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Portofoliu lucrări relevante

1. **Ciocîrlan, E.**, Sofletea, N., Ducci, F., Curtu, A.L. *Patterns of genetic diversity in European beech (Fagus sylvatica L.) at the eastern margins of its distribution range*. iForest-Biogeosciences and Forestry 10 (6), 916; 2017. WOS:000417296700001, IF: 1.246. https://doi.org/10.3832/ifor2446-010. http://www.sisef.it/iforest/contents/?id=ifor2446-010. https://drive.unitbv.ro/s/RmdQLxSrxEgJ7Ax

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"Strengthening conservation: a key issue for adaptation of marginal/peripheral populations of forest trees to climate change in Europe (MaP-FGR)"
Guest Editors: Fulvio Ducci, Kevin Donnelly

Patterns of genetic diversity in European beech (*Fagus sylvatica* L.) at the eastern margins of its distribution range

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Populations located at the periphery of the species' distribution range may play an important role in the context of climate change. These peripheral populations may contain specific adaptations as a result of extreme environmental conditions. The aim of this paper was to assess within population genetic diversity and among population differentiation in one of the most important forest tree species in Europe, European beech (*Fagus sylvatica*), at the eastern margins of its natural range. We analysed four peripheral, isolated populations and five core populations from the continuous natural range along the Carpathian Mountains using a set of microsatellite markers. Higher levels of genetic diversity as measured by allelic richness (7.34 *vs*. 6.50) and observed heterozygosity (0.71 *vs*. 0.59) were detected in core populations than in peripheral ones. Population differentiation was slightly higher among peripheral populations than among core, Carpathian populations. There was strong evidence of bottleneck effects in two out of the four peripheral, isolated populations. Both core, Carpathian populations and peripheral, lowlands populations share the same chloroplast haplotype suggesting a common geographical origin from the putative Moravian refuge area. Past long distance founding events with material from the Carpathian mountain chain might explain the occurrence of small, isolated beech populations towards the steppe in the south-east of Romania. Our genetic data may contribute to a better understanding of the evolutionary history of the remnants of beech scattered occurrences at the eastern margins of species' distribution range.

Keywords: ***Fagus sylvatica*, Genetic Diversity, Peripheral Populations, Bottleneck Effect**

Introduction

Populations residing at the current low-latitude and low-altitude margins of species' distribution range are particularly important in the context of climate change (Borovics & Mátyás 2013, Hampe & Petit 2005). Peripheral populations, which currently face higher risks of extinction, may play a critical role in determining species responses under climate change (Fady et al. 2016, Hampe & Petit 2005). These peripheral populations are typically smaller and isolated from the continuous distribution range of the species and, as a result, are likely to experience increased genetic drift and to receive less immigrants than core populations (Channell & Lomolino 2000). Moreover, peripheral populations at the warmer margins of the species distribution may experience higher selection pressure exerted by the warmer climate and thus harbor valuable adaptations. Lower neutral genetic variation as well as higher differentiation rates are expected in peripheral, isolated populations than in core populations from the continuous distribution range (Eckert et al. 2008). In addition to climate change, peripheral tree populations may be affected by human activities, such as browsing by cattle, deforestation, and improper forest management.

So far, there are a limited number of studies on genetic diversity of peripheral *versus* core populations of forest tree species. Significantly higher allelic and genotypic diversity in peripheral populations than in core populations was reported in Eastern white pine (Chhatre & Rajora 2014). However, similar values for heterozygosity across peripheral and core populations were observed in the same study as well as in Scots pine (Wójkiewicz et al. 2016), eastern white cedar (Pandey & Rajora 2012) and Sitka spruce (Gapare et al. 2005). Evidence of bottlenecks was reported in peripheral populations of Sitka spruce but not in Scots pine populations. Generally, the differences between peripheral and core populations as estimated using neutral genetic

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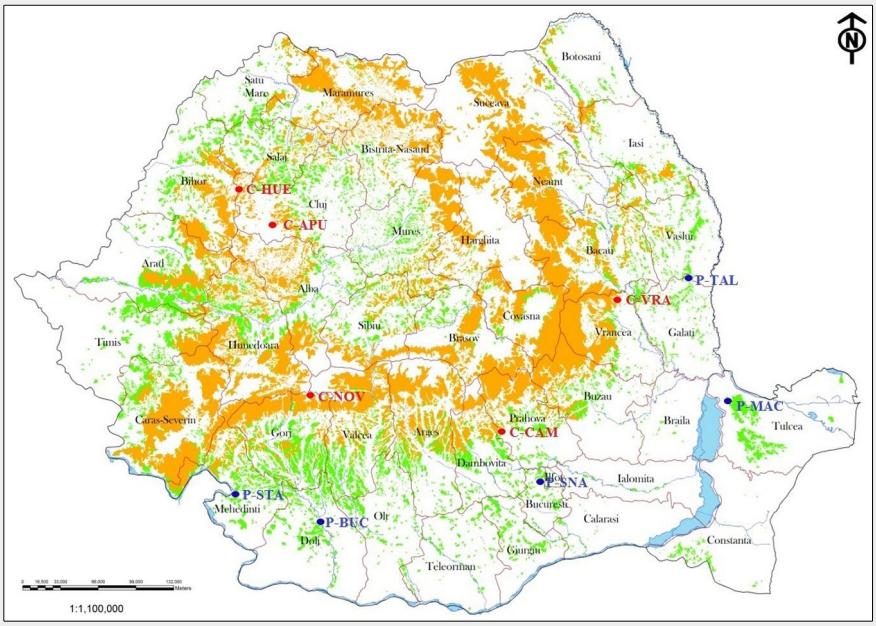

Fig. 1 - Geographic location of the sampled beech populations. (P): peripheral; (C): core. Abbreviations and geographic coordinates of the populations are given in Tab. 1. (Orange area): continuous range of European beech in the Carpathian Mountains; (green area): oak forests.

markers were statistically significant only for certain diversity indices which vary from one study to another.

In this study, we focused on common beech (*Fagus sylvatica*), one of the most important forest trees in Europe. Common beech occupies about 21 million hectares (Forest Europe/UNECE/FAO 2011) and plays a crucial role for the society, economy and environmental health. It is also a keystone species that fulfills central functions within its ecosystems and interacts with hundreds of associated plant, animal and fungal species. At present, it is the most common forest tree species in Romania, occupying approximately 33% of the forest area and representing 40% of the growing stock (Biris 2014). Unlike in western and central Europe, beech was not planted for forestry purposes in Romania and, consequently, the current populations are autochthonous (Stanescu et al. 1997). Only a few studies included samples of beech from Romania (Comps et al. 1990, Gömöry et al. 2003, Magri et al. 2006) and no investigation was done on peripheral, small, disjunct populations from the south-eastern part of the country where European beech reaches the eastern edge of its range (Stanescu et al. 1997). The most isolated peripheral beech population is located close to the Danube Delta, in the old Macin Mountains (maximum altitude: 467 m a.s.l.) formed in the second part of Paleozoic, during the Hercynian orogeny. This population grows only along a deep valley and on a north facing slope, and might be of Tertiary origin (Georgescu 1928). A Balkan origin of this particular beech population was also assumed (Diaconeasa 1977). This is in contrast with the geographical origin of existing beech populations in the nearby Carpathian Mountains, which may originate from the Moravian refugium. A secondary refugium might have been in Apuseni Mountains (western Romania), but did not contribute to the colonization of the Carpathians (Magri et al. 2006).

Understanding the evolutionary history of these peripheral beech populations, which are currently threatened by climate change and human activities, can help undertake appropriate management and conservation measures.

In this study, we assessed the genetic diversity of European beech at its eastern margins of the distribution range using a set of microsatellite markers. The specific questions of this paper are: (i) Is within population genetic diversity lower and population differentiation higher in peripheral, isolated populations than in core populations from the continuous Carpathian range? (ii) Is there any evidence based on chloroplast DNA variation of a different geographical origin of existing peripheral and core populations? (iii) Do peripheral, isolated populations show a significant bottleneck signature?

Material and methods

Study site and sampling

Our analysis included nine populations of European beech sampled throughout the natural range of the species in Romania (Fig. 1). The sampled populations were grouped into two categories (Tab. 1): four peripheral, isolated populations (P-MAC, P-SNA, P-STA and P-TAL) and five core populations (C-HUE, C-APU, C-NOV, C-CAM and C-VRA). The core populations are located on both sides of the South-Eastern Carpathian Mountains in the continuous natural range of beech. We also sampled the last four remaining living individuals from a small, isolated beech population (Bucovat, P-BUC) located in the lowlands of southern Romania (Fig. 1). Two peripheral populations (P-SNA and P-TAL) were sampled exhaustively. Material was collected from 50 individual trees in the remaining populations. The sampled individuals were at least 30 m apart in the populations that were not exhaustively sampled. A total of 462 individual trees were sampled in 2012. Only three randomly chosen beech individuals per population, except for population P-MAC with five individuals, were analyzed at

Tab. 1 - Geographic location, sample size and climate conditions of the sampled beech populations. Sampling of all remaining trees was done in two populations (P-SNA and P-TAL). (P): peripheral; (C): core.

Label	Population	No. of samples	Latitude N	Longitude E	Altitude (m)	Annual average temperature (°C)	Annual rainfall (mm)
P-MAC	Macin	100	45°16′	28°10′	125	10.5	479
P-SNA	Snagov	37	44°43′	26°09′	92	10.6	585
P-STA	Stârmina	50	44°29′	22°45′	77	10.4	591
P-TAL	Talasmani	21	46°07′	27°50′	230	9.4	538
C-HUE	Huedin	50	46°58′	22°43′	446	8.7	672
C-APU	Apuseni	50	46°40′	23°01′	1130	5.9	821
C-NOV	Novaci	50	45°13′	23°40′	560	8.3	702
C-CAM	Câmpina	50	45°06′	25°43′	520	8.3	673
C-VRA	Vrancea	50	46°05′	27°01′	315	9.6	559

chloroplast DNA level. The material consisting of buds or leaves was stored at -60 °C before further analyses.

Genotyping

DNA was extracted from buds or leaves using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1990). DNA concentration and purity were determined spectrophotometrically with a Nanodrop 8000. Seven gSSRs originally developed for *F. sylvatica* (*FS3-04*, *FS4-46*, *Mfs11* – Pastorelli et al. 2003, Vornam et al. 2004) and *F. crenata* (*Sfc0018*, *Sfc0161*, *Sfc1063* and *Sfc1143* – Asuka et al. 2004) and one EST-SSRs (*Fir065*) originally developed for *Quercus* spp. (Durand et al. 2010) were used. *FS4-46* was excluded from further analysis because of some ambiguities in its interpretation and due to the presence of a large number of null alleles. Polymerase chain reaction (PCR) was carried out in Corbett and Eppendorf Thermal Cyclers. The amplification was performed in 10 µL of reaction mixture consisting of: 5× PCR Buffer (Promega), 0.2 mM each of dNTP, 2.5 mM MgCl2, 0.4-0.5 µM each of primers and 1.0 U of Taq DNA polymerase (Promega). The PCR profile was as follows: 15 minutes of initial denaturation at 95 °C followed by 30 cycles of 1 min denaturation at 94 °C, a 30 s annealing step at 47 °C (for multiplex 2 – *Mfs11*, *Fir065*, *FS4-46* and *Sfc1143*) or 55 °C (for multiplex 1 – *Sfc0018*, *Sfc0161*, *Sfc1063* and *FS3-04*), a 1 min elongation step at 72 °C and a 20 min final extension step at 72 °C.

Three polymorphic chloroplast microsatellites (*ccmp-4*, *ccmp-7* and *ccmp-10*) were also amplified. The PCR reactions and performed in a 15 µL volume containing 5× PCR Buffer (Promega), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 0.25 units of Promega Taq DNA polymerase. The PCR protocol consisted of one cycle of initial denaturation at 94 °C for 15 min, followed by 35 cycles (*ccmp-7* and *ccmp-10*) or 30 cycles (*ccmp-4*) of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. A final extension cycle at 72 °C for 10 min followed. Amplified PCR products were diluted and were than run on a GemoneLab GeXP Genetic Analyser® using Frag-3 method and Size Standard 400. The products were further analyzed using Fragment Analysis Software using default parameters and PA ver. 1.0 dye correction.

Microsatellite markers were tested for genotyping errors due to large allele dropout, scoring of stutter peaks and non-amplified alleles using Micro-Checker® ver. 2.2.0.3 (Van Oosterhout et al. 2004). The software indicated the presence of null alleles at very low frequencies (less than 2%) for three markers (*Sfc0018*, *Sfc0161* and *Fir065*) in only two populations (C-APU and C-NOV). No evidence of large allele dropout or scoring of stutter peaks was found in the populations.

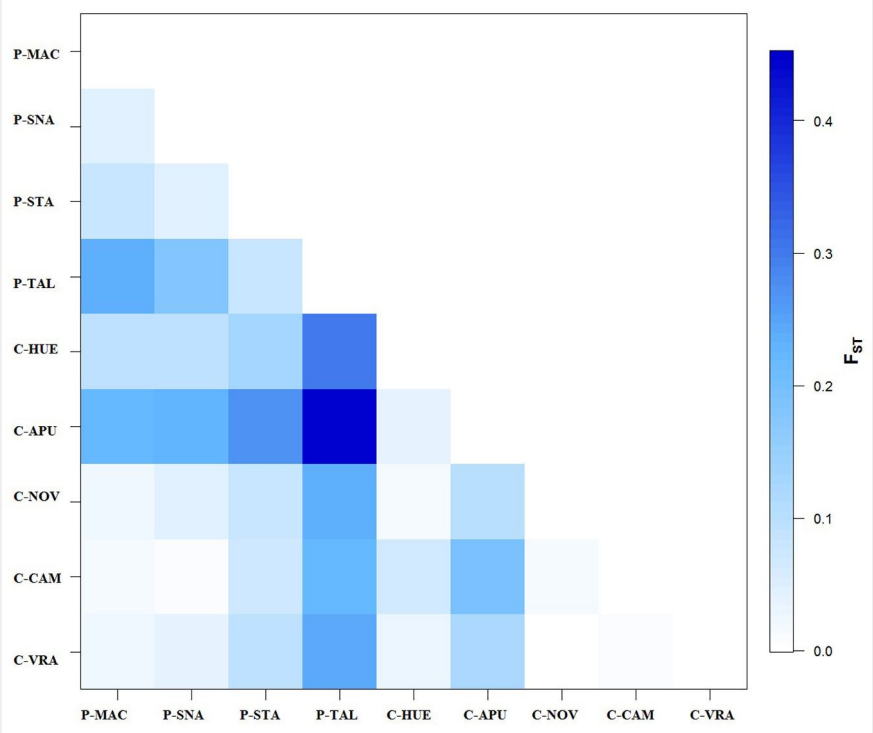

Fig. 2 - Matrix of pairwise genetic differentiation (F_{ST}) among beech populations.

Genetic diversity and differentiation

The software GenAlEx® ver. 6.5 (Peakall & Smouse 2006) was used to estimate allele frequencies and standard genetic diversity indices: average number of alleles per locus (N_a), effective number of allele (N_e), observed heterozigosity (H_o), expected heterozigosity (H_e) and fixation index (F). Allelic richness (A_R), a measure that is independent of sample size, was estimated with FSTAT ver. 2.9.3 (Goudet 1995). Student's *t*-test was used to examine differences between mean values of genetic diversity measures. Analysis of Molecular Variance (AMOVA) was performed using the software Arlequin® ver. 3.5.2.2 (Excoffier et al. 2005). A matrix of pairwise genetic differentiation measures between all populations pairs was computed. For genetic differentiation among beech populations, pairwise F_{ST}'s were computed using the same software. The significance of the F_{ST} statistics was tested by 10,000 permutations. The graphical representations of all pairwise F_{ST} were done using the Rfunction of the package "pairFstMatrix.r" (Schneider et al. 2000) implemented in Arlequin *via* Rcmd (Fig. 2). An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was computed with 100 bootstrap replications, based on Nei's standard genetic distance (Nei 1987) using the software Populations ver. 1.2.31 (Langella 2000) and TreeView ver. 1.6.6 (Page 2001).

Genetic assignment

The Bayesian clustering method implemented in the software STRUCTURE ver. 2.3.3 (Pritchard et al. 2000) was used to genetically assign individuals to clusters. Simulations were run for 100,000 steps following a burn-in period of 50,000 steps, considering values of *K* (number of clusters) from one to 10, with 3 replications for each value of *K*. The analysis was performed using admixture, correlated allele frequencies and no prior information on sampling location. The number of population clusters was estimated using Δ*K* parameter according to Evanno et al. (2005) using the STRUCTURE HARVESTER program (Earl & Von Holdt 2012).

Bottleneck analysis

The software BOTTLENECK (Cornuet & Luikart 1996) was used to test for recent change in population size. We tested all beech populations for a bottleneck signature under the stepwise mutation model (SMM), infinite alleles model (IAM) and two phase model (TPM). We tested the significance levels using 1000 simulation iterations and both Wilcoxon's signed rank test and standardized differences test.

