acetate**	**383.4**	***	**45.6 ± 6.7**	**24.6 ± 8.1**	**1.8 ± 4.0**
13	Germacrene D	4.4	***	0.8 ± 0.6 ^{ab}	0.6 ± 0.3 ^{a}	1.1 ± 0.8 ^{b}
14	γ-Cadinene	6.177	***	0.4 ± 0.3 ^{b}	1.2 ± 1.2 ^{a}	0.8 ± 0.9 ^{ab}
15	Elemol	6.5	***	1.5 ± 1.6	0.5 ± 0.6 ^{a}	0.7 ± 0.9 ^{a}
16	Germacrene B	4.6	***	0.4 ± 0.6 ^{a}	0.6 ± 0.6 ^{ab}	0.9 ± 0.9 ^{b}
17	Germacrene D-4-ol	6.1	***	4.1 ± 2.2 ^{a}	3.9 ± 2.9 ^{a}	5.9 ± 2.5
18	β-Oplopenone	2.051	-	0.9 ± 0.5 ^{a}	0.7 ± 0.4 ^{a}	0.9 ± 0.4 ^{a}
19	α-Cadinol	11.4	***	2.7 ± 1.0 ^{a}	2.8 ± 1.2 ^{a}	3.8 ± 0.8
20	Abietadiene	3.5	***	1.0 ± 0.5 ^{a}	1.3 ± 0.7 ^{ab}	1.5 ± 1.2 ^{b}
21	Abieta-7,13-dien-3-one	3.6	***	4.4 ± 3.6 ^{b}	7.0 ± 5.7 ^{a}	4.7 ± 3.4 ^{ab}

F—ANOVA F value; *p* Level of significance for ANOVA (*** $p < 0.001$); Contents are given as percentages (mean ± SD) of the total essential oil composition, means with the same superscript letters within the same row did not differ significantly (Tukey's pairwise test).; SA—*trans*-sabinyl acetate chemotype; I—intermediate chemotype; S—sabinene chemotype. Compounds that significantly differed between all three chemotypes in post-hoc test are marked in boldface.

The separation of three chemotypes was confirmed with MANOVA (F = 34.0, $p < 0.001$), including the post hoc pairwise Hotelling's test ($p < 0.001$) and Discriminant analysis (DA). The DA Scatter plot (Figure 3a) showed clear separation. The first eigenvector accounted for 84.4% of the total variability, strongly differentiating the sabinene (S) from the *trans*-sabinyl acetate (SA) chemotype. The separation of the SA chemotype and the intermediary chemotype (I) was more pronounced on the second axis, though most of the individuals could be separated on the first one as well.



(**a**) (**b**)

Figure 3. Discriminant analysis scatter plot of leaf essential oil composition of *Juniperus sabina* var. *balkanensis;* (**a**) putative chemotypes as groups; symbols correspond to different chemotypes, while dots with same colour belong to same population; (**b**) populations as groups. —Central Balkan, —Resava gorge, —Mt. Bistra, —Mt. Korab, —Mt. Prokletije, —Mt. Biokovo, —Mt. Velebit. Principle components given in the left-hand side corner: S—Sabinene, SA—*trans*-Sabinyl acetate, So—*trans*-Sabinol, T—terpinen-4-ol, A—Abieta-7,13-dien-3-one.

The present analysis (PCA, not shown) did not show the correlation of individual samples' EO composition with either location, geological substratum, region or sex. Additionally, individuals sampled in different years from the same population showed similar EO composition. The same was confirmed in MANOVA, with the exception of the populations. MANOVA was able to differentiate populations based on the EO profile (F = 13.12, $p = 2.2 \times 10^{-99}$), while the post hoc Hotelling's pairwise test differentiated most of the populations. The pairwise test was not able to separate Mt. Bistra and Mt. Biokovo ($p > 0.05$) on one side, nor Central Balkan, Mt. Korab and Mt. Velebit ($p > 0.05$), on the other (Supplemental Table S2). Discriminant analysis (Figure 3b) with populations as groups showed the formation of three clusters that differed somewhat from MANOVA: (1) Mt. Velebit and Resava gorge, (2) Central Balkan, Mt. Korab, Mt. Prokletije, and (3) Mt. Bistra and Mt. Biokovo. These results correspond with the mean values of sabinene and *trans*-sabinyl acetate for the studied populations. Hierarchical cluster analysis (Sq. Mahalanobis distances, UPGMA) (Figure 4) also confirmed the results of the DA. As the obtained cladogram did not correspond to the geographic distribution (Mantel test, $R = -0.4$, $p > 0.05$), the distribution of chemotypes across populations was also considered (Figure 2). The ratio of the three chemotypes influenced the mean population EO profile strongly, with populations from Mt. Biokovo and Mt. Bistra being the only populations with just two chemotypes detected.