Results

Within population genetic diversity

Eight to 22 alleles were observed per locus, with a total of 98 alleles across all populations and loci. Private alleles (6) were found in a single peripheral population (P-STA). Five private alleles were detected in the core populations, as follows: three in C-APU, one allele in C-HUE and one in C-VRA. Most of the private alleles except for one (*Fir65-183bp*) in population P-STA were rare ($p<0.05$). The values for the basic statistics of genetic diversity are listed in Tab. 2. Only two parameters showed significant differ-

Tab. 2 - Basic genetic statistics averaged across seven microsatellite loci for each population and group (peripheral and core). (N): sample size; (N_a): mean number of alleles per locus; (N_e): number of effective alleles; (A_R): allelic richness; (H_o): observed heterozygosity; (H_e): gene diversity; (F): heterozygote deficit; (SE): standard error.

Population		N	N_a	N_e	A_R	H_o	H_e	F
P-MAC		85.7	9.14	4.61	6.697	0.686	0.718	0.041
P-SNA		34.1	7.57	4.56	6.603	0.641	0.728	0.116
P-STA		46.4	9.57	4.34	7.438	0.584	0.693	0.139
P-TAL		19.1	5.57	2.58	5.273	0.433	0.491	0.116
C-HUE		48.9	10.29	3.87	7.328	0.583	0.682	0.127
C-APU		47.4	9.86	3.87	7.164	0.644	0.686	0.073
C-NOV		49.0	9.57	5.23	7.788	0.734	0.752	0.029
C-CAM		47.7	9.71	4.79	7.481	0.729	0.751	0.023
C-VRA		49.1	9.14	4.87	7.076	0.771	0.728	-0.047
Peripheral populations	Mean	46.3	7.96	4.02	6.503	0.586	0.658	0.103
	SE	0.6	1.15	0.81	0.107	0.071	0.073	0.055
Core populations	Mean	48.4	9.71	4.53	7.367	0.712	0.720	0.041
	SE	0.4	1.35	0.78	0.350	0.064	0.058	0.058
Overall	Mean	47.5	8.94	4.30	6.983	0.645	0.692	0.069
	SE	2.1	0.44	0.27	0.278	0.025	0.023	0.018

Tab. 3 - Analysis of molecular variance (AMOVA) at seven nuclear microsatellite loci. (a): All nine populations; (b): peripheral populations; (c): core populations; (df): degrees of freedom.

Test	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Prob.
(a)	Among populations	8	31.432	0.03628	9.77	<0.001
	Within populations	899	301.231	0.33507	90.23	<0.001
(b)	Among populations	3	42.349	0.13785	5.36	<0.001
	Within populations	406	893.919	2.43592	94.64	<0.001
(c)	Among populations	4	51.026	0.10389	4.13	<0.001
	Within populations	493	1187.683	2.40909	95.87	<0.001

ences ($p< 0.05$) between peripheral populations and core populations. Thus, the value of allelic richness (A_R), a genetic parameter without population size bias, and observed heterozygosity (H_o) was higher for core populations than for peripheral ones. Gene diversity and fixation index showed similar values (Tab. 2).

Genetic differentiation among populations

The analysis of molecular variance (AMOVA) showed that the majority of the variance is within populations (Tab. 3). The differentiation among beech populations was moderate (F_{ST}=0.0978). AMOVA also indicated a slightly higher population differentiation among peripheral populations than among core populations (Tab. 3). The matrix of pairwise F_{ST} values (Fig. 2) revealed that differentiation between two populations of the same category (*e.g.*, peripheral) was usually lower than differentiation between populations of different categories. The vast majority of the population pairs (more than 89%) were significantly differentiated from each other ($p<0.001$). The strongest differentiation was found between a core (C-APU) and a peripheral (P-TAL) population (F_{ST}=0.4526). No genetic differentiation was observed between pairs of neighboring peripheral (P-SNA and P-MAC) and core populations (C-VRA and C-MAC).

Population genetic structure

The dendrogram constructed using Nei's genetic distances between pairs of populations revealed two main groups, one for peripheral and one for core populations, respectively. However, there was a small reliability of nodes (22) based on bootstrap resampling. The most south-eastern peripheral populations (P-MAC and P-SNA) and north-western core populations appear to be very similar (Fig. 3).

The most probable number of genetic clusters identified by the Bayesian analysis, using the *ad hoc* statistic ΔK, was four (additional data is given in Fig. S1 and Fig. S2 – Supplementary material). However, for K = 2, one cluster corresponds to peripheral populations (green color in Fig. 4) and one to core populations (red color). When having a third cluster (K = 3), peripheral populations remain together in one cluster while core populations are divided in two clusters in accordance with their geographic location: within Carpathian region, on the inner side of the South-Eastern Carpathian Mountains (blue color) and outside Carpathian ridge (green color). For K = 4, the initial group of peripheral populations is splitted into two clusters: the first cluster corresponds to populations P-MAC and P-SNA, which is in good agreement with the UPGMA dendrogram, and the second one to the rest of peripheral populations (Fig. 4).

Chloroplast DNA analysis

The three chloroplast microsatellite makers were monomorphic in all populations. Allele 118bp, 146bp, and 109bp were observed at *ccmp-4, ccmp-7*, and *ccmp-10*, respectively. The same haplotype was observed at the chloroplast level across peripheral and core beech populations, revealing the same geographical origin.

Test for bottleneck signature

We observed evidence for recent bottle-

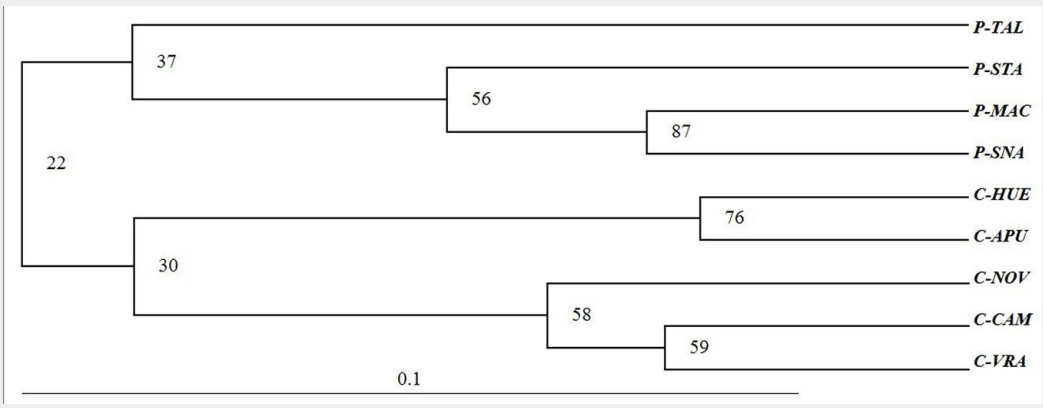

Fig. 3 - UPGMA based on Nei (1987) genetic distance between beech populations.

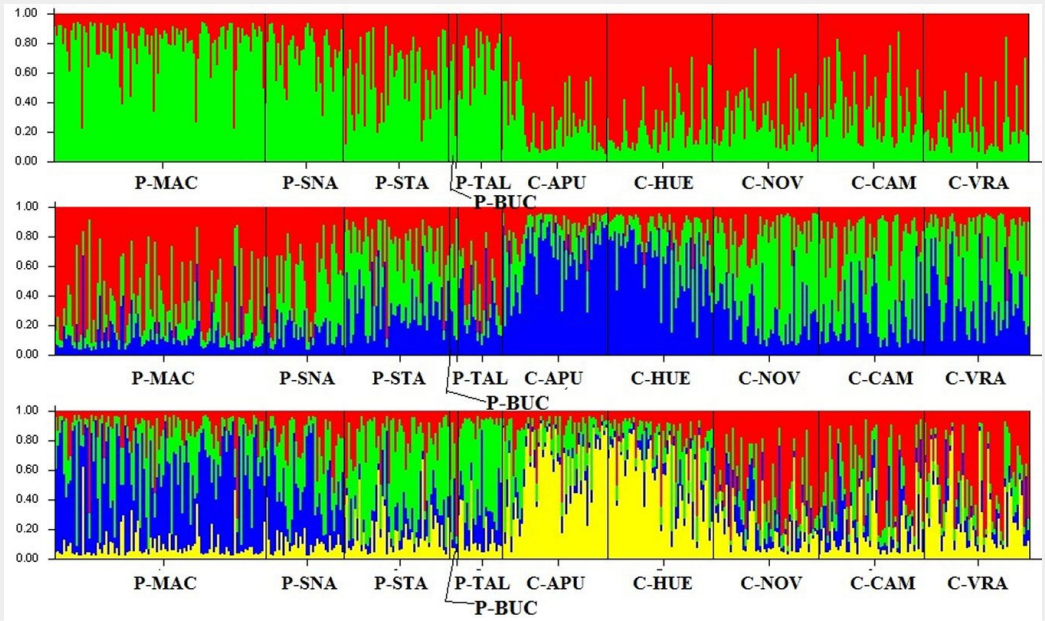

Fig. 4 - Genetic structure revealed by seven microsatellite markers. Each individual tree is represented by a thin vertical line which is divided into color segments that are proportional to its membership in the genetic clusters (k=2, top panel; k=3, middle panel; k=4, lower panel) inferred in the Bayesian analysis. Membership values are averaged across three runs. Populations are separated by a thin black line. (P): peripheral; (C): core.

necks or reductions in effective population size in two out of nine beech populations (Tab. 4). The two populations were both classified as peripheral: P-MAC under all three models (IAM, SMM and TPM) and P-SNA under the SMM model.

Tab. 4 - Test for recent bottlenecks in beech populations under stepwise mutation model (SMM), infinite alleles model (IAM) and two phase model (TPM). Significance was tested according to the Wilcoxon sign-rank test. Significant *p*-values are indicated with an asterisk (*).

Population	SMM	IAM	TPM
P-MAC	0.0016*	0.0078*	0.0016*
P-SNA	0.0251*	0.0546	0.1093
P-STA	0.1078	0.9375	0.1093
P-TAL	0.0756	0.3750	0.0546
C-HUE	0.0718	0.6875	0.0656
C-APU	0.0738	0.1875	0.0790
C-NOV	0.1093	0.1290	0.5781
C-CAM	0.0656	0.1056	0.6875
C-VRA	0.1093	0.2156	0.5781

Discussion

Our set of seven nuclear microsatellite markers indicates a lower level of genetic diversity as measured by allelic richness, which was corrected for variation in sample size, and observed heterozygosity in peripheral, isolated beech populations than in core populations from the continuous Carpathian range. Expected heterozygosity and inbreeding coefficient were similar (no significant differences) between populations classified as peripheral or core based on the beech distribution range in Romania. Similarity in expected heterozygosity but marked differences in observed heterozygosity and inbreeding coefficients between peripheral and core populations were reported in an American conifer species, Sitka spruce, using sequence tagged site loci (Gapare et al. 2005). Very recently, similar levels of genetic diversity between core and peripheral populations were also reported in Scots pine using nuclear microsatellite loci (Wójkiewicz et al. 2016). In terms of private alleles there was nearly a balance between the peripheral and core populations (6 *vs.* 5). However, private alleles were found in only one peripheral population (P-STA) which is located in the south-western part of the country in the proximity of the Balkan Mountains. Surprisingly, no private alleles were found in the other peripheral, isolated populations located much farther from the continuous natural distribution range than the population P-STA. Most of the private alleles detected in core populations are from the two beech populations located in Western Carpathians (Apuseni Mountains) on the other side of the Carpathian arc as the majority of the studied populations. The presence of private alleles in Apuseni Mountains might be explained by the existence of a refuge area for beech in that region (Magri et al. 2006). A slightly larger population differentiation was observed among peripheral populations than among core, Carpathian populations, although the latter ones are spread on a larger area and on both sides of the Carpathian mountain chain. This is consistent with the pattern observed in Sitka spruce and may be the result of more substantial gene flow among core populations than among peripheral ones or shared ancestral polymorphism (Gapare et al. 2005). The clustering of populations and the outcomes of the Bayesian analysis partially reflect the geographic relationships between populations. Thus, the two populations from the Western Carpathians are grouped together as well as the three core populations from the other side of the Carpathian Mountains. The cluster with two peripheral populations, P-MAC and P-SNA, has strong bootstrap support (87%). These two isolated populations might have been the remnants of a migration wave coming from the Carpathian Mountains towards the Danube valley when the climate conditions were more favorable to beech. Mountain ranges were colonized first and valleys (*e.g.*, Danube valley) were colonized rather late in the Holocene (Magri et al. 2006).

All sampled beech populations share the same chloroplast haplotype. A single haplotype was reported based on the same set of chloroplast microsatellite markers for the Carpathian region in a study on the entire natural distribution range of European beech (Magri et al. 2006). However, no sampling was done at that time in peripheral, isolated lowlands populations from south-eastern Romania. The hypothesis that one of the isolated, peripheral populations (P-MAC) located in the Macin Mountains might originate from a distinct (Balkan) glacial refugium (Diaconeasa 1977), and not from the same putative refuge area as of the Carpathian populations (Moravian area – Magri et al. 2006) is not supported by our data. Numerous other haplotypes were detected based on the same set of three chloroplast microsatellites in the Balkan Mountains and in Greece, but none of these haplotypes were spread northwards (Hatziskakis et al. 2009, Magri

et al. 2006, Papageorgiou et al. 2008) or observed in our sample. The fact that beech individuals from P-MAC share the same chloroplast haplotype with the Carpathian core populations indicates rather a common geographical origin (Moravian area) of the analyzed populations.

The occurrence of *Fagus orientalis* and *F.* x *taurica* individuals, hybrids between *F. sylvatica* and *F. orientalis*, were documented in the peripheral Macin (P-MAC) population (Dumitriu-Tataranu & Ocskay 1952, Oprea et al. 2011). The larger proportion of oriental-like beech individuals suggested a different evolutionary history of this remote beech population. However, first observations made on the site indicated the presence of *F. sylvatica*-like individuals. Moreover, typical ground flora species for beech stands in the nearby Carpathian Mountains were also identified in the beech stand from Macin (Georgescu 1928). Recent statistical analyses of leaf morphology of individual trees sampled in Macin population suggested that most of the individuals are *F. sylvatica*-like and only a few show characters of *F. orientalis* (Ciocîrlan 2014).

A long distance founding event may explain the origin of the beech population (P-MAC) in Macin Mountains. This event implies the existence of bottleneck signatures which was actually found under either IAM or SMM model in population P-MAC. Moreover, evidence of a bottleneck was observed in a second peripheral population of very small size (P-SNA). The rest of the peripheral populations may also have experienced bottlenecks, but given the limited sample size of individual trees and loci used in our study, microsatellite-based bottleneck tests often have a limited power to detect recent declines of populations (Peery et al. 2012). An advance of beech front from the Carpathian Mountains towards the south-eastern lowlands (steppe) of Romania along river valleys or during periods of a more humid climate and long distance dissemination events is the most plausible hypothesis for the origin of the current peripheral populations. Actually, what we see at present is only a small portion of the isolated, peripheral beech stands that existed before in the region (Enculescu 1923, Floricica 1973, Georgescu 1928). A forest site with typical ground flora for beech stands but no beech individuals were identified in the Macin Mountains at the beginning of the 20th century. The lack of beech individuals on a typical site might be explained by wood extractions made by the local population (Georgescu 1928). Evidence of remote populations, located several hundred kilometers away from the main species distribution range and which may originate as a result of long distance dissemination events are also found in species of related genera such as in *Quercus pubescens* at the northern edge of the distribution range (Chybicki et al. 2012).

In conclusion, our data suggest that the existing peripheral beech populations located at the eastern edge of the species distribution range are remnants of a wider array of small beech populations having the same geographical origin as those from the Carpathian Mountain chain. These peripheral populations are less variable than the core populations from the continuous distribution range in terms of allelic richness and observed heterozygosity. Moreover, the population differentiation among peripheral populations is higher than among core populations. This may be mainly explained by bottleneck effects in the past, of which we found evidence in two peripheral populations, and restricted gene flow with the putative origin populations from the Carpathian Mountains. The survival of these peripheral populations under extreme ecological conditions (increased temperatures, prolonged drought) makes them particularly important for research and conservation purposes.

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Supplementary Material

Fig. S1 - Estimation of number of clusters (*K*) from the rate of change in the log probability of data L(*K*) between successive *K* values, as measured by Δ*K*.

Fig. S2 - Number of clusters (*K*) derived from the Bayesian analysis implemented in STRUCTURE software.

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Genetic diversity and structure of Silver fir (*Abies alba* Mill.) at the south-eastern limit of its distribution range

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Abstract. In the Romanian Carpathians, Silver fir covers about 5% of the forest area and is the second most important conifer species. Although there are a number of genetic studies concerning the distribution of genetic diversity of *Abies alba* in Europe, populations from the south-eastern limit of the distribution range have been studied less. The aim of the present study was to assess the genetic diversity and differentiation in 36 silver fir populations along the Carpathian Mountains in Romania, using seven microsatellites loci. High levels of genetic diversity (H_e = 0.779 to 0.834 and A_R = 11.61 to 14.93) were found in all populations. Eastern Carpathians populations show higher levels of diversity, both in allelic richness and expected heterozygosity and higher degrees of genetic differentiation compared to southern populations. Bayesian clustering analysis revealed the existence of two genetically distinct groups for silver fir populations, one larger cluster which comprises the Inner Eastern Carpathians, Curvature Carpathians, South Carpathians and the Banat Mountains and the second cluster contained most of the North and Outer Eastern Carpathians population. Both AMOVA and Barrier analysis supported genetic differentiation among geographical provenance regions. The high genetic diversity of silver fir populations from the eastern limit of its distribution provide high potential to mitigate the negative effects of climate warming being valuable genetic resources in the context of global change. The distribution pattern of genetic variation at local, regional and country scale could and should be considered for the preservation of the forest genetic resources.

Keywords: *Abies alba*, nuclear microsatellites markers, genetic diversity, differentiation, genetic structure

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Introduction

In the context of global warming, the populations living at edges of the natural species range will be the first facing climate change effects (Mátyás et al. 2009), there the main constrains are extreme temperatures, drought, edaphic conditions and water availability, which often require specific mechanisms of adaptation, resulting in natural selection of various specific quantitative traits (Howe et al. 2003). The adaptation of populations to stress conditions depends not only on selection, but also on other evolutionary factors such as migration and genetic drift (Savolainen 1996). As the evolutionary potential of species depends on spatial genetic structure and on the level of genetic diversity among and within populations, it is of the utmost importance to know the extent of genetic diversity in natural populations and the environmental determinism in assessing the species' response to expected climate change (Marchi et al. 2016; Ortego et al. 2012).

Evaluating the role of geography and environment in shaping the current genetic structure can largely contribute to disentangling the effects of local adaptive processes and spatial isolation (Wang 2013), while the assessment of the genetic structure and the identification of populations with adaptive value are a significant challenge in preserving species genetic resources (Eckert et al. 2008). In contrast to the expanding edge, the low-latitude limit (rear edge) of the species' ranges remains understudied, despite the critical importance of rear edge populations as long-term storage of species genetic diversity and foci of speciation, which has been little acknowledged (Hampe & Petit, 2005).

The distribution of genetic variability for marginal populations was described for some species such as Scots pine (Savolainen 1996) and European beech (Ciocîrlan et al. 2017), while for Silver fir, recent studies indicate its higher adaptive capacity to mitigate climate change effects, in comparison to other coniferous species (e.g. Norway spruce, Scots pine) (Tinner et al. 2013, Rousch et al. 2016), but also the possibility of a severe growth decline in warm and dry regions of Europe (Bosela et al. 2018). At the Eastern edge of the species, the most vulnerable populations are located at low altitudes, with a pronounced precipitation deficit, such as those from the edge of the Eastern (Romanian) Carpathians and those from Banat Mountains (Mihai et al. 2018).

Genetic and palynological studies have identified three glacial refuges for Silver fir in Europe: Southern Italy, the North-West of Greece and the Pyrenees Mountains (Konnert & Bergmann, 1995; Terhürne-Berson et al. 2004; Cheddadi et al. 2014). It is assumed that the Silver fir of Eastern Europe mostly comes from the glacial refuges located in the South of the Balkan Peninsula (Konnert & Bergmann, 1995). The postglacial migration routes assessed by palynology concluded that Silver fir reached the Romanian range through the south of the Carpathians, occupying firstly the Western Carpathians (Apuseni and Banat Mountains), from where it subsequently migrated to the Eastern Carpathians (Diaconeasa & Fărcaş 2001, Feurdean & Willis 2008). Silver fir reached the Eastern Carpathians about 2500 years later than the south-west of Romania (Fărcaş et al. 2013, Tanţău et al. 2003). The hypotheses regarding the existence of glacial refuges in the Southern Carpathians (Retezat Mountain) (Magyari et al. 2012) are not supported (Postolache et al. 2016), but the existence of small/micro-refuges contributing to a rapid expansion of the species during the Holocene cannot be entirely dismissed (Vitasse et al. 2019), given the fact that a new glacial refuge was identified in the North of the Apennines and Euganean Hills in Northern Italy (Samartin et al 2016, Gubler et al. 2018).

Even though previous studies have analysed the capacity of Silver fir populations to adapt to climate change, Silver fir populations from the south-eastern edge of its distribution (e.g

the Romanian Carpathians) still remain understudied. This location, at the convergence of three biogeographic regions (Southern, Central and Eastern Europe), together with its very diverse ecological and climatic features, make it one of the centres of highest diversity in Europe (Feurdean & Tanțău 2017). In the Romanian Carpathians, Silver fir grows in various climatic conditions (e.g. sub-Mediterranean climate in south-west and continental climate in north) and lithological substrates. In the Eastern Carpathians, the natural distribution of silver fir overlaps with Paleocene and Cretaceous formations (marl, sandstone, clay), whereas in the Western Carpathians the metamorphic and eruptive structure of rocks (crystalline shale, granite, etc.) entail the scarcity of the Romanian silver fir (Lucău-Dănilă 1991). Pollen analysis indicates an obvious anthropogenic influence, suggesting that the substantial reduction of the area occupied by silver fir, at least in the north-eastern area, is exclusively due to clear-cutting on large surfaces in the past (Tanţău et al. 2011). The human impact on the distribution, structure and functionality of the forest has become even more noticeable in the last 300 years, the proportion of silver fir decreased by approximately 15% during this period (Barbu et al. 2015).

The aim of the paper is to assess the level of genetic diversity and genetic structure in the Silver fir populations at its Eastern distribution limit (the Romanian Carpathians), including the relative contribution of history, environmental factors and geography on current genetic variation, as a prerequisite to establishing sustainable measures for the conservation of species biodiversity. More specifically, we will try to answer the following questions. (i) Is the genetic structure of Silver fir populations homogenous, in accordance with the postglacial migration history? (ii) To what extent did anthropogenic and environmental factors influence the diversity and the current genetic structure of Silver fir? (iii) Do the Silver fir populations of the Eastern distribution limit have the capacity to persist under the expected climate changes?

Materials and methods

Sampling and genotyping

In the Romanian Carpathians, Silver fir covers about 5% of the forest area and is the second most important conifer species (Barbu & Barbu, 2005). Almost two-thirds of the distribution area are located in the Eastern Carpathians, at altitudes spanning from 400 m to 1200 m a.s.l where it forms pure or mixed stands with European beech and Norway spruce. In the rest of the Romanian Carpathians, Silver fir has a scattered distribution and occurs in mixed Fagus-Picea forests (Șofletea & Curtu, 2001). In order to capture the whole ecotype variability of Silver fir in the Romanian range–i.e. thermophilic populations of Banat Mountains (Southwestern Carpathians), the populations of continental climate in Bucovina (Northern Carpathians) or those adapted to a cold climate and rainfall deficitin the Moldavian hills (Eastern Carpathians) (Șofletea & Curtu, 2001) - we have sampled 36 Silver fir populations across the Romanian Carpathians during 2014-2016. All the sampled populations were naturally regenerated, putatively autochthonous, most being designated as Forest Genetic Resources (RGF) (Pârnuţă et al. 2012) or selected seed sources (Table 1). An extensive genetic analysis was conducted on populations from the Eastern distribution range (Figure 1), mainly because this region represents the Eastern rear edge of the species distribution in Europe and, according to future projections, will be the most vulnerable in the context of climate change.

Needle or bark disks with cambium from 35 to 40 individuals, located at a distance of at least 30 m, were collected for DNA extraction. To minimize the effect of the age factor as far as possible, sampled trees were approximately

Table 1 Description of the 36 investigated Silver fir (*Abies alba* Mill.) populations

No	Code	Population	Geographic coordinates		Altitude (m a.s.l)	Bedrock	Provenance region*
			Latitude	Longitude			
1	POI	Poieni	47°52’	24°36’	950	Crystalline shale	A1
2	STB	Strâmbu Băiuţ	47°38’	24°00’	800	Igneous	A1
3	LIB	Liban	46°28’	25°22’	720	Igneous	A1
4	TOP	Topliţa	47°00’	25°16’	1100	Igneous	A3
5	FRU	Frumoasa	46°21’	25°41’	920	Marl	A3
6	TUS	Tuşnad	46°09’	25°51’	700	Igneous	A3
7	DEM	Demăcuşa	47°41’	25°25’	1100	Flysch	A2
8	STU	Stulpicani	47°25’	25°39’	900	Phyllite	A2
9	BRA	Brateş	46°50’	26°10’	850	Flysch	A2
10	MOI	Moineşti	46°37’	26°24’	800	Clay	A2
11	PUT	Putna	47°51’	25°38’	650	Flysch	A2
12	MAR	Marginea	47°48’	25°42’	670	Flysch	A2
13	SOL	Solca	47°45’	25°48’	500	Marl	A2
14	GHU	Gura Humorului	47°38’	25°47’	730	Flysch	A2
15	MAL	Mălini	47°24’	26°01’	550	Sandstone	A2
16	RAS	Râşca	47°20’	26°07’	500	Marl	A2
17	VAR	Văratec	47°08’	26°15’	600	Flysch	A2
18	GAR	Gârcina	46°58’	26°19’	550	Marl	A2
19	TAZ	Tazlău	46°41’	26°22’	550	Flysch	A2
20	MNC	Caşin	46°05’	26°45’	520	Clay	A2
21	CAI	Căiuţi	46°05’	26°50’	500	Clay	A2
22	SOV	Soveja	47°59’	26°38’	550	Sandstone	B2
23	VID	Vidra	46°00’	26°44’	520	Sandstone	B2
24	VIN	Vintileasca	45°37’	26°38’	1050	Flysch	B2
25	CHE	Cheia	45°27’	25°55’	920	Flysch	B2
26	AZU	Azuga	45°25’	25°32’	950	Flysch	B2
27	COV	Covasna	45°53’	26°15’	900	Flysch	B1
28	AVR	Avrig	45°39’	24°29’	750	Crystalline shale	C1
29	LAP	Lapusnic	45°18’	22°43’	1200	Sandstone	C1
30	LUP	Lupeni	45°18’	23°02’	800	Crystalline shale	C1
31	TIS	Tismana	45°08’	22°54’	720	Granite	C2
32	ANI	Anina	45°04’	21°53’	650	Calcareous	D1
33	DOB	Dobra	45°40’	22°30’	1100	Crystalline shale	D2
34	RMO	Rusca Montană	45°39’	22°22’	1000	Crystalline shale	D2
35	REM	Remeţi	46°46’	22°34’	850	Igneous	E2

Table 1 (continuation)

36	ABR	Abrud	46°20’	23°06’	950	Flysch	E3

Note. Abbreviations: A1 – Inner Eastern Carpathians; A2 – Outer Eastern Carpathians; A3 – Eastern Carpathians: Giurgeu-Ciuc Depression; B1 – Curvature Carpathians: Braşov Depression; B2 – Curvature Carpathians; C1 – Southern Carpathians: northern part; C2 – Southern Carpathians: southern part; D1 – Banat Mountains: Mehedinţi-Cerna-Semenic; D2 – Banat Mountains: Ţarcu-Poiana Ruscă; E2 – Apuseni Mountains: western part; E3 – Apuseni Mountains: eastern part

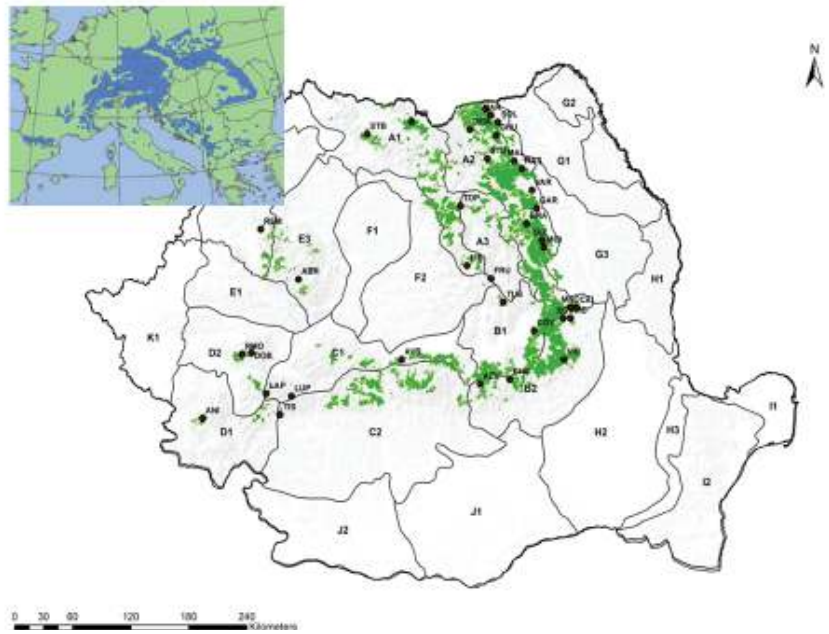

Figure 1 Current natural distribution range of *Abies alba* in Romania and geographical locations of 36 populations used in the present study. The inset shows the species range in Europe (EUFORGEN). The code of the populations and provenance region are given in the Table 1. The distribution map were generated according to the main type of the silver fir forest ecosystem in Romania.

100 years old. The material was dried in silica gel and stored at -70°C before analysis.

Genomic DNA was extracted following methods of Dumolin et al. (1995). Genotyping was based on seven neutral microsatellites markers: four developed for *Abies nordmanniana* (NFH3, NFH15, NFF7 and NFF3) (Hansen at al. 2005) and three developed for *Abies alba* (Sf1, Sfb4 and Sf78) (Cremer et al. 2006). These seven microsatellite loci were separated into two multiplex combinations. For the PCR reactions we used the Qiagen Type-it Microsatellite PCR Kit (Qiagen, Germany) which is optimized and ready-to-use master mix. The multiplex reactions were set up as 15 µl PCR mix containing 7.5 µl Qiagen multiplex PCR buffer (2x), 1.5 µl primer mix, 1µlDNA (30-50 ng genomic DNA) and 5 µl Rnase free-water. Concentrations of primers were 0.05 µmol NFF7, 0.40 µmol NFH3 and 0.40 µmol NFF3 in multiplex A; 0.10 µmol Sfb4, 0.20 µmol Sf78,0.20 µmol Sf1 and 0.20 µmol NFH15. The PCR reactions were run in Palm-Cycler (Corbbet) using the following program: 15 min. at 95°C, followed by 27 cycles for 30 sec at 94°C, 1.30 min at 57°C and 30 sec at 72°C and a final extension step for 30 min at 60°C. Obtained PCR products were analysed by a GeXP Genetic Analysis System (Beckman Coulter Inc., USA). Genotypes were scored using Genomelab software ver. 10.2.3 (Beckman Coulter). The used microsatellites have dinucleotide repeats, except Sf1 with trinucleotide repeats.

Microsatellite data were first checked regarding the presence of null alleles and genotyping errors through Micro-Checker 2.2.3

software (van Oosterhout et al. 2004). Linkage disequilibrium and significant deviations from Hardy-Weinberg equilibrium in nSSR loci were tested with Genepop (Rousset 2008) and FSTAT ver. 2.9.3 (Goudet 1995), respectively. We estimated main parameters of genetic diversity: mean number of alleles per locus: (N_a), rarefied allelic richness for 35 diploid individuals; (A_R), number of private alleles (A_P), observed heterozygosity; (H_o), expected heterozygosity; (H_e) and inbreeding coefficient; (F_{IS}) with the software FSTAT ver. 2.9.3 (Goudet 1995) and Arlequin ver. 3.5 (Excoffier & Lischer 2010). The significance level of F_{IS}'s deviation from zero was calculated based on 5000 randomization using FSTAT ver. 2.9.3 (Goudet 1995).

Genetic structure

Genetic differentiation among the 36 Silver fir populations was estimated by pairwise F_{ST} (Weir & Cockerham, 1984) using Arlequin 3.5.2 (Excoffier et al. 2005), graphically represented with the web server tool Heatmapper (Babicki et al. 2016); the F_{ST} significance was tested by 10000 permutations. Based on pairwise F_{ST} value, a neighbour-joining tree was constructed in the software SPLITSTREE 4.13.1 (Huson, 1998). Further, to determine the percentage of within and among population genetic variation, we conducted an analysis of molecular variance (AMOVA). Then, the populations were grouped as follows: (i) by the different geographical regions of the Carpathians: NEC – North-eastern Carpathians, CC – Curvature Carpathians, SC – Southern Carpathians and SWC – South-western Carpathians) and (ii) by the homogeneous groups resulted from STRUCTURE version 2.3.4 (Pritchard et al. 2009), where K = 2 and the cluster affiliation of individuals was greater than 0.6. Based on this, three levels of AMOVA were performed and the molecular variation was partitioned among groups (F_{CT}), among populations within groups (F_{SC}) and among populations (F_{ST}). The individual-based genetic structure was assessed by the Bayesian clustering method, as implemented in STRUCTURE; simulations were run 5 times for each value of K (1-10) for 100000 iterations after a burn-in period of 100000 iterations with the LOCPRIOR model and admixture ancestral model. The best number of different clusters using ΔK parameter (Evanno et al. 2005) was determined with STRUCTURE HARVESTER v. 0.6.94 (Earl & von Holdt 2012), while the graphical representation of STRUCTURE results was based on the web application POPHELPER (Francis 2016).