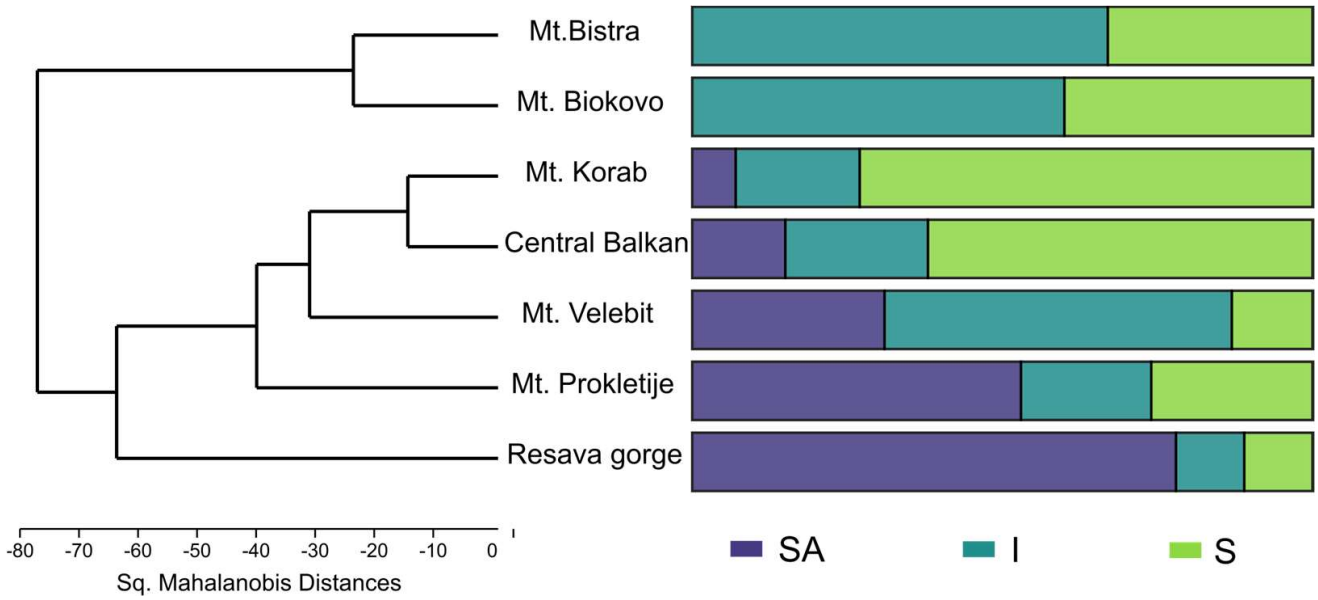


Figure 4. Dendrogram (UPGMA, Sq. Mahalanobis' Distances); right hand side shows distribution of chemotypes in populations; SA—*trans*-Sabinyl chemotype; I—Intermediate chemotype; S—Sabinene chemotype.

3.3. Genetic Variability

Individuals from different populations that had different chemotypes were used in molecular analyses. The PCR amplification and sequencing yielded PCR products between 795 and 802 bp long. The obtained sequences were aligned with the NCBI accessions of both *J. sabina* var. *sabina* and *J. sabina* var. *balkanensis* (Table 3) and were trimmed before further analysis. Analysis of the obtained chloroplast intergenetic spacer *trn*G-*trn*S sequences showed that all of the tested individuals share SNPs and indels congruent with *J. sabina* var. *balkanensis*, irrespective of their chemical composition (Table 3).

Table 3. SNPs and indels in chloropast *trn*G-*trn*S intergenetic spacer of *Juniperus sabina* var. *balkanensis* populations.