Landscape genetics

The isolation by distance (IBD) hypothesis was tested by performing a Mantel test between the matrix of pairwise F_{ST} and geographical distances, with 9999 random permutations, using GenAlEx 6.5 (Peakall & Smouse 2012). To avoid problems caused by population structure, because the test of IBD could be strongly influenced by it (Meirmans, 2012), we tested the IBD separately for each cluster obtained by STRUCTURE analysis.

To identify important discontinuities and barriers in the distribution of genetic diversity we used two methods of boundary detection (Blair et al. 2012). First, a Genetic Landscape Shapes analysis was conducted using the software ALLELES IN SPACE (AIS) (Miller, 2005), by constructing a Delaunay triangulation network between Silver fir populations based on geographic coordinates, and the calculation of average genetic distances, followed by an interpolation procedure to infer genetic distances to sample locations. Within the resulting three-dimensional plot, X and Y are the sample coordinates and Z (surface plot heights) correspond to the genetic distances.
The Monmonier's maximum distance algorithm, as implemented in BARRIER version 2.2 (Manni et al. 2004), was also employed to search for genetic barriers. The geographical

coordinates of each sampled Silver fir population were connected by a Delauney triangulation and the corresponding Voronoï tessellation was derived. Further, the pairwise associated Nei's genetic distances (D_A) were calculated in MSA software (Dieringer & Schlötterer 2003) and 100 bootstrap replicates of the distance matrix were used to calculate the statistical significance of the predicted barriers. We tested $N = 6$ barriers, in accordance with the maximum possible number of clusters obtained from the STRUCTURE analysis (see Figure 4). Finally, only the genetic barriers with over 80% bootstrap support were represented.

Results

Population genetic diversity

Across all the populations, the seven loci analysed were highly polymorphic, with a total number of alleles of 214. The number of alleles per locus (N_a) ranged from 9 (locus Sf1) to 58 (locus Sf78), while the observed heterozygosity (H_o) value varied from 0.452 (Sf1) to 0.905 (NFF7) (Table 2). Data analysis of each population revealed presence of null alleles for three different loci (Sf1, Sfb4 and Sf78), within nine populations, and for two loci (Sfb4 and NFH15) within two populations (data not shown). The frequency of null alleles was less than 10%, with an average of 7%, and all loci were maintained for further analysis. Significant linkage disequilibrium ($p < 0.001$) was detected for three of the marker combinations: NFH3 and Sf78 in population BRA, NFH3 and NFH15 in population SOV, and NFH3 and NFF7 in population VID.

The parameters of genetic diversity of the silver fir populations are presented in Table 3. The mean number of detected alleles per locus (N_a) ranged from 11.85 (VID) to 15.57 (AVR), with an average value of 14.02. The lowest value of rarefied allelic richness (A_R) was found in VID (11.61) and the highest of 14.93 in AVR. The observed heterozygosity (H_o) value ranged from 0.779 (AZU population) to 0.834 (REM population). Private allele analysis revealed 21 alleles, the maximum number of 2 alleles being found in the northern population POI, eastern population VID and in three populations of the south-west region (TIS, ANI and ABR). The lowest values of the inbreeding coefficient (F_{IS}) value (-0.072) were in the Inner Eastern Carpathians (TUS and COV) which indicates a significant excess of heterozygotes ($p<0.01$) (Table 3), and the highest (0.081, $p<0.001$) in south-western Carpathians (Banat Mountains, population ANI).

In general, a higher level of genetic diversity, both allelic richness and expected heterozy-

Table 2 Characterization of nSSR loci used in analysis

No	Locus	Observed fragment length (bp)	N_a	H_o	H_e	F_{IS}	F_{ST}
1	NFH3	91-191	19.69±0.26	0.894±0.008	0.883±0.005	-0.012±0.008	0.026
2	NFH15	98-140	11.69±0.23	0.810±0.012	0.801±0.008	-0.011±0.012	0.030
3	NFF3	111-157	9.47±0.21	0.814±0.010	0.800±0.005	-0.017±0.012	0.028
4	NFF7	116-176	17.19±0.28	0.905±0.008	0.898±0.003	-0.007±0.009	0.023
5	Sf78	161-291	20.72±0.36	0.890±0.011	0.907±0.003	0.020±0.011	0.028
6	Sfb4	143-199	15.36±0.33	0.845±0.011	0.853±0.004	0.010±0.013	0.027
7	Sf1	206-230	4.02±0.18	0.442±0.015	0.452±0.009	0.022±0.028	0.021

Note. Abbreviations: N_a - number of alleles per locus, H_o - observed heterozygosity, H_e - expected heterozygosity, F_{IS} - inbreeding coefficient, F_{ST} - coefficient of differentiation.

Table 3 Genetic diversity statistics on the basis of seven nuclear microsatellites loci (nSSR)

Populations	N	N_a	A_R	A_P	H_o	H_e	F_{IS}
POI	40	14.85	14.36	2	0.840	0.833	-0.008
STB	40	14.00	13.47	1	0.814	0.828	0.017
LIB	40	14.00	13.52	0	0.786	0.798	0.016
TOP	40	14.14	13.69	0	0.785	0.799	0.019
FRU	40	13.57	13.16	0	0.823	0.805	-0.021
TUS	40	14.00	13.39	0	0.846	0.789	-0.072**
TOM	40	14.42	13.86	1	0.786	0.822	0.045*
STU	40	14.28	13.73	0	0.775	0.807	0.040
PUT	40	13.85	13.41	0	0.846	0.819	-0.033
MAR	40	14.71	14.12	0	0.814	0.817	0.004
SOL	40	13.71	13.14	1	0.793	0.827	0.042
GHM	40	14.14	13.66	0	0.775	0.820	0.055*
MAL	40	13.00	12.65	0	0.836	0.813	-0.028
RAS	40	13.28	12.81	1	0.793	0.822	0.036
VAR	40	14.14	13.60	1	0.804	0.814	0.013
GAR	40	14.28	13.77	0	0.796	0.831	0.043*
BRA	40	15.28	14.69	1	0.811	0.816	0.007
TAZ	40	13.57	13.16	0	0.789	0.831	0.051*
MOI	40	13.42	12.93	0	0.800	0.815	0.020
CAS	35	13.00	13.00	0	0.788	0.789	0.003
CAI	40	13.85	13.28	0	0.814	0.800	-0.017
SOV	40	14.28	13.74	0	0.793	0.806	0.017
VID	40	11.85	11.61	2	0.781	0.797	0.021
VIN	40	14.00	13.40	0	0.772	0.813	0.051*
CHE	40	14.28	13.82	0	0.810	0.803	-0.008
AZU	40	14.00	13.44	0	0.766	0.779	0.018
COV	40	14.14	13.58	1	0.868	0.809	-0.072**
AVR	40	15.57	14.93	1	0.828	0.821	-0.009
LUP	40	14.57	13.91	1	0.821	0.809	-0.015
LAP	40	14.14	13.56	1	0.793	0.804	0.015
TIS	40	13.85	13.32	2	0.782	0.784	0.003
ANI	40	14.42	13.85	2	0.729	0.794	0.083***
DOB	40	13.28	12.88	0	0.800	0.801	0.002
RMO	40	14.28	13.61	0	0.775	0.789	0.018
ABR	40	14.57	13.87	2	0.761	0.793	0.041
REM	40	14.00	13.51	1	0.800	0.834	0.041

Table 3 (continuation)

Overall	14.02	13.51		0.800	0.810	0.012

Note. Abbreviations: N - sample size, N_a - mean number of alleles per locus, A_R- rarefied allelic richness, A_P - number of private alleles, H_o - observed heterozygosity, H_e - expected heterozygosity, F_{IS} - inbreeding coefficient. Significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

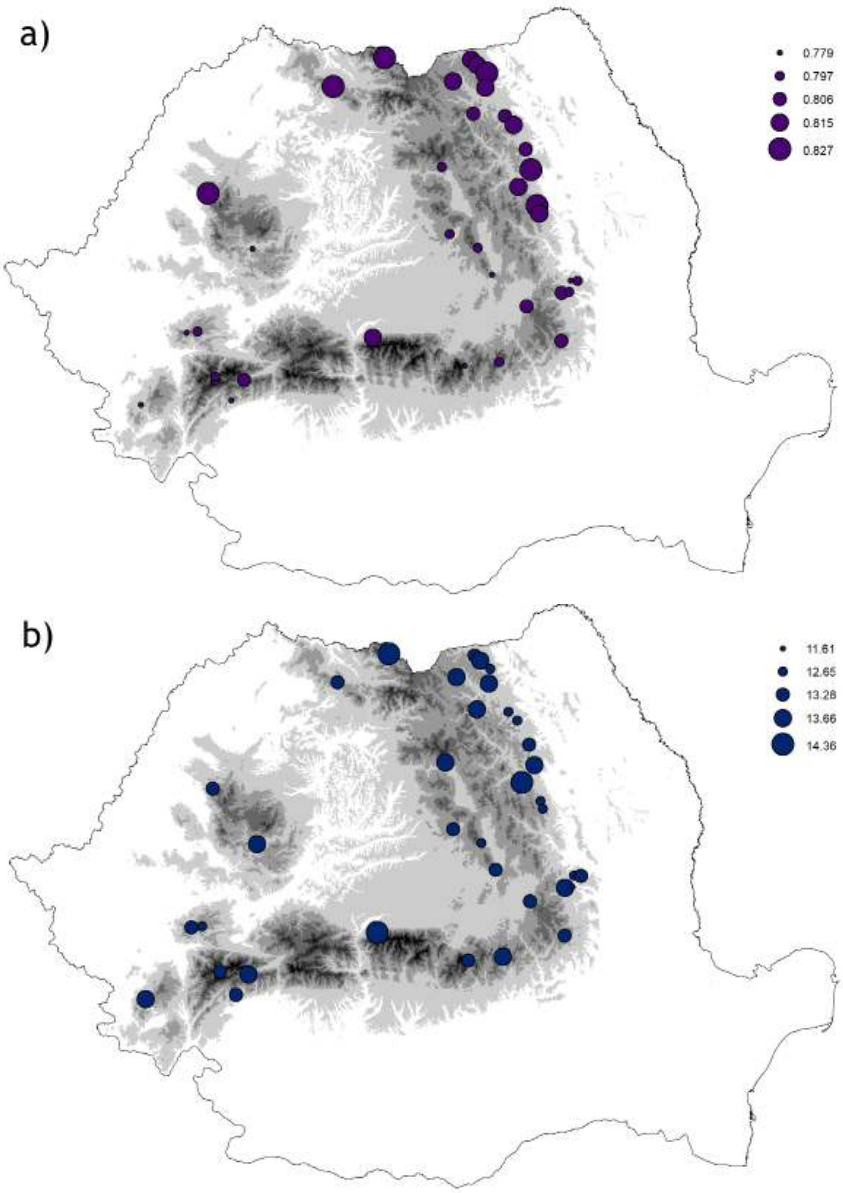

Figure 2 Spatial distribution of genetic diversity across Romanian silver fir populations. a) Expected heterozygosity (H_e); b) allelic richness (A_R)

gosity (H_e) was found in the Eastern Carpathian populations (Figure 2), with the highest expected heterozygosity (H_e) occurring in the Northern and Outer Eastern Carpathians (Figure 2a). The same pattern was revealed by allelic richness (A_R), but the differences were not as striking as in the case of expected heterozygosity (H_e) (Figure 2b).

Population differentiation

The level of genetic differentiation among the analysed populations was relatively low (F_{ST} = 0.014) (Table 4, Figure 3), though more than half (65%) of the pairwise F_{ST} ramping were significant (p<0.001), from 0.001 to 0.036. The highest differentiation was found between eastern population CAS and south population TIS. The lowest values were obtained between nearby populations such as MAL and RAS, CAS and CAI as well as between the western population RMO and four populations from the south-western region (AVR, LUP, ANI and DOB). The matrix of pairwise F_{ST} (Figure 3) shows that the differentiation between populations in the interior of the north-eastern and the

Table 4 Analysis of hierarchical molecular variance (AMOVA) for 36 silver fir populations based on nSSR (a) all populations, (b) among provenances region and c) among genetic clusters inffered by STRUCTURE

Source of variation	*df*	Sum of squares	Variance components	% variation	*F*-statistics
a)					
Among populations	35	215.447	0.04176	1.452	0.014***
Within populations	2830	8012.751	2.833.62	98.547	
b)					
Among provenances (F_{CT})	3	38.400	0.01264	0.438	0.004***
Among populations within provenances (F_{SC})	32	177.084	0.03394	1.178	0.011***
Within populations (F_{ST})	2830	8012.751	2.83362	98.383	0.016***
c)					
Among clusters (F_{CT})	1	33.445	0.02193	0.760	0.007***
Among populations within clusters(F_{SC})	31	164.145	0.03097	1.073	0.010***
Within populations (F_{ST})	2593	7338.076	2.83242	98.16	0.018***

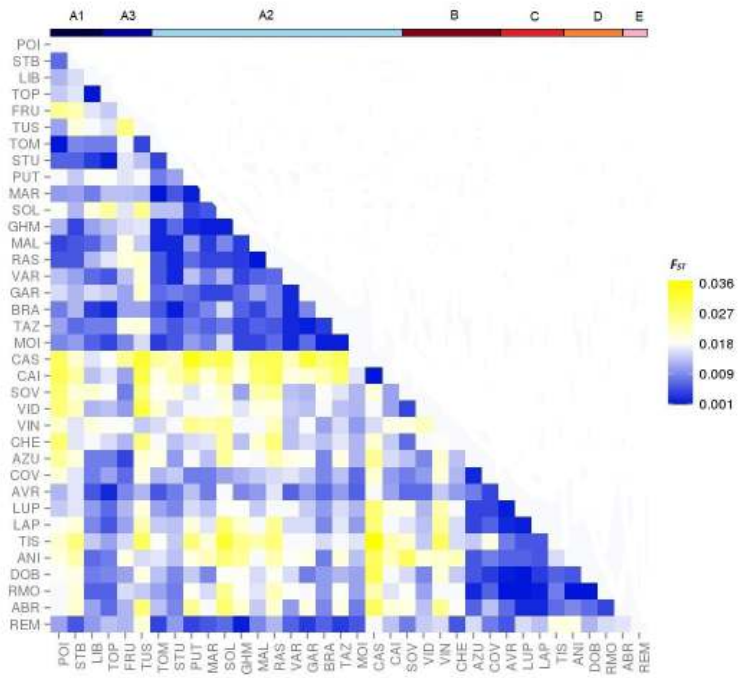

Figure 3 Pairwise genetic differentiation among silver fir populations (A1 – Inner Eastern Carpathians; A2 – Outer Eastern Carpathians; A3 – Eastern Carpathians: Giurgeu-Ciuc Depression; B – Curvature Carpathians; C – Southern Carpathians; D – Banat Mountains; E – Apuseni Mountains)

south-western groups is lower, in comparison with the central range (Southern Carpathians).

The analysis of molecular variance revealed that only a small proportion of the genetic diversity was found among populations, 98% value of genetic diversity being found within populations. The AMOVA analysis, performed after grouping silver fir populations according to the provenance regions and genetic clusters respectively, shows similar results, with only 0,43%, respectively 0,76% of the variation distributed among groups, and the same significant proportion (p<0.0001) of the genetic variation found within populations (~98%) (Table 4).

The STRUCTURE analysis identified two distinct genetic clusters (K=2) (Figure 4a). The

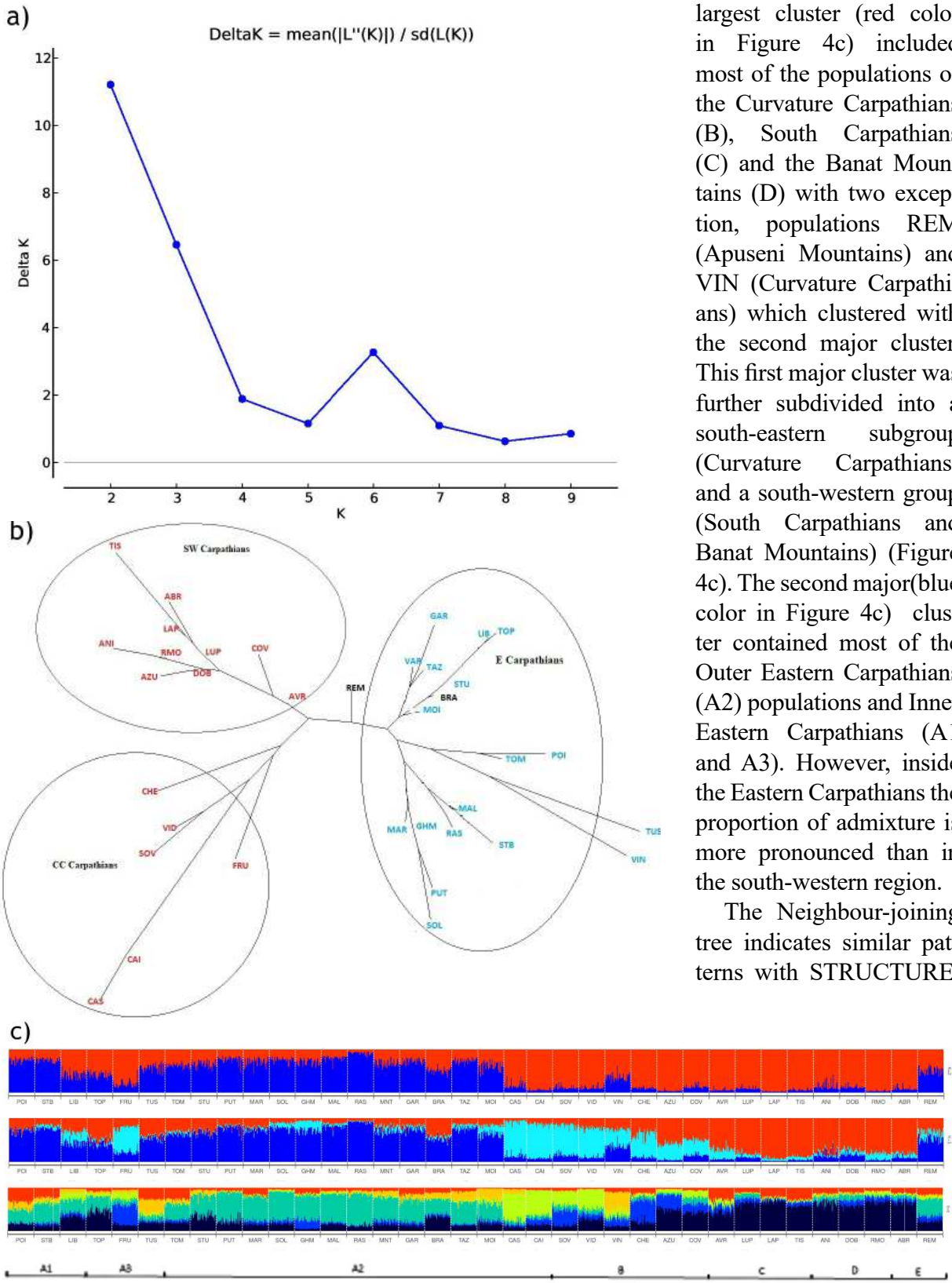

Figure 4 Population structure inferred from a model based Bayesian cluster analysis and Neighbor-joining tree a) graph of delta K with a distinct peak at K = 2 two minor peaks at K = 3 and K = 6, b) neighbor-joining phylogenetic tree, c) genetic structure of the 36 silver fir populations for different inferred K. Different inferred populations are distinguished by different colors

largest cluster (red color in Figure 4c) included most of the populations of the Curvature Carpathians (B), South Carpathians (C) and the Banat Mountains (D) with two exception, populations REM (Apuseni Mountains) and VIN (Curvature Carpathians) which clustered with the second major cluster. This first major cluster was further subdivided into a south-eastern subgroup (Curvature Carpathians) and a south-western group (South Carpathians and Banat Mountains) (Figure 4c). The second major(blue color in Figure 4c) cluster contained most of the Outer Eastern Carpathians (A2) populations and Inner Eastern Carpathians (A1 and A3). However, inside the Eastern Carpathians the proportion of admixture is more pronounced than in the south-western region.

The Neighbour-joining tree indicates similar patterns with STRUCTURE,

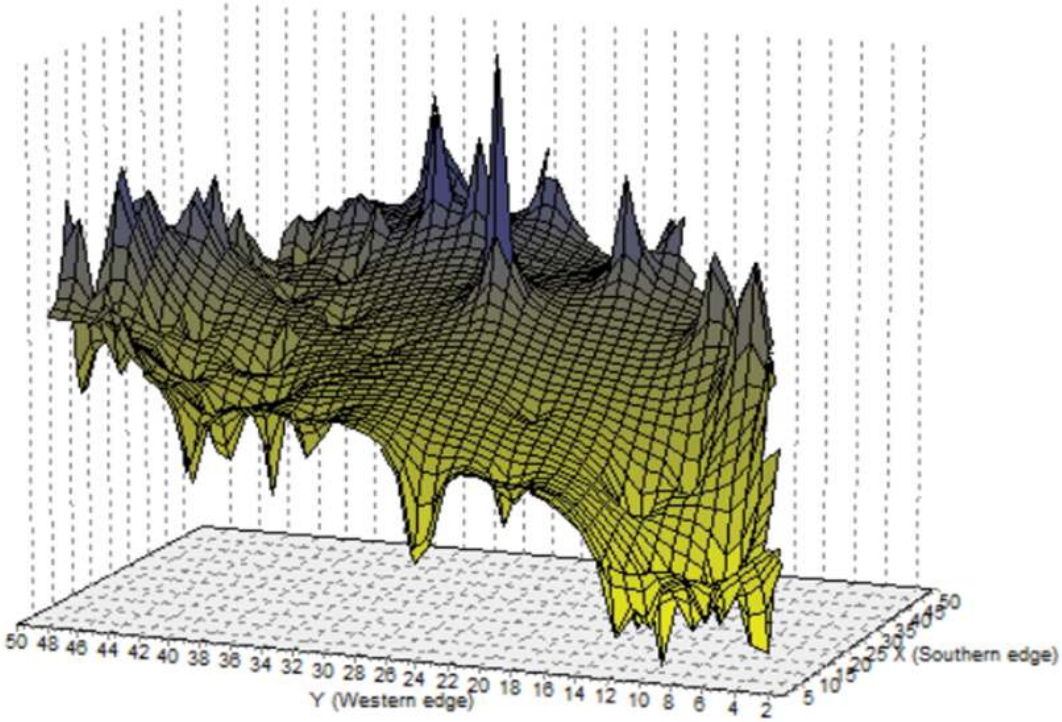

Figure 5 Landscape genetic shape plot of 36 silver fir population. X and Y axes correspond to geographic coordinates and the Z axis correspond to genetic distances between individuals. Blue peaks indicate areas with high pairwise genetic distances and yelow valleys indicate areas with low pairwise genetic distances

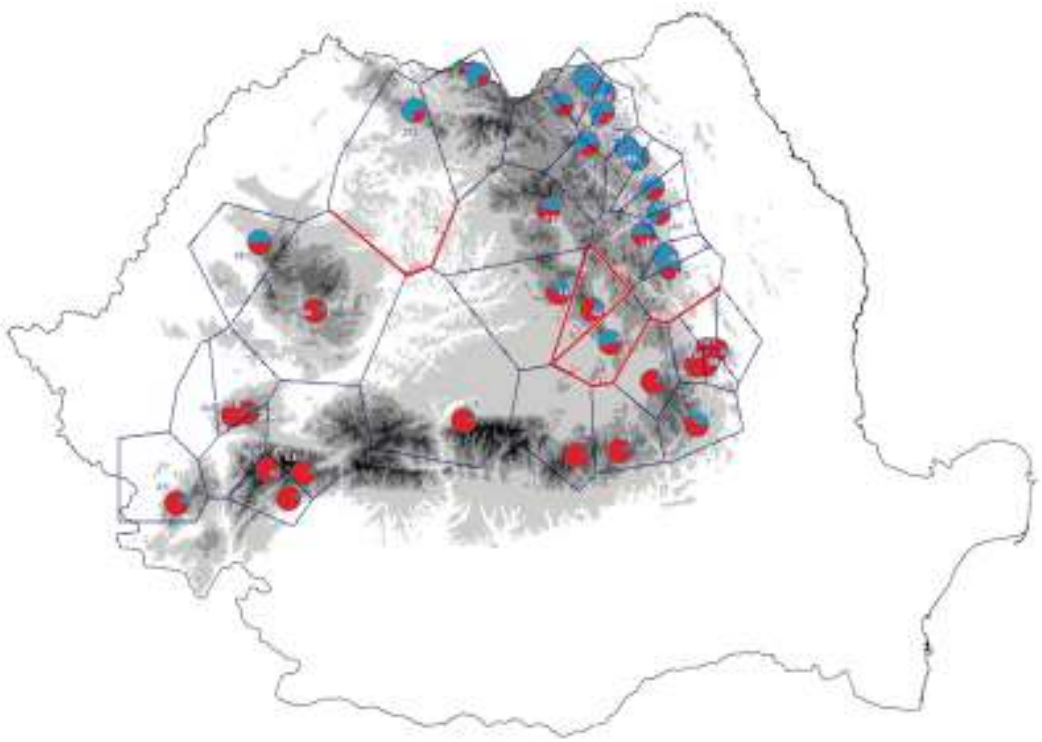

Figure 6 Map shows estimated populations structure for K = 2 and the detected genetic barrier. Red and blue clusters represent the genetic ancestry groups according to STRUCTURE analysis. Red lines show the main detected barriers with >80% bootstrap support

with two larger clusters containing populations from Curvature Carpathians and Banat Mountains and other four subgroups related to the Eastern Carpathians region; otherwise, the highest number of clusters inferred by STRUCTURE is six (Figure 4b).

Landscape genetics

The IBD pattern revealed by Mantel test indicated a weak, but significant correlation between genetic differentiation (F_{ST}) and geographic distances (r = 0.359, P = 0.010) when considering all the populations, slightly higher and significant for populations of cluster blue (Eastern Carpathians) (r = 0.490, P = 0.0001), and lower, but also significant for cluster red (South-western Carpathians) (r = 0.297, P=0.005).

A pattern which is largely consistent with the Neighbour-joining analysis resulted from the genetic landscape shape analysis (Figure 5), with the south-western populations (Banat Mountains) showing a weak differentiation (e.g. the surface plot of genetic distances drop in this group), than the two other regions with a pronounced differentiation, one in the south-eastern part of distribution (CC Carpathians) and another in the north-eastern part.

Results from the STRUCTURE and NJ tree revealed clear genetic differentiation between north-eastern and southern populations (Figure 6) and in order to detect genetic barriers between these groups of populations the Barrier analysis based on Nei's genetic distances (D_A) was used. The results indicate the existence of two major barrier. The first and most significant barrier (100% bootstrap probability) (Figure 6) appeared between south-western and north-eastern populations and is concordant with the Transylvanian Plateau. The second barrier separated the Inner Eastern population FRU from all the others with 100% support, this barrier going further (80% bootstrap support) and dividing the eastern populations, from those of the Curvature Carpathians.

Discussion

Population genetic diversity

Our study on the genetic variation of Silver fir in the Romanian Carpathians, which overlaps with the south-eastern species distribution limit, revealed high genetic diversity and low genetic differentiation, as expected in conifer populations (Petit & Hampe, 2006). Although different sets of microsatellites were employed, most of the populations revealed higher levels of genetic diversity, compared to those previously reported for Silver fir in Europe (Cvrckova et al. 2015, Popovic et al. 2017, Piotti et al. 2017). The expected heterozygosity (H_e=0.810) obtained in the present study was higher than values reported by Gömöry et al. (2012) for Carpathians silver fir populations which found 0.680 for Balkan lineage, respectively 0.641 for Central European lineage. The recent study of Belleti et al. (2017), comprising 45 populations from Italy, the mean expected heterozygosity was 0.724, while the observed heterozygosity was only 0.563. In terms of private alleles, the same authors identified only two out of 45 populations that harboured two private alleles and 12 populations harboured one allele.

Sancho-Knapik et al. (2014) studied the genetic structure of ten populations from the western rear edge in the Spanish Pyrenees and compared them to two German populations and found that Spanish populations show a lower genetic diversity and a high genetic differentiation.

Based on the fixation index (F_{ST}) the overall observed genetic differentiation in our study is relatively low, only 1,4 % of the total genetic variation being due to differences among populations. For Italy, Belleti at al. (2017) found that about 8% of the genetic diversity is among populations, but this is explained by the fact that many populations have remained isolated for a long time, which is not the case for the Romanian silver fir populations. For Czech

silver fir populations the F_{ST} value ranged from 0.015 to 0.036, comparable (Cvrčková et al. 2015) our values. For 14 Romanian population and one Bulgarian populations, Postolache et al. (2016) found a mean value for F_{ST} of 0.018. AMOVA confirmed the low degree of differentiation between provenances and genetic clusters, but the variance among populations within clusters (F_{SC}) is almost equal to that obtained by Gömöry et al. (2012) 1,35% for the two different mitochondrial lineage.

The geographical distribution of genetic diversity contradicts the core - peripheral hypothesis, according to which peripheral populations are expected to hold lower levels of genetic diversity within populations (Lesica & Allendorf 1995, Eckert et al. 2008, Duncan et al. 2015), our eastern peripheral populations retaining the highest level of genetic diversity, both allelic richness and heterozygosity. However, some exceptions are registered in the populations sampled in the south-eastern region (CAI, CAS, VID and SOV) and in other few peripheral populations (MAL, RAS and MOI), as allelic richness is below general average. Identification of centers of neutral diversity, as in the eastern Carpathians, suggesting that these peripheral populations possess high evolutionary potential. In fact, some of this populations (SOL, RAS, FRU) recognized as edaphotypes (high adaptability to hydrophilic soil) could show the adaptative imprint on a specific environment and the selection pressure could generate local adaptation. This would be an interesting case to be looked at using adaptative markers.

A decrease in genetic diversity along the Southern Carpathians (from east to west) coincides with a stronger fragmentation of the distribution area, which makes the populations more susceptible to the loss of genetic diversity. With only two exceptions (AVR and LUP), all the southern populations show a slight excess of homozygotes, which implies increased mating between relatives.

Landscape genetics

The distribution of the genetic clusters is related to the main bio-geographical regions described for the Romanian Carpathians (Georgescu & Doniţă 1965) and corresponds broadly with the phylogeographical model identified for alpine plants (Ronikier 2011, Hurdu et al. 2016). Only a few studies investigated genetic differentiation within the South-Eastern Carpathians (the largest part of the Carpathians).

The genetic landscape shape analysis revealed an area with a high degree of differentiation, the second barrier between the genetic groups (more complex than the first), being largely congruent with the border between the Curvature Carpathians and Eastern Carpathians.This region is characterized by lower altitude, which does not generate a barrier to gene flow; a potential explanation could be the existence of cryptic microrefugia in the Eastern and Southern Carpathians and limited gene flow between these regional gene-pools.

The studies conducted so far (Konnert & Bergmann, 1995; Fady et al., 1999; Liepelt et al., 2009; Gömöry et al., 2004, 2012., Piotti et al., 2017 etc.) consider that the current genetic structure of Silver fir populations has been shaped by the postglacial recolonization routes, and the migration history seems to be the main factor driving genetic differentiation rather than recent adaptation (Konôpková et al. 2019) or human activities. It is commonly accepted that for Silver fir the Balkan refugium down to Southern Greece is the source for the recolonization of South-east Europe (Konnert & Bergmann 1995; Terhürne-Berson et al. 2004). Silver fir from this region represents a separate mitochondrial lineage (Ziegenhagen et al.2005, Liepelt et al. 2009). On the border between Romania and Ukraine a contact zone with the North-Apennine mitochondrial lineage is formed (Ziegenhagen et al. 2005, Liepelt et al. 2009) with few populations harbouring both mitochondrial lineages (Gömöry et al. 2012). Strong differentiation found between

Bulgarian silver fir populations (southern Balkans) and the Romanian Carpathians (Konnert & Bergmann, 1995, Postolache et al., 2016) and the geographical position of the western refugia leads to the hypothesis that probably only the northwest Balkan refugium determined the actual gene-pool of the Carpathian silver fir (Gömöry et al. 2012). It cannot be ruled out that a different refugium or even microrefugia have contributed to the colonization of Eastern Europe (Dobrowolska et al. 2017).

Palynological evidence indicates that silver fir appeared first in the Apuseni Mountains (Fărcaş et al. 2007; Feurdean & Willis, 2008), from where they migrated to the north and east to the Carpathians. What is particular in the case of fir expansion in Romania is the late arrival and low postglacial migration rate, a distance of only 200 km between Apuseni Mountains and Eastern Romanian Carpathians being covered in about two millennia (Feurdean & Willis, 2008), and probably there were other factors responsible for the slow eastward expansion, like competition with *Picea abies*, disturbances and last but not least, climate change.

Nuclear microsatellite data revealed admixture of two gene pools in the Northern Carpathians with one gene pool increasing and the other decreasing from north-west to south-east (Gömöry et al. 2012).

In the present study, the genetic signature of migration routes is very well highlighted by the genetic differences observed between the north-eastern Carpathians populations and south-western Carpathians ones. Based on nuclear microsatellite data and Bayesian analysis, two genetic clusters were identified in the Romanian Carpathians, one homogeneous cluster which corresponds to the southern part of the distribution range, and the second one, more heterogeneous which includes north-eastern populations. Therefore, north-eastern Carpathians populations have a high degree of admixture, most likely due to pronounced gene flow through pollen from west glacial refugium favoured by the orography of these mountains (NW to SE orientation) and wind directions (predominantly from the North). Similar patterns in genetic structure along the Carpathians have been observed in other conifer species. In *Picea abies*, Tollesfrud et al. (2008) indicated the existence of a glacial refuge in southern Carpathians, hypothesis also supported by palynological evidence (Feurdean et al. 2007), while in Scots pine, the populations of Apuseni Mountains are differentiated from the populations of Eastern Carpathians, with the identified barriers suggesting an existence of a refuge in the Eastern Carpathians (Gy Toth et al. 2017).

Recolonization of Romanian Carpathians most probably occurred through seed from Balkan refugia (Balkan mitochondrial lineages) northward until the border to Ukraine forming a contact zone to the Apennine mitochondrial lineages. Pollen flow from the north might have led to the current picture of decreasing admixture proportions in silver fir populations from Northern to Southern Romanian Carpathians.