Ind.	Chemotype	LT [1]	Position in Alignment								GenBank Accession Numbers
			7	148	460	466..483	664	709	752	761	
V1	SA	795	G	C	G	+ [2]	G	-	G	A	OP297053
B1	S	802	G	C	G	+	G	-	G	A	OP297042
B4	I	802	G	C	G	+	G	-	G	A	OP297043
P10	SA	798	G	C	G	+	G	-	G	A	OP297049
P4	I	800	G	C	G	+	G	-	G	A	OP297050
K3	S	802	G	C	G	+	G	-	G	A	OP297046
K8	SA	802	G	C	G	+	G	-	G	A	OP297047
M1	-	799	G	C	G	+	G	-	G	A	OP297048
R1	S	802	G	C	G	+	G	-	G	A	OP297051
R7	SA	798	G	C	G	+	G	-	G	A	OP297052
C1	S	797	G	C	G	+	G	-	G	A	OP297044
C8	SA	802	G	C	G	+	G	-	G	A	OP297045
var. *balkanensis* [3]		-	G	C	G	+	G	-	G	A	MT136665.1
var. *sabina* [3]		-	A	T	T	-	A	A	A	T	MT136634.1

Alignment length 781 bp; [1] Total readable length in bp; [2] indel: AAAAATAGAATACATAAA; [3] Sequences downloaded from NCBI; V—Mt. Velebit, B—Mt. Biokovo, P—Mt. Prokletije, K—Mt. Korab, M—Mt. Bistra, R—Resava gorge, C—Central Balkan; Chemotypes—SA—sabinene acetate, S—sabinene, I—intermediate.

Three individuals per population with different chemotypes were selected for ISSR analyses, 21 individuals in total. The number of products generated by ISSR primers ranged from three to six, ranging from 250 to 2000 bp. Primers JS17 and JS16 gave the most fragments (6), while JS3 had the least (3). These five primers generated a total of 24 fragments (Table 4). Each ISSR primer had an average of 4.4 polymorphic bands per

assay unit. The percentage of polymorphism ranged from 66.7% for JS3 to 100% for JS17 and JS16. PIC values ranged from 0.195 for ISSR01 to 0.334 for ISSR17.

Table 4. Marker parameters calculated for each ISSR primer used with *Juniperus sabina* var. *balkanensis*.

Name	Primer Sequence	T_m [°C]	TB	PB	MB	%PB	PIC
JS 17	5′-TCC TCC TCC TCC TCC TCC-3′	61.3	6	6	0	100	0.334
JS 01	5′-GAG AGA GAG AGA GAG AC-3′	49	4	3	1	75	0.195
JS 16	5′-AGA GAG AGA GAG AGA GCC-3′	53.8	6	6	0	100	0.227
JS 03	5′-CAC ACA CAC ACA CAC AG-3′	53	3	2	1	66.7	0.311
JS 04	5′-AGC AGC AGC AGC GT-3′	54.9	5	5	0	100	0.298
Total			24	22	2	88.3	0.273

T_m primer melting temperature; TB—total bands; PB—number of polymorphic bands; MB—number of monomorphic bands; %PB—percentage of polymorphic bands; PIC—polymorphic information content calculated according to Roldan-Ruiz et al., 2000 [39].

Analysis of molecular variance (AMOVA) showed that most of the genetic variability was within the populations (72.8%), whereas the rest was between populations (20.4%) and the three geographic regions (Table 5).

Table 5. Analysis of molecular variance (AMOVA) in 7 populations of *Juniperus sabina* var. *balkanensis*.

Source	d.f. *	S.s.	VC	TV (%)	*p*
Within population	14	36.67	2.62	72.8	
Among populations	4	19.28	0.73	20.4	0.001
Among regions	2	13.01	0.25	6.8	0.004

* Degrees of freedom; S.s.—sum of squares, VC—variance components, TV—total variation.