Management and conservation implications

Our study suggests three distinct hot-spots of genetic diversity in the (Romanian) Eastern Carpathians: the northern group (STB, POI, DEM, PUT, MAR), the eastern group (BRA, GAR, MOI, TAZ) and the southern population AVR. Although the genetic differentiation is low, the genetic structure of Silver fir populations is not as homogeneous as expected (especially in the NE Carpathians), therefore any management and conservation strategy - including *ex situ* conservation, breeding programs, translocation and rezilience programs need to consider this distinctiveness of the identified genetic groups. Due to its relatively low genetic diversity, populations from Banat Mountains (ANI) which might be vulnerable to future environmental change, require a special attention (e.g. an intensive genetic monitoring and urgent measures to increase the genetic diversity).

A mix of environmental conditions and ge-

netic specificity must be considered for delineating seed zones and designation of seed sources. Our study shows that the diversity of climatic, edaphic and topographic conditions can influence the population differentiation at regional scales and, although the provenance regions are well represented across Romania and the existing Silver fir seed sources are more than enough (about 4000 ha of selected seed sources) (Pârnuţă et al. 2012), the ecological particularities of the place where the forest reproductive material is to be used are not always considered. As pointed out before, the edaphic ecotypes could have certain genetic features which can be analyzed in more detail based on adaptive markers.

Conclusions

The genetic structure of the Eastern peripheral populations suggests an introgression zone with decreasing admixture proportion from north-west to south-east of the Romanian Carpathian mountain with topography and the regional climate playing an important role.
The silver fir populations from the eastern limit of its distribution hold high potential to mitigate the negative effects of climate warming, being valuable genetic resources in the context of global change.

Despite slight gradients of genetic diversity werefound and weak genetic differentiation was very weak, the distribution pattern of genetic variation at local, regional and country scale could and should be considered for appropriate management of forest genetic resources and development of strategy for conservation of valuable gene pools.

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Article

Genetic Diversity and Spatial Genetic Structure in Isolated Scots Pine (*Pinus sylvestris* L.) Populations Native to Eastern and Southern Carpathians

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Abstract: Small, isolated populations are more vulnerable to natural disturbances and loss of genetic diversity. Scots pine, an abundant tree species in the boreal forest of Eurasia, has a scattered natural distribution across Eastern and Southern Carpathian Mountains, where only a few relict populations still exist. We estimated genetic diversity and spatial genetic structure in Scots pine on the basis of microsatellite nuclear markers (nSSR) data. We found a relatively high level of genetic diversity (*He* = 0.697) within populations and no evidence of recent bottlenecks. Genetic diversity was lower in peat bog populations, as compared to populations that grow on rocky slopes or acidic soils and nutrient-poor sites. Population genetic structure was weak, and genetic discontinuities among populations were detected. Spatial genetic structure (SGS) was observed in nearly all Scots pine populations. The strength of SGS, quantified by Sp statistics, varied greatly among populations, ranging from 0.0011 to 0.0207, with an average of 0.01. Our study highlights that Eastern and Southern Carpathian populations still possess high within-population diversity in spite of the recent fragmentation and reduction of the Scots pine natural distribution range. We discuss the importance of spatial patterns of genetic diversity for developing strategies of conservation and sustainable use of Scots pine genetic resources in the Carpathian region.

Keywords: relict populations; island-like populations; spatial genetic structure; forest genetic resources

1. Introduction

Relict forest tree populations, i.e., populations that are presently confined to a small territory, but whose original distribution range was much larger in the past, may contribute substantially to the genetic diversity of a particular species [1]. Geographically isolated populations, which are located at the margins of species distribution range, may harbor rare, unique genetic variants that might be of importance for species survival under changing environmental conditions [2,3]. The genetic diversity of geographically marginal populations, which are typically small and island-like, may be reduced due to higher genetic drift, increased inbreeding, limited or lack of gene flow from other populations, and natural selection after long periods of time of survival in new ecological settings [4,5].

Spatial genetic structure (SGS) within natural tree populations derives from a series of interacting genetic and demographic processes, which may be difficult to disentangle [6–8]. Limited gene dispersal via pollen and seed is the prevalent cause of occurrence of SGS at a fine spatial scale or stand level. For example, a stronger SGS is expected in gravity-dispersed than in wind-dispersed tree species [9]. Wind-pollinated and wind-dispersed tree species (e.g., Aleppo pine) usually show a weak SGS [10].

The isolation by distance theory can predict patterns of SGS at the drift–dispersal equilibrium [11]. Other factors such as life stage or age, population density, spatial configuration of the population, and natural disturbances may influence SGS [9,10,12]. Substantial variation among populations within species shows the importance of local environmental factors in shaping fine-scale SGS in four Alpine conifer species [13]. The existence of significant SGS within forest tree populations may support the hypothesis of natural origin of forest stands [14]. Furthermore, forest management practices appear to have changed SGS when comparing mature managed stands with an unmanaged one in Scots pine [15].

Scots pine (*Pinus sylvestris* L.) is the most common Eurasian conifer species, with a distribution range that stretches from Western Europe to the Eastern parts of Siberia. It has great ecological and economic importance and is adapted to a variety of soil and climate conditions [16,17]. Scots pine is a monoecious, wind-pollinated, pioneer, and light-demanding species [18–21]. Its present distribution range is the result of recolonization events and postglacial retraction of a once larger distribution range [22,23]. Although Scots pine was very common in the Carpathian region during the last glaciation [24,25], it currently has a scattered, disjunct occurrence across Carpathian Mountains [26]. During Holocene, Scots pine showed resilience to climate variability, but low competition ability compared to other tree species [27]. As a consequence of the expansion of other tree species, the natural distribution area of Scots pine in Romania was greatly reduced, being now estimated at approximately 9000 ha [28]. In the Carpathian territory, three Scots pine ecotypes can be distinguished according to the habitat characteristics: (1) on rocky and steep mountain slopes, (2) on nutrient-poor and very acidic soils and (3) on peat bogs [28].

Molecular studies indicate relatively high levels of genetic diversity in Scots pine populations in Western and Eastern Carpathian Mountains [23,29]. The postglacial reduction and fragmentation of Scots pine natural range, which is confirmed by palynological records [30], do not seem to have affected the magnitude of genetic diversity in Scots pine. Previous studies that sampled Scots pine populations in Romania analyzed natural populations along with plantations established with material of unknown origin [29] or only natural populations located inside the arch of the Romanian Carpathians, with a strong focus on Eastern Carpathians and Apuseni Mountains [23]. Moreover, the sample size per population was relatively small (8–30, mostly 20 individuals/population) and the spatial distribution of individual trees within populations was not correlated with genetic diversity.

In this study, we (1) analyze the level and geographic distribution of nuclear genetic diversity in natural Scots pine populations located in Eastern and Southern Carpathian Mountains, and (2) assess within population spatial genetic structure (SGS) in relict populations that grow under different site conditions, from rocky slopes to peat bogs.

2. Materials and Methods

2.1. Study Populations

Eight natural populations were sampled in the highly fragmented distribution range of Scots pine in Romania (Table 1 and Figure 1a). In contrast to previous studies [23,29], more populations located in the Southern Carpathian Mountains and populations on the outward-oriented side of the Southeastern Carpathian arch were sampled. The sample size consisted of 96 adult trees per every population. The sampling scheme strongly depended on the spatial configuration of each population. Sampling along two transects disposed along a cross (two perpendicular lines) was used whenever possible. Because we did not find enough individuals to be sampled at one site, two or more subpopulations were sampled in the Retezat (S-RE) and Valea Sebesului (S-VS) populations, respectively. The distance between sampled individual trees was at least 15–20 m to minimize the possibility of sampling closely related individuals. The Scots pine populations were located in three site conditions: (i) rocks lying on steep slopes (S-RE, S-VS, S-LO, and E-CB populations); (ii) on acidic and nutrient-poor soils, in areas without rock or skeleton in the upper horizons of the soil (E-TU and E-BI populations); (iii) peat

bogs (E-FB and E-PS populations). Plant material (1-year-old needles) was stored at −60 °C until DNA extraction.

Table 1. Geographic location of the sampled Scots pine population (S—Southern Carpathian Mountains; E—Eastern Carpathian Mountains; R—rocky slopes; A—acidic and nutrient-poor soils, in areas without rock or skeleton in the upper horizons of the soil; PB—peat bog).

No.	Population	Acronym	Ecotype	Sample Size	Geographic Location		
					Latitude	Longitude	Altitude (m)
1.	Retezat	S-RE	R	96	45°26′ 45°24′	22°46′ 22°46′	680–750 890–925
2.	Valea Sebeșului	S-VS	R	96	45°42′ 45°42′	23°36′ 23°35′	750–1070 730–780
3.	Lotrișor	S-LO	R	96	45°18′	24°16′	340–510
4.	Cheile Bicazului	E-CB	R	96	46°49′	25°49′	1060–1110
5.	Tulnici	E-TU	A	96	45°55′	26°36′	580–610
6.	Bisoca	E-BI	A	96	45°33′	26°40′	930–950
7.	Fântâna Brazilor	E-FB	PB	96	46°30′	25°15′	950–960
8.	Poiana Stampei	E-PS	PB	96	47°18′	25°07′	920

2.2. DNA Extraction, Amplification, and Sizing

DNA was extracted from 20 to 25 mg of plant material using the CTAB (cetyl trimethylammonium bromide) method [27,31]. Initially, 10 nuclear microsatellites were used (SPAG 7.14, SPAC 11.4, SPAC 11.6, SPAC 11.8, SPAC 12.5 [32] psyl16, psyl17, psyl42, psyl44, and psyl57 [17]). Two multiplex reactions for the PCR amplification were performed: multiplex A—psyl16, psyl17, psyl42, psyl44, and psyl57; multiplex B—SPAG 7.14, SPAC 11.4, SPAC 11.6, SPAC 11.8, and SPAC 12.5. The PCR reaction was carried out in a total volume of 15 µL (first multiplex), containing 7.2 µL of Qiagen Multiplex PCR Master Mix 2×, 5.36 µL of primer mix, 0.34 µL of Qsolution, 0.6 µL of RNase-free water, and 1.5 µL of DNA or 10 µL (second multiplex), containing 2 µL of buffer 5× (Promega), 1 µL of $MgCl_2$, 1.5 µL of dNTPs (deoxyribonucleotide triphosphate, Promega), 3 µL of primer mix, 0.1 µL of Taq polymerase, 0.9 µL of RNase-free water, and 2 µL of DNA.

The PCR profile consisted of 15 min of initial denaturation at 95 °C followed by 30 cycles of 1 min denaturation at 94 °C, a 30 s annealing step at 47 °C (for multiplex A) or 55 °C (multiplex B), a 1 min elongation step at 72 °C, and a 20 min final extension step at 60 °C. Amplified PCR products were diluted and were then run on a GemoneLab GeXP Genetic Analyzer and analyzed using the Frag-3 method and Size Standard 400.

2.3. Genetic Data Analysis

Micro-Checker [33] was used to test all markers for null alleles and possible scoring errors derived from large allele dropout and the presence of microsatellite stutter bands. The software indicated the presence of null alleles at high frequencies for two microsatellite markers (SPAC 11.4 and SPAC 11.6), which were excluded from further analysis. No evidence of large allele dropout or scoring of stutter peaks was found in the populations. Standard population genetic diversity indices (number of effective alleles (*Na*), number of effective alleles (*Ne*), expected heterozygosity (*He*), observed heterozygosity (*Ho*), inbreeding coefficient (F_{IS}), and private allele number (*Pa*)) were calculated for each population using GenAlEx v.6.5 [34,35]. To test for differences between ecotypes one-way ANOVA was performed using the STATISTICA software v.10 [36].

To assess population genetic structure, the Bayesian clustering method implemented in Structure software v.2.3.4 [37] was used. Simulations were run for 100,000 steps following a burn-in period of 50,000 steps, considering values of *k* (number of clusters) from 1 to 8, with 10 replications for each value of *k*. The analysis was performed using an admixture, correlated allele frequencies, and no prior information on sampling location. The most likely number of clusters was assessed on the basis of log

likelihoods (*ln Pr(X|k)*) and the Δ*k* method of [35,38] using the STRUCTURE HARVESTER software v.0.6.94 [39].

The pairwise F_{ST} between all populations and analysis of molecular variance (AMOVA) were computed with ARLEQUIN software 3.5.2.2 [40] using 1000 permutations. BOTTLENECK software v.1.2.02 [41] was used to test for recent population bottlenecks on the basis of the stepwise mutation model (SMM) and the two-phase model (TPM). Statistical significance was determined by the sign and Wilcoxon tests with 1000 iterations.

To explore the existence and location of barriers to gene flow, the BARRIER software v.2.2 [42] was used. The software uses the Monmoniers maximum difference algorithm [43], designed to visualize on a geographic map (represented by geographical coordinates) the trend of data constrained in a matrix. A matrix of Nei's genetic distance between all populations sampled was used. Nei's genetic distances (D_A) were calculated in MSA software [44], and 100 bootstrap replicates of the distance matrix and three barriers were used to calculate the statistical significance of the predicted barriers.

2.4. Spatial Genetic Structure (SGS)

To assess patterns of SGS within populations, a spatial autocorrelation analysis was performed using the multivariate method by [45] implemented in GenAlEx v.6.5 software [34,35]. Geographical distances between individuals within each population were calculated according to latitude and longitude coordinates recorded with GPS Garmin 62s for every sample (except for population E-CB). The range of expected genetic similarity under random association was estimated using 999 random permutations; 95% confidence intervals around each value of *r* were estimated using 999 bootstraps. The *r*-values were plotted using the option of even distance classes, and the five classes were examined by distances of 25 m. The statistic $S_p = -b_F/(1 - F_1)$, using SPAGeDi v.1.5 [46], where F_1 is the mean Nason's kinship coefficient [47] between all pairs of individuals in the first distance class (0–25 m), and b_F (*b*-log) is the slope of the regression of kinship versus the log of distance [9], was calculated. The significance of the slope of the regression analysis was determined after 10,000 permutations. Their significance was tested with a one-tailed *t*-test using STATISTICA software v.10 [36].

3. Results

3.1. Genetic Diversity

The eight nSSR loci were highly polymorphic in all Scots pine populations (Table 2), with the mean number of detected alleles per locus (Na) ranging from 8.750 (E-FB) to 11.750 (S-LO). The mean number of effective alleles (*Ne*) had the lowest values in the two peat bog populations (E-PS and E-FB). Moreover, the mean value of expected heterozygosity (*He* = 0.645) in peat bogs populations was significantly lower ($p = 0.045$) compared to the other Scots pine populations. The highest value of the expected heterozygosity (*He* = 0.733) was recorded in one Southern Carpathian population, which grow on rocky slopes (population S-VS). There was an excess of homozygotes across all populations (the mean value of inbreeding coefficient was 0.122) with one exception (peat bog population E-FB), in which there was a slight excess of heterozygotes ($F_{IS} = -0.046$). However, F_{IS} values were significantly different from zero in two populations only (Table 2). Most of the private alleles (16 out of 18 alleles) were observed in four populations that grow on rocky sites. Three out of these four populations were located in the Southern Carpathian Mountains (Figure 1).

Table 2. Standard genetic parameters in Scots pine population (*Na*—number of alleles; *Ne*—number of effective alleles; *He*—expected heterozygosity; F_{IS}—inbreeding coefficient; *Pa*—number of private alleles; SE—standard error).

Population	Ecotype		*Na*	*Ne*	*He*	F_{IS}	*Pa*
S-RE	R	Mean	9.750	4.553	0.724	0.162	3
		SE	0.977	0.698	0.062	0.104	
S-VS	R	Mean	10.500	5.165	0.733	0.049	4
		SE	2.062	1.114	0.057	0.090	
S-LO	R	Mean	11.750	6.226	0.731	0.168 *	4
		SE	2.289	1.590	0.078	0.061	
E-CB	R	Mean	10.750	5.222	0.672	0.154	5
		SE	2.250	1.468	0.087	0.088	
E-TU	A	Mean	10.375	5.290	0.710	0.164	0
		SE	1.936	1.136	0.088	0.080	
E-BI	A	Mean	10.250	5.246	0.711	0.138	0
		SE	1.980	1.096	0.088	0.083	
E-FB	PB	Mean	8.750	3.488	0.658	−0.046	2
		SE	1.934	0.463	0.065	0.073	
E-PS	PB	Mean	9.375	4.214	0.635	0.187 *	0
		SE	2.299	1.093	0.094	0.066	
Total		Mean	10.188	4.925	0.697	0.122	18
		SE	0.677	0.391	0.027	0.029	

* Significant F_{IS} values ($p < 0.05$) are indicated by an asterisk.

Analysis of molecular variance (AMOVA) revealed that within-population variation accounted for most of the total variance (Table 3). The genetic differentiation among populations, as measured by the F_{ST} value, was 0.047. The population bottleneck analyses showed no evidence of recent genetic bottlenecks in the studied Scots pine populations.

Table 3. Analysis of molecular variance (AMOVA).

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percent of Variation
Among population	7	385.255	55.036	0.495	6%
Within population	760	5711.833	7.516	7.516	94%
Total	767	6097.089		8.011	100%

3.2. Population Genetic Structure

A two-cluster structure had the strongest statistical support in our sample (Figure 1b and Figure S1, Supplementary Materials). The highest value of Δk statistics (19.9) was obtained for $k = 2$ (Figure S1, Supplementary Materials). Two Scots pine populations located on the outward-oriented side of the Southeastern Carpathian arch (E-BI and E-TU) showed the highest membership values in one of the two genetic clusters (in green color in Figure 1). In contrast, the two peat bog populations (E-FB and E-PS) and one Southern Carpathian population located on rocky slopes (S-VS) showed a higher membership in the second genetic cluster (in red color in Figure 1). The other three populations were very admixed. However, when having three genetic clusters ($k = 3$), the two peat bog Eastern Carpathian populations split from the Southern Carpathian population (Figure 1b).

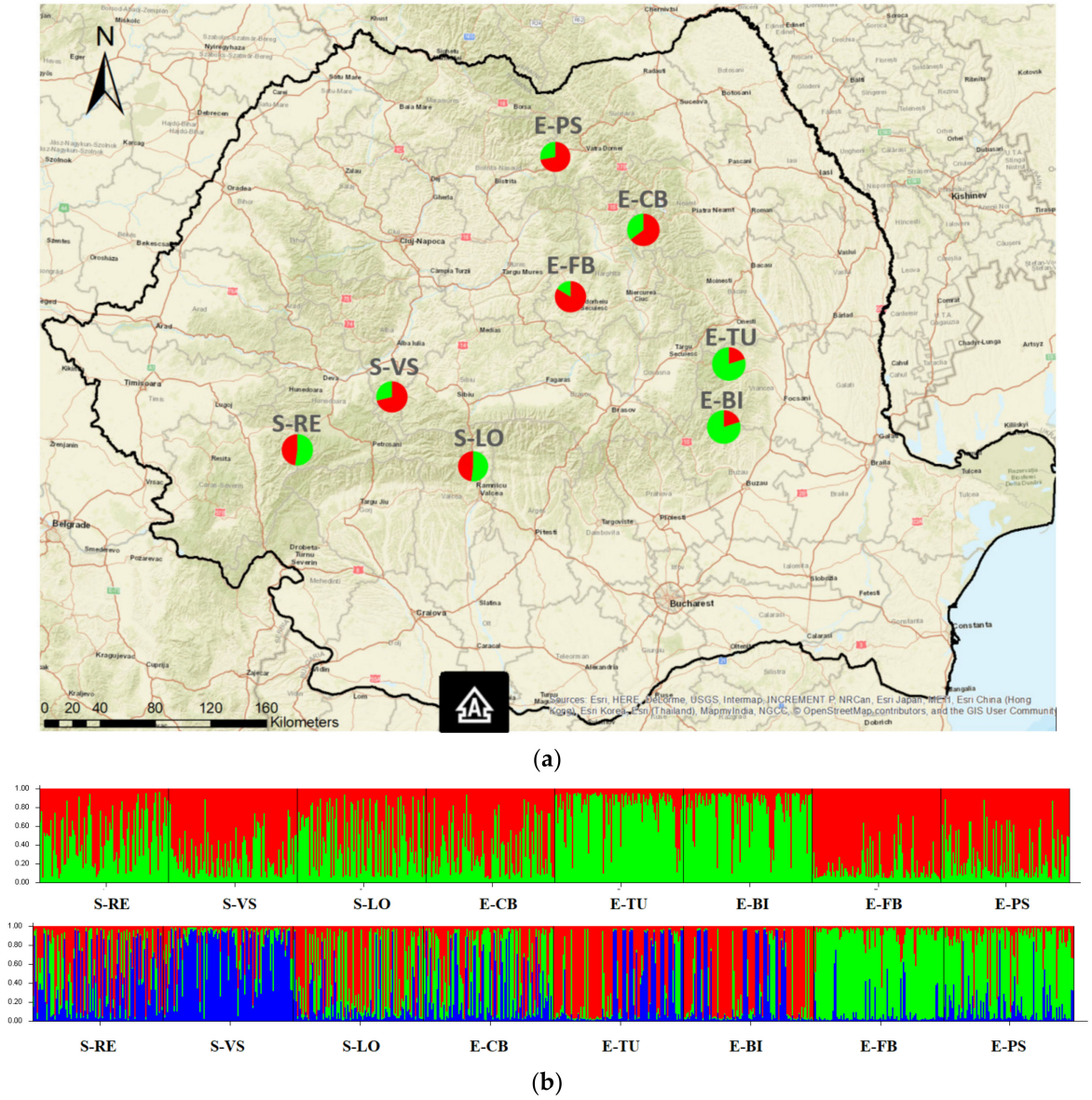

Figure 1. Genetic structure and geographical distribution of eight natural Scots pine populations (**a**). Geographic location of each sampled populations and their color-coded grouping. The acronyms stand for the population code in Table 1. (**b**) Estimated population structure for $k = 2$ (the upper part) and $k = 3$, assignment.

A genetic barrier prediction analysis detected one barrier against gene flow with strong bootstrap support (61–79%) (Figure 2), which delimited a group of four Eastern Carpathian populations (E-PS, E-CB, E-TU, and E-BI). A second but weak barrier (26% bootstrap support) separated two Eastern Carpathian populations (E-TU and E-BI) located on the outward-oriented side of the Eastern Carpathian arch. A third very weak barrier (12% bootstrap support) was detected between two populations from the Southern Carpathian Mountains (S-RE and S-LO).

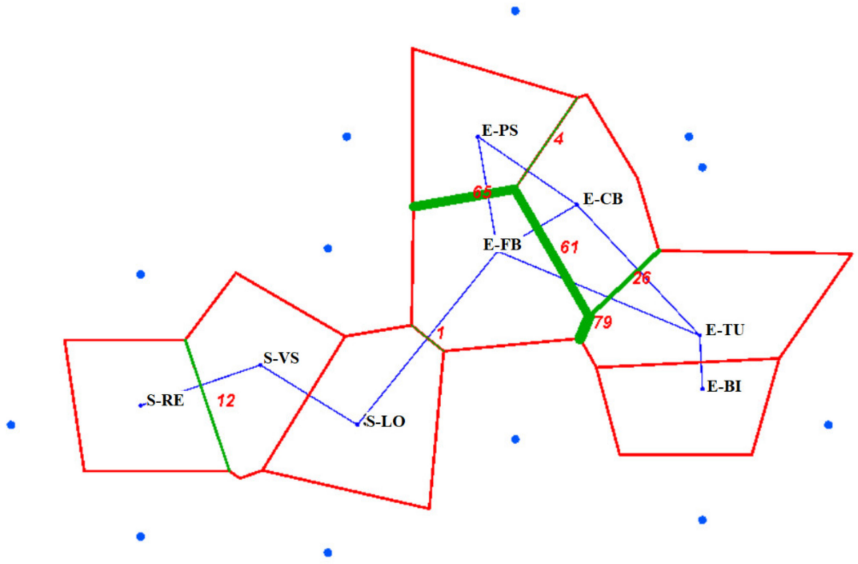

Figure 2. Identification of predicted genetic barriers among eight Scots pine populations, predicted by BARRIER v.2.2 software (the genetic barriers are shown in green bold lines with bootstrap values) on the basis of Nei's genetic distance matrix.

3.3. Spatial Genetic Structure

A nonrandom spatial distribution of genotypes within Scots pine populations was found at six out of the seven locations (Figure 3). Values of the correlation coefficient *r* were positive and significant in the first distance class (0–25 m) for six populations. In two populations (S-VS and E-FB), the correlation coefficient was significantly positive for the first two distance classes (up to 50 m). The spatial distribution of Scots pine genotypes appeared to be random only in one peat bog population (E-PS).

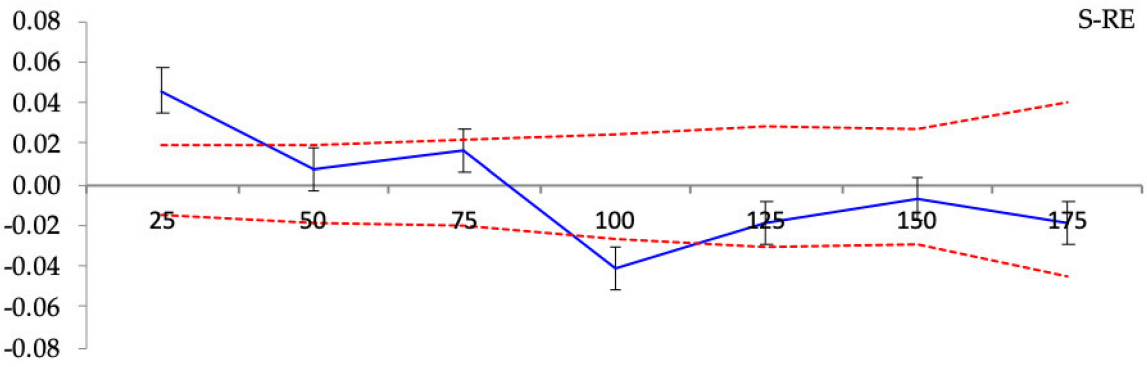

Figure 3. *Cont.*

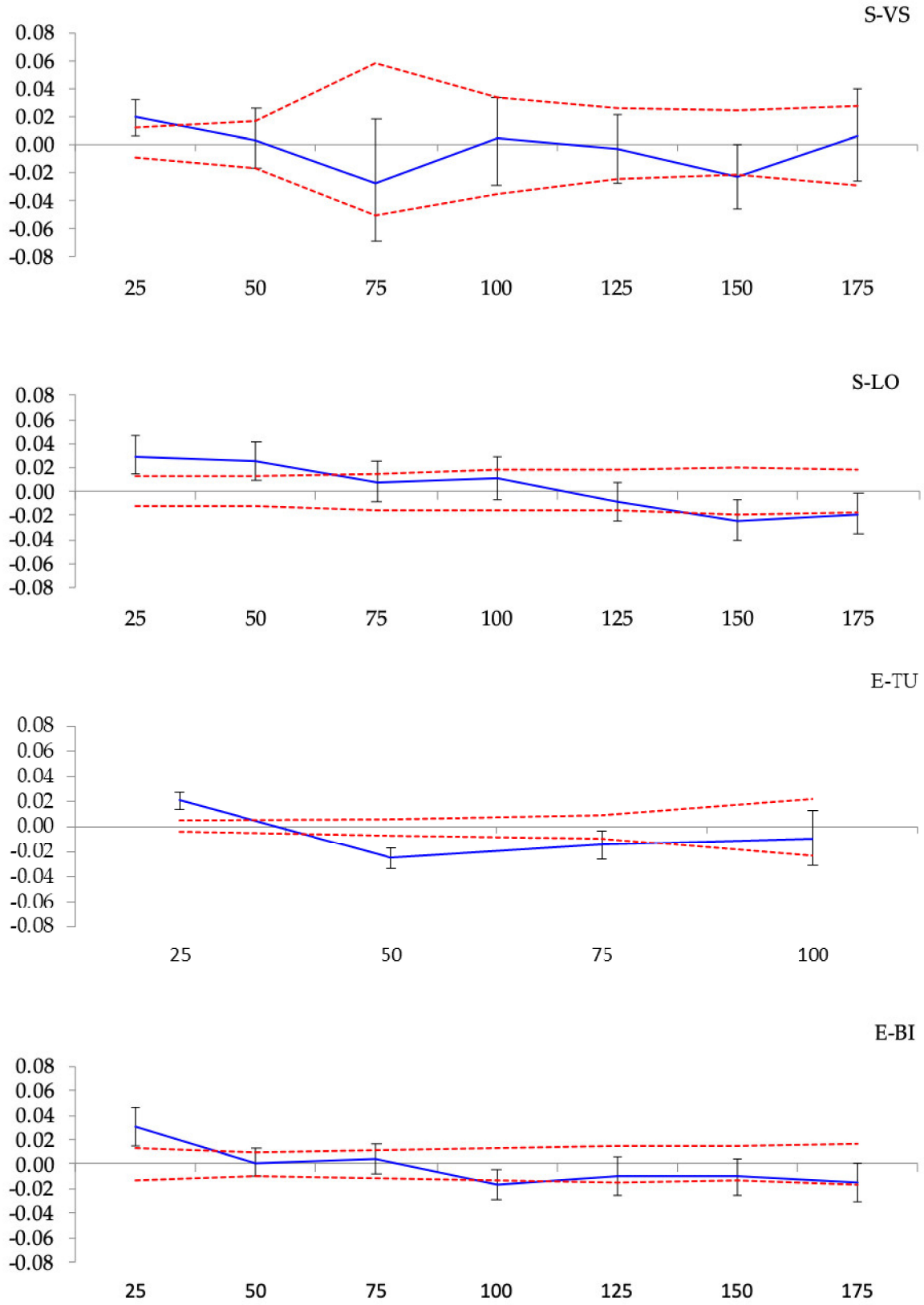

Figure 3. *Cont.*

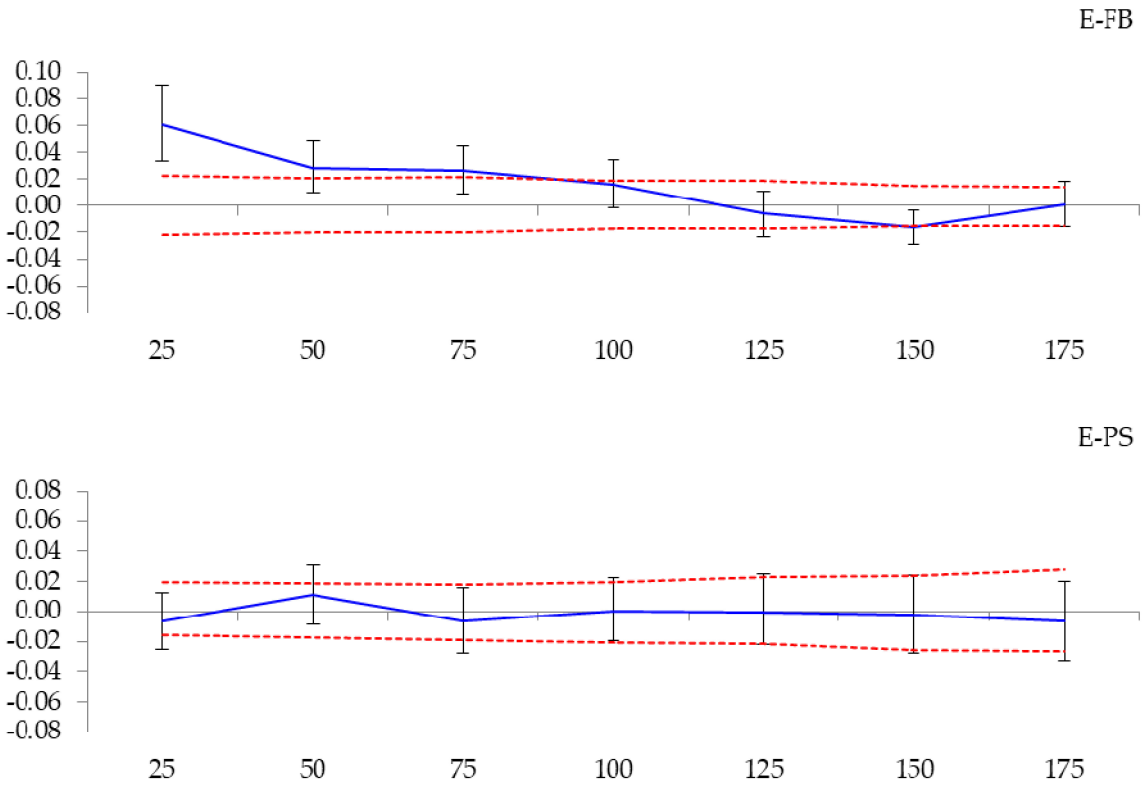

Figure 3. Multilocus spatial–genetic correlograms of genetic and geographic distance in seven Scots pine populations. The *y*-axis is the genetic correlation coefficient (*r*), and the *x*-axis is the distance class (m); confidence intervals (95%) were calculated using permutation tests (red lines), and bootstrapped 95% confidence error bars around *r* are also shown.

The value of the S_p statistic varied greatly, ranging from 0.0011 to 0.0201 in populations E-PS and E-TU, respectively, with an average value of 0.0100. The value of b_F was significantly different from zero in all populations (Table 4).