The principle coordinate analysis (PCoA) scatter plot showed the separation of most populations only on the second axis (Figure 5a). This was because, in all populations, one-third differed strongly from the rest of the populations. Nevertheless, the neighbour-joining (NJ) tree (Nei's genetic distances) showed a grouping of populations based on the geographic distribution (Figure 5b). In the NJ cladogram, three groups can be distinguished: (1) populations from the Resava gorge and Central Balkan that belong to the same mountain range; (2) populations from the northern and central Dinaric mountains; and (3) Prokletije and Korab, two populations from the southern Dinaric mountains. Interestingly, the population from Mt. Bistra grouped with the Central Balkan populations, rather than the much closer Mt. Korab and Mt. Prokletije. In addition, with the exception of the Mt. Bistra population, a strong north-south grouping is apparent. The genetic distances were rather low, ranging between 0.079 and 0.340, further suggesting a close relationship between populations. Structure analysis could not separate gene pools, nor could it separate the populations, suggesting the same origin of all populations and/or the constant gene flow between these populations. The Mantel test did not show a correlation with the geographic region ($R = 0.2$, $p > 0.05$). This is most probably due to the populations from Mt. Bistra and Mt. Korab, which are geographically the closest populations, being very different.

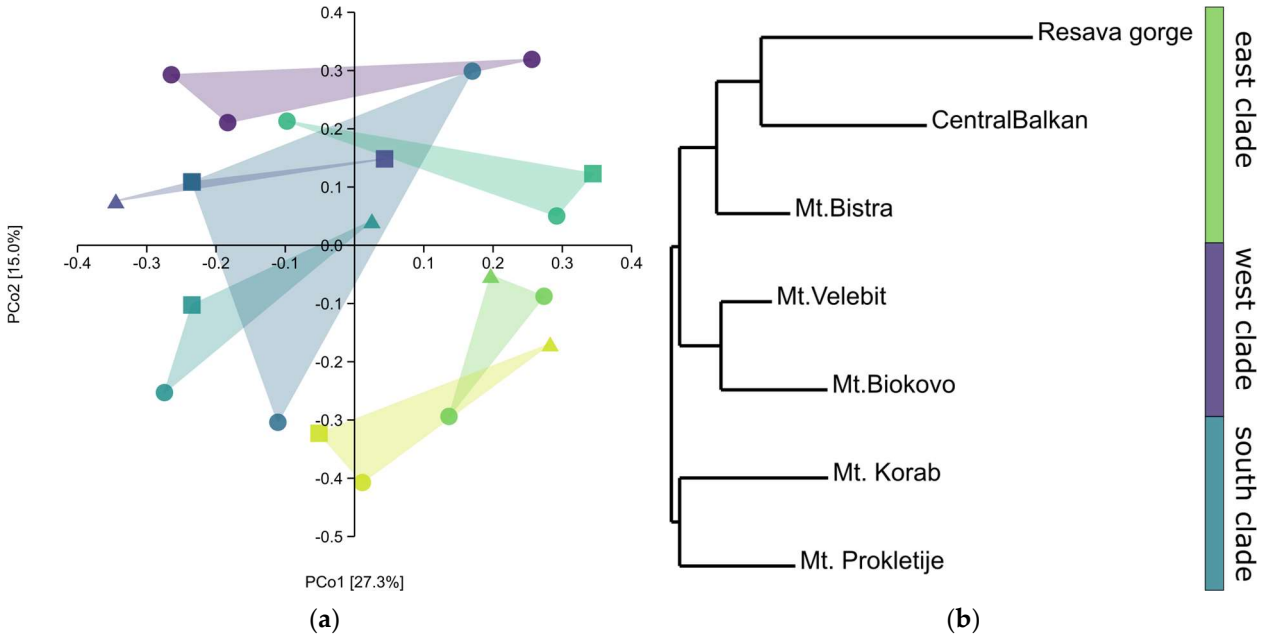


Figure 5. (**a**) PCoA scatter plot (Jaccard's distances), —Central Balkan, —Resava gorge, —Mt. Bistra, —Mt. Korab, —Mt. Prokletije, —Mt. Biokovo, —Mt. Velebit; triangles —sabinene chemotype, squares—intermediate chemotype, dots—*trans*-sabinyl acetate chemotype; (**b**) NJ tree, Nei's genetic distances.

3.4. Environmental Data

PCA analysis of 19 bioclimatic factors showed three groups of localities: (1) Mt. Korab; (2) Resava gorge & Central Balkan; and (3) all others based on the minimum temperatures of the coldest month and the mean temperature of the wettest quarter. Additionally, the localities Resava gorge and Central Balkan were the driest, with at least 20% less precipitation than the others. Linear correlation analysis (Pierson correlation) with the raw data did not show a correlation between any of the 21 EO components with the environmental factors, with the exception of terpinen-4-ol with the precipitation seasonality ($R = 0.6$, $p < 0.01$) and summer precipitation ($R = -0.7$, $p < 0.01$). However, when the mean population values were tested, several compounds strongly correlated with the environmental factors (Supplemental Table S3). The concentration of the dominant compounds sabinene and *trans*-sabinyl acetate did not correlate with any environmental parameter. Some compounds, such as *trans*-sabinol, positively correlated only with the inclination, i.e., the steeper the locality, the more abundant the *trans*-sabinol, on average, in the population. Limonene, *trans*-thujone, citronellol, methyl citronellate, bornyl acetate, and β-oplopenone correlated negatively with the temperature parameters (i.e., the higher the temperature, the less abundant these compounds were). Additionally, terpinen-4-ol, citronellol and methyl citronellate were also positively correlated with the precipitation parameters (i.e., the higher the precipitation, the more abundant these compounds were). While some compounds were correlated with the environmental parameters, there is no evidence that the distribution of chemotypes (i.e., concentration of dominant compounds) is related to the tested environmental factors. This was also confirmed by both the Mantel test ($R = 0.2$, $p > 0.05$) and partial Mantel test ($R = 0.3$, $p > 0.05$).

4. Discussion

Analysis of EOs from 100 individuals from seven populations showed high variability in the EO composition between individuals from the same population that grow only a few meters apart, which corroborates the chemophenetic significance of EOs. This also suggests that the environment does not strongy influence EO composition, as different chemotypes

could be detected on the same substratum, growing under the same conditions. Statistical analysis confirmed strong and statistically significant negative correlation of sabinene and *trans*-sabinyl acetate, thus confirming the hypothesis of their biosynthethic connection. Three chemotypes were detected in the studied populations. Our data correspond to the available literature data (Table 6). Previous reports show that sabinene was the dominant compound in most of the populations. However, *trans*-sabynil acetate was dominant in the populations from Canada, Switzerland, and Iran. Only in one sample, from Bulgaria, was myrtenyl acetate the dominant compound [9]. Our population from Bulgaria was very similar to those EOs obtained by Zheljazkov et al. [9]. Aside from the dominant compounds, the overall relatively high variability of the EO corresponds to the literature data.

Table 6. Three dominant compounds in *Juniperus sabina* leaf essential oil composition from the literature.

Taxon [1]	Origin	Sex	Dominant Compounds (%)	Ref.
J. sabina	Canada	-	*trans*-Sabinyl acetate (38.0), sabinene (30.5), myrcene/cadinene (4.5)	[46]
	Spain	-	Sabinene (38.1), *trans*-sabynil acetate (16.2), terpinen-4-ol (6.9)	[47]
		-	Sabinene (54.9), limonene (2.4), terpinen-4-ol (7.2)	[47]
	Switzerland	-	*trans*-Sabinyl acetate (35.0), sabinene (34.8), limonene (3.0)	[47]
	Slovakia [2]	m	Sabinene (24.5), myrtenyl acetate (20.8), elemol (12.2)	[9]
		m	Sabinene (28.2), methyl eugenol (13.5), terpinen-4-ol (12.4)	[9]
	Bulgaria [2]	f	Myrtenyl acetate (23.0), sabinene (16.7), elemol (13.2)	[9]
		m	Sabinene (31.0), elemol (13.7), terpinen-4-ol (13.6)	[9]
	Iran	m	*trans*-Sabinyl acetate (46.2), sabinene (24.3), δ-cadinene (5.5)	[14]
		m	*trans*-Sabinyl acetate (30.3), sabinene (26.7), δ-cadinene (6.9)	[14]
		m	Sabinene (21.5), α-pinene (14.7), γ-terpinene (6.8)	[16]
		f	Sabinene (24.3), 4-terpineol (8.1), myrcene (5.4)	[16]
	Kazakhstan	-	Sabinene (42.6), *trans*-sabynil acetate (15.9), α-pinene (15.8)	[47]
	Mongolia	-	Sabinene (36.8), α-pinene (17.2), cedrol (15.2)	[48]
		-	Sabinene (46.5), *trans*-sabynil acetate (15.9), cedrol (13.2)	[47]
		-	Sabinene (50.0), *trans*-sabinyl acetate (18.3), terpinen-4-ol (3.1)	[47]
		-	Sabinene (56.7), terpinen-4-ol (4.7), methyl citronellate (3.1)	[47]
	China	-	Sabinene (39.0), *trans*-sabynil acetate (17.5), cedrol (15.8)	[49]
		-	Sabinene (46.5), *trans*-sabinyl acetate (15.9), cedrol (13.2)	[50]
var. *arenaria*	China	-	sabinene (57.1), α-pinene (3.8), germacrene D-4-ol (3.5)	[47]
var. *erectopatens*	China	-	Sabinene (33.3), unknown (13.2), elemol (3.7)	[48]

[1] Taxon as described in the reference, [2] Potentially *J. sabina* var. *balkanensis*.

In the present analysis, three chemotypes were more-or-less equally distributed in the sample; however, the distribution of chemotypes differed between populations. In the populations from Mt. Bistra and Mt. Biokovo, *trans*-sabinyl acetate chemotype was not detected. While in the case of Mt. Biokovo, this could be due to a sampling artefact ($n = 5$), this cannot be the case with the Mt. Bistra population, particularly considering the almost equal distribution of chemotypes in the whole sample. Additionally, ISSRs show that Mt. Bistra has more-or-less the same genetic diversity as most of other populations (Figure 5a). In the model, a single gene is crucial for the transformation of sabinene into *trans*-sabinyl acetate; it follows classic Mendelian dominant/recessive inheritance that the amount of these compounds would correspond to the number of alleles present in the genotype. However, isolation of the enzymes responsible for biosynthesis and the identification of the genes are necessary to confirm this. Previous reports on *Juniperus deltoides*, *J. communis* var. *communis* and *J. communis* var. *saxatilis* show different distributions of chemotypes across the Balkans [18,19,21,22]. In *J. deltoides*, the limonene chemotype was more common in coastal populations, while α-pinene was more common in the continental populations [21]. A similar pattern was observed in the two varieties of *J. communis* where five different chemotypes were detected [18,19]. In. *J. communis* var. *communis*, several chemotypes were detected in the literature [19,51–54], which showed different abundances related to

the geographic distribution. While the distribution range of *J. communis* var. *communis* and *J. deltoides* is somewhat continuous in this region, *J. communis* var. *saxatilis* has a more fragmented distribution, as it grows above the tree line in high mountains. Nevertheless, in this taxon, the geographic distribution of chemotypes was also apparent, where *δ*-3-carene chemotype was most common in the areas with greater precipitation, and *α*-pinene and sabinene were more frequent in the more arid areas of the Balkans. In this research, the sabinene chemotype was more common in the areas with lower temperatures [18]. On the other hand, in more closely related species (*J. phoenicea* var. *turbinata*), EO was strongly dominated by a single compound (*α*-pinene), although several other compounds were present in higher concentrations (e.g., *δ*-3-carene, caryophyllene oxide, germacrene D, limonene and myrcene). No apparent pattern was noticed in the geographic distribution [20 and refs. cited therein].

The detected genetic variability was on par with the available literature data. To the best of our knowledge, ISSRs have never previously been used in *J. sabina*. However, Boogat et Salehi [28] used 12 ISSR primers to analyse five populations of *Juniperus polycarpos*, obtaining PIC values between 0.38 and 0.5, which was higher than our results. Since ISSRs are dominant genetic markers, their highest PIC value cannot be greater than 0.5; therefore, the obtained PIC values suggest that selected ISSRs were moderately informative in our study. *J. sabina* genetic diversity was previously studied using SSRs and RAPDs [2,55]. For SSRs, authors obtained PIC values between 0.179 and 0.751. ISSRs were used to study the genetic diversity and differentiation of populations of *J. polycarpos*, *J. brevifolia*, *J. phoenicea, J. chinenesis,* and *J. excelsa* [28,30,56–58]. In all of these studies, ISSRs showed a high number of polymorphic bands. Most of the studies of different junipers, however, relied on the SSRs [2,56,59–64] or AFLPs [18,65–69], which have shown to have high correlation with the ISSRs [56]. Furthermore, studies performed by several authors showed high correlation of SSRs and ISSRs with other genetic markers [58,60,70].

Juniperus sabina from Asia showed that 88% of variability lies within populations, while only 12% exists between populations [2]. These results are somewhat lower than ours, particularly when taking the area of study into consideration. Bettencourt et al. [56] showed that ISSRs tend to have a lower population variability than SSRs. Similar results of genetic diversity were obtained for other junipers, where intrapopulation variability accounted of between 50.9% in *J. communis* [69] and 98% in *J. oxycedrus* [71], depending on both the geographic area analysed and the species. However, in most cases, the population variability was around 90% [18,56,59,61–63,66,68]. Conifers, and juniper among them, tend to show high genetic diversity within populations, and low separation of populations and regions. This can be explained by the biology of conifers. Junipers are long living species, the seeds of which are dispersed by birds, and the pollen is carried by wind. As one can see from the widely available data, genetic distances between populations in junipers are usually low, even when the distribution is fragmented and populations live at great distances.

Both ISSRs and EOs showed high intrapopulation variability with moderate separation of populations. Additionally, both sets of markers did not show a correlation with the geographic region, according to the Mantel test. However, the grouping of the populations differed widely, and no correlation was found between genetic and phenotypic markers. Molecular markers showed that populations from the same mountain ranges were grouped closely together. The only exception was the population from Mt. Bistra, which grouped with the east clade, rather than the south. This could be due to the different origins of these two populations, with the population from Mt. Bistra coming from the east, rather than from Albania and Montenegro, but this could also be due to the low sample size in the present study and the high genetic diversity within this taxon. Additionally, the present genetic pattern could also be explained by the distribution of this taxon during Pleistocene. Savin juniper grows in very arid areas, where there is no competition from other trees and woody species. As pollen cannot be used in the reconstruction of savin juniper's historic distribution, a precise distribution range cannot be reconstructed. Nevertheless, based on

the biology of the species, one can assume that the areas suitable for this taxon were more abundant during the Pleistocene in the Balkans. The genetic data suggest the possibility of two refugia, although further studies are necessary to test this. The EO composition showed different groups than the molecular data, which were not geographically correlated. For example, populations from Mt. Biokovo and Mt. Bistra were grouped close to each other, as well as the populations from Mt. Velebit and Resavska gorge. The chemical composition of the leaf essential oils and the distribution of the chemotypes were not correlated with the environmental data and geological substratum, suggesting that other ecological factors might be at play, e.g., selection due to herbivory. The high genetic diversity detected in the studied populations suggests that the sexual reproduction is more prevalent than the vegetative, with the exception of the population from Resavska gorge, which had the lowest genetic diversity of the studied populations, indicating that this population might have gone through a genetic bottleneck.

5. Conclusions

A comprehensive survey of the diversity of endemic *Juniperus sabina* var. *balkanensis* from its range showed high genetic diversity, as indicated by both the molecular and phytochemical markers. The molecular markers suggest some slight separation of the populations in three regions, high genetic diversity, and quite possibly, strong genetic flow between populations. The high genetic variability also indicates that, in most populations, clonal reproduction is not common. On the other hand, the leaf essential oil showed the separation of populations based on the distribution of chemotypes, although no correlation with geographic, environmental and genetic parameters was found. However, the essential oil composition corroborated the biosynthetic link between sabinene and *trans*-sabinyl acetate. A more detailed population study using multiple genetic markers is necessary to fully describe and understand the genetic diversity within this taxon, particularly taking into consideration the loss of natural habitats due to the succession of vegetation and decrease in domestic grazing.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121062/s1, Figure S1: PCA scatter plot of leaf essential oil composition of *Juniperus sabina* var. *balkanensis*; Table S1: Chemical composition of leaf essential oil of analysed *Junuperus sabina* var. *balkanensis* from the Balkans; Table S2: Pairwise Hotelling's *p* values between EO composition of the studied populations of *Juniperus sabina* var. *balkanensis* from the Balkans; Table S3: Simple correlation between environmental factors and mean essential oil components.

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