Table 4. Parameters describing spatial genetic structure (F_1—average of kinship coefficient between individuals of the first distance class (0–25 m); b_F (*b*-log)—slope of the regression of kinship coefficient F_{ij}; S_p—intensity of spatial genetic structure; (±SE)—standard error; (95% CI)—95% confidence intervals; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Population	F_1	b_F (*b-log*) (±SE)	$S_p = -b_F/(1 - F_1)$ (95% CI)
S-RE	0.0137 *	−0.0036 ± 0.0041 ***	0.0036 (0.0004–0.0081)
S-VS	0.0875 **	−0.0046 ± 0.0041 ***	0.0049 (0.0021–0.0087)
S-LO	0.0187 **	−0.0071 ± 0.0047 ***	0.0072 (0.0026–0.0168)
S-TU	0.0277 *	−0.0201 ± 0.0493 **	0.0207 (0.0021–0.0239)
S-BI	0.0227 *	−0.0136 ± 0.0102 **	0.0139 (0.0031–0.0356)
S-FB	0.0446 **	−0.0175 ± 0.0102 **	0.0183 (0.0036–0.0303)
S-PS	−0.0007	−0.0011 ± 0.0052 ***	0.0011 (−0.0033–0.0131)

4. Discussion

A relatively high level of genetic diversity (*He* = 0.697) was observed in relict Scots pine populations sampled in Eastern and Southern Carpathian Mountains. A similar value (*He* = 0.681) was obtained for one Scots pine population in Scandinavia [48], and higher genetic diversity was observed in Central Europe (*He* = 0.859), as well as in Italy (*He* = 0.810–0.847) and the Iberian Peninsula (0.810), on the basis of nSSR markers [49–52]. Slightly lower values were reported for Scots pine in previous studies on Romanian populations including both natural populations and plantations with unknown material [29] or only natural populations from the Eastern Carpathians and Apuseni Mountains, except for one population that was located in the Southern Carpathians [23]. However, these differences have to be treated with caution since different nSSRs were employed in every study. For example, our set of nSSRs had five out of the eight markers used in a previous study on natural Scots pine populations [23] and was almost completely different compared to [29].

Scots pine peat bog populations (E-PB and E-PS) show lower genetic diversity, *He* = 0.658 and *He* = 0.635, respectively, compared with populations that grow on nutrient-poor soils or on rocks in the Carpathian Mountains. Interestingly, the same pattern can be observed for populations sampled in the same region in a previous study [23]. A lower genetic diversity of Scots pine in peat bog populations might be explained by extreme environmental conditions, small population size, and human interventions. Scots pine peat bog populations are found on flat terrain and are more accessible compared to natural Scots pine populations located on rocky steep slopes in Eastern and Southern Carpathian Mountains [28]. The mean number of alleles and the number of private alleles also have the lowest values in the peat bog populations. These populations grow in habitats with extreme natural conditions, where a strong selection pressure is assumed, which might potentially lead to a reduction of genetic diversity. No signs of recent bottlenecks were revealed in our analysis but we used only eight nSSR markers. Interestingly, most of the private alleles were observed in the Southern Carpathian populations that grow on rocky slopes. Sampling in such small, relict populations was very difficult because of the rocky terrain. This fact supports the hypothesis that sampled Southern Carpathian populations are untouched by man and may harbor rare variants. In a recent study [53], the only Scots pine population sampled in the Southern Carpathians belongs to a gene pool typical for populations from the Western Carpathians in Hungary and the Apuseni Mountains, and not to a second gene pool corresponding to Eastern Carpathian populations. The region of Eastern Carpathians is considered a distinct glacial refugium for Scots pine [23].

The values of the fixation index (F_{IS}) observed in our study are in agreement with those previously reported in Scots pine [49,51]. Moreover, only in two out of the eight populations, the F_{IS} values were positive and differed significantly from zero, thus indicating an excess of homozygote individuals. Homozygote excess is a common phenomenon in conifer species and may be the result of selection against heterozygotes, assortative mating, or the presence of null alleles [54]. However, isolated and relict Scots pine populations from the Apennine Mountains do not show any significant excess of homozygotes [49], which is also the case in the majority of our Carpathian populations.

A relatively high level of genetic differentiation among Eastern and Southern Carpathian populations was revealed by AMOVA (6%), which is consistent with previous reports on Scots pine peripheral populations from Southeastern Europe [23,49,50]. Past demographic events rather than limited recent gene flow may explain this pattern of among population differentiation at nuclear level [23].

A weak geographic structure, with many admixed populations, was revealed by our analysis. No information about the geographic location of the populations was taken into consideration when running STRUCTURE software, compared to a previous study that used this kind of data (with LocPrior) [23]. The present-day population structure is a consequence of interglacial and postglacial evolutionary history of Scots pine in the Carpathian region. The existence of glacial refugia in the Carpathians [24,25], an admixture of phylogenetic lineages, and population expansions and contractions may have influenced the current gene pool of the species [23,29]. At present, Scots pine is

able to survive only in extreme site conditions (e.g., peat bogs, rocks), i.e., ecological niches in which it still remains more competitive than other broadleaved and conifer species [28].

According to STRUCTURE analysis, the two Scots pine populations located in the outward-oriented side of the Eastern Carpathian arch (E-TU and E-BI) are genetically very similar. A genetic discontinuity between this group of two populations and the rest of the Eastern Carpathian populations is supported by BARRIER analysis. Furthermore, the group of the two peat bog populations seems very distinct in STRUCTURE analysis (without LocPrior) but there is apparently a relatively strong genetic discontinuity between the two peat bog populations according to BARRIERS. However, when information on the geographic location was given in STRUCTURE, the peat bog population E-FB appeared to be in a different genetic cluster than the peat bog population E-PS (data not shown); thus, the results of two analyses were eventually congruent. Moreover, the same peat bog population E-FB is located to the inside of the Carpathian arch, being the most central Scots pine population in our sample and, thus, more isolated from the other Eastern Carpathian populations. As suggested by previous results [23], no strong barrier was detected between Eastern and Southern Carpathian populations.

Spatial Genetic Structure

A statistically significant SGS was detected in nearly all studied Scots pine populations. Limited gene dispersal by pollen and seed in accordance with isolation by distance hypothesis may explain the pattern of SGS [9]. Most seeds fell under the canopy of mother trees in relict, mountainous Scots pine populations in Southern Spain [55]. The existence of SGS is expected in untouched, natural populations, even at the adult stage, as was the case in our study. For example, SGS was detected in an old-growth Eastern white pine forest [56] or in a natural, mixed oak forest [12]. The lack of SGS in one peat bog Scots pine population (E-PS) might be explained by the history of the stand, including human interventions, as well as by the sampling design. Thus, sampling of nearby, presumably related individuals up to 15–20 m was, in general, avoided. This fact might have been influenced the strength of SGS in sampled Scots pine populations.

The strength of the SGS, as indicated by the S_p statistics, varied greatly among our Scots pine populations. However, the mean value across populations (0.0100) obtained in our study is consistent with S_p values reported for outcrossing (0.0126) and tree species (0.0102), respectively [9]. The strength of SGS was slightly lower in two mature managed stands of Scots pine (range: 0.0045–0.0098) [15]. A weaker SGS (Sp = 0.0018) was reported for Alpine populations of *P. cembra*, a species with bird-mediated seed dispersal [13]. The variation in S_p value may be connected with the sampling scheme and population density. Thus, sampling of groups of individuals at different locations hundreds of meters apart within two mountainous Scots pine populations, because not enough individual trees were found at each location, might explain the lower values for the S_p statistic, 0.0036 and 0.0049, in populations S-RE and S-VS, respectively. The S_p value is lower in high-density as compared with low-density populations [9]. This might be an explanation for the lowest S_p value (0.0012) obtained in the relatively high-density population E-PS. The highest S_p value was calculated for population E-TU (0.0207), which had a lower density when compared to other sampled populations. Similar values (0.02–0.026) were reported in small, isolated remnants of maritime pine in the Iberian Peninsula [6]. Deviations from random mating, a lower population density, and potential grouping of reproductive individuals might explain a significant and stronger SGS in small and isolated populations compared to continuous ones [6].

A limitation of our study is the low number of nuclear genetic markers used. However, the number of SSR makers we employed is very similar to recent studies on population genetic structure in Scots pine [23,29]. Furthermore, different sampling schemes (e.g., along one or two transects, consisting of more subpopulations) within Scots pine populations were adopted because of both the spatial configuration of the terrain in the Carpathian Mountains and the scattered distribution of native Scots pine individual trees.

5. Conclusions

The present study, along with previous reports on genetic diversity in Carpathian populations [23], may contribute to the development of a strategy for sustainable management and conservation of the last remnants of Scots pine in the Romanian Carpathians. Scots pine plantations were established with seed imported from other regions (e.g., presumably Central Europe) without a strict record of this transfer [28]. Unfortunately, the use of local seed as reproductive material for forestry purposes was completely neglected. The conservation of Scots pine genetic resources in the Carpathian Mountains should not rely exclusively on in situ conservation units. The establishment of ex situ conservation stands should be an alternative for a better conservation and use of this unique gene pool.

Our study, which was based on the sampling of native populations to both sides of the Carpathian Arch, confirms previous reports that indicate relatively high genetic diversity within populations in spite of a reduction and recent fragmentation of the Scots pine distribution area. We found evidence for lower genetic diversity in peat bog Scots pine populations, compared to populations that grow on nutrient-poor soils and rocky slopes in the Carpathian Mountains. A weak geographic structure of genetic diversity along Southern and Eastern Carpathians was revealed, which may be explained by the postglacial admixture of populations originating from different glacial refugia that existed in the region. The patterns of SGS detected in natural Scots pine populations can be explained by limited seed dispersal, as well as by other factors such as spatial configuration of the population, sampling scheme, and population density.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/10/1047/s1: Figure S1. Estimation of population structure using *LnP(D)*-derived Δk for determining the optimum number of subpopulations. The maximum value of delta k was found to be at $k = 2$.

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Chloroplast DNA Diversity in Populations of *P. sylvestris* L. from Middle Siberia and the Romanian Carpathians

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Abstract: Scots pine is one of the dominant conifer species in forest ecosystems of the boreal zone in Eurasia. Knowledge of the genetic structure and the level of genetic variability of Scots pine populations is relevant for the development of measures aimed at conservation of species' diversity. In this study, we used ten paternally inherited chloroplast microsatellite loci to investigate the genetic diversity of nineteen Scots pine populations from Middle Siberia and the Romanian Carpathians. The results of the study showed high genetic diversity (H_{CP} = 0.91–1.00) in all of the investigated populations. The cpSSR analysis yielded a total of 158 haplotypes. The majority of the haplotypes (85%) were detected only once (unique haplotypes). Three common haplotypes were found between the Carpathian and the Siberian populations of Scots pine. Analysis of molecular variance (AMOVA) showed that only 3% of the variation occurred among populations from Middle Siberia and 6% of the variation existed among populations from the Carpathian Mountains. Overall, we found a weak geographic population structure in Scots pine from Middle Siberia and the Romanian Carpathians. The present study on genetic diversity in the Siberian and the Carpathian populations of Scots pine may contribute to the sustainable management and conservation of Scots pine genetic resources in Middle Siberia and the Romanian Carpathians.

Keywords: Scots pine; chloroplast DNA; relict populations; genetic diversity; Siberia; Carpathians

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1. Introduction

Genetic diversity is the basis for biological stability; it allows species to evolve and to adapt to changing environmental conditions [1]. Knowledge of the genetic structure and the level of genetic variability of populations is relevant for the development of measures aimed at the conservation of species' genetic diversity [2].

Scots pine (*Pinus sylvestris* L.) is one of the keystone species in forest ecosystems of the boreal regions in Eurasia. It is of great ecological and economic importance and is adapted to a variety of environmental conditions [3,4]. Scots pine is a monoecious, wind-pollinated and predominantly outcrossing conifer [5–7]. It usually forms extensive pure forests or mixed stands with birch and other conifers [8].

In Russia, Scots pine is one of the main forest-forming tree species and it covers 15.6% of Russian forests [9]. A significant part of its distribution is located in Siberia, where it reaches the northern border of woody vegetation distribution (bog massifs in the West Siberian plain), the zone of dry steppes in the south (isolated groves in Altay, Khakassia,

Tuva, and Buryatia regions) and rocky mountain slopes (Kuznetsk Alatau Mts. and the Sayan Mts.) [10–12]. In Siberia, Scots pine forests form a complex spatial mosaic, which is determined by an adaptive compromise of Scots pine among the phytocenotic competition with the other forest-forming species and its edaphic preferences. In the Angara River basin, Scots pine forms a continuous distribution range, occupying a variety of soils [12]. Long-term intensive exploitation of pine forests in Russia has led to their fragmentation across the whole territory [13,14]. Over the last decade, the area of Scots pine forests in Russia has decreased by 2 million ha [15]. Consequently, the characterization of the genetic variability of natural Scots pine populations in Russia is an important prerequisite for better use of their genetic resources in breeding and biodiversity conservation programs. Unlike Russia, where Scots pine has a vast distribution area, in Romania it occurs sparsely across the Eastern and Southern Carpathian Mountains, where a few relict populations still exist. These populations grow in ecologically extreme habitats, where a strong selection pressure is assumed [7]. Knowledge about the level of genetic diversity present in relict populations is important for preparing and implementing protective measures for these small and endangered populations of Scots pine in the Romanian Carpathians [16].

In recent years, different types of molecular markers have been used for the assessment of the genetic diversity and genetic structure in tree species [7,17–20]. Among them, chloroplast simple sequence repeats (a.k.a SSRs or microsatellites) are useful markers in population genetic diversity evaluation, population structure analysis and phylogenetic studies [4,16,21–27]. Chloroplast microsatellites are characterized by high polymorphism, uniparental inheritance, and a lack of sexual recombination [28]. For many years, chloroplast simple sequence repeats (cpSSRs) have been one of the tools used to characterize tree genetic resources, including Scots pine [29–32].

A number of studies, based on chloroplast microsatellites, have shown that the genetic variation of Scots pine is generally high and accumulated mainly within populations, while genetic differences among populations are fairly small [4,5,21]. This homogeneity appears particularly as an effect of common ancestry in a recent evolutionary history, as well as an extensive gene flow, especially through pollen. Since the pollen of Scots pine has a great mobility potential, the homogeneity could be easily maintained over long distances [33].

In this study, we used chloroplast microsatellite markers to characterize the level of genetic diversity of Scots pine populations in two geographic regions of its Eurasian natural range with different ecological settings and evolutionary history. Specifically, we aimed to address the following questions: (1) What is the pattern of genetic structure and diversity in Scots pine populations from Middle Siberia and the Romanian Carpathians? (2) How large is the degree of genetic differentiation between the two Scots pine distribution regions at the chloroplast DNA level?

2. Materials and Methods

2.1. Plant Material

Nineteen native populations of Scots pine were chosen within the natural distribution range of the species in Middle Siberia and the Romanian Carpathians (Table 1, Figure 1).

Scots pine is a dominant species in all studied populations. Four of them (Pop-A:Pop-D,) are located in taiga forest zone, three (Pop-E:Pop-G) grow in forest-steppe zones and eight (Pop-H:Pop-O) are distributed in the Southern Siberian mountain zone [34]. Four relict populations of Scots pine (Pop-P:Pop-S) are located in the Southern and Eastern Carpathian Mountains. Ten adult trees were randomly chosen in each population. Consequently, the total number of analyzed individuals was 190. Needles collected from the trees were stored in silica gel until DNA extraction was carried out.

Table 1. Geographical location of 19 Scots pine populations in Middle Siberia and the Romanian Carpathians.

No.	Population/Name/ Federal Region	N	Forest Zone	Latitude/Longitude	Altitude (m)
	Middle Siberia				
1	Pop-A/Vanavara_2/Krasnoyarsk krai	10	T	60.36/102.37	260–270
2	Pop-B/Vanavara_1/Krasnoyarsk krai	10	T	60.24/102.43	350–360
3	Pop-C/Chunoyar/Krasnoyarsk krai	10	T	57.44/97.37	165–170
4	Pop-D/Borzovo/Krasnoyarsk krai	10	T	57.17/97.27	275–280
5	Pop-E/Sukhobuzimskoye_2/Krasnoyarsk krai	10	Fs	56.50/93.22	155–160
6	Pop-F/Sukhobuzimskoye_1/Krasnoyarsk krai	10	Fs	56.30/92.97	280–290
7	Pop-G/Zeledeevo/Krasnoyarsk krai	10	Fs	56.22/92.24	325–330
8	Pop-H/Uyar/Krasnoyarsk krai	10	SSm	55.89/93.82	360–370
9	Pop-I/Shalo/Krasnoyarsk krai	10	SSm	55.76/93.76	340–350
10	Pop-J/Narva/Krasnoyarsk krai	10	SSm	55.45/93.73	360–380
11	Pop-K/Sarala_2/Republic of Khakassia	10	SSm	55.00/89.40	550–560
12	Pop-L/Sarala_1/Republic of Khakassia	10	SSm	54.87/89.22	500–520
13	Pop-M/Yrban/Republic of Tuva	10	SSm	52.72/95.74	860–980
14	Pop-N/Balgazyn_1/Republic of Tuva	10	SSm	51.08/95.09	970–1060
15	Pop-O/Balgazyn_2/Republic of Tuva	10	SSm	51.02/95.28	880–890
	Romanian Carpathians				
16	Pop-P/Poiana Stampei	10	ECm	47.30/25.12	920
17	Pop-Q/Cheile Bicazului	10	ECm	46.83/25.80	1060–1110
18	Pop-R/Retezat	10	SCm	45.44/22.78	680–750
19	Pop-S/Lotrişor	10	SCm	45.30/24.28	340–510

Note: Sample size (N); Taiga forest zone (T); Forest-steppe zone (Fs); Southern-Siberian mountain zone (SSm); Southern Carpathian Mountains (SCm); Eastern Carpathian Mountains (ECm).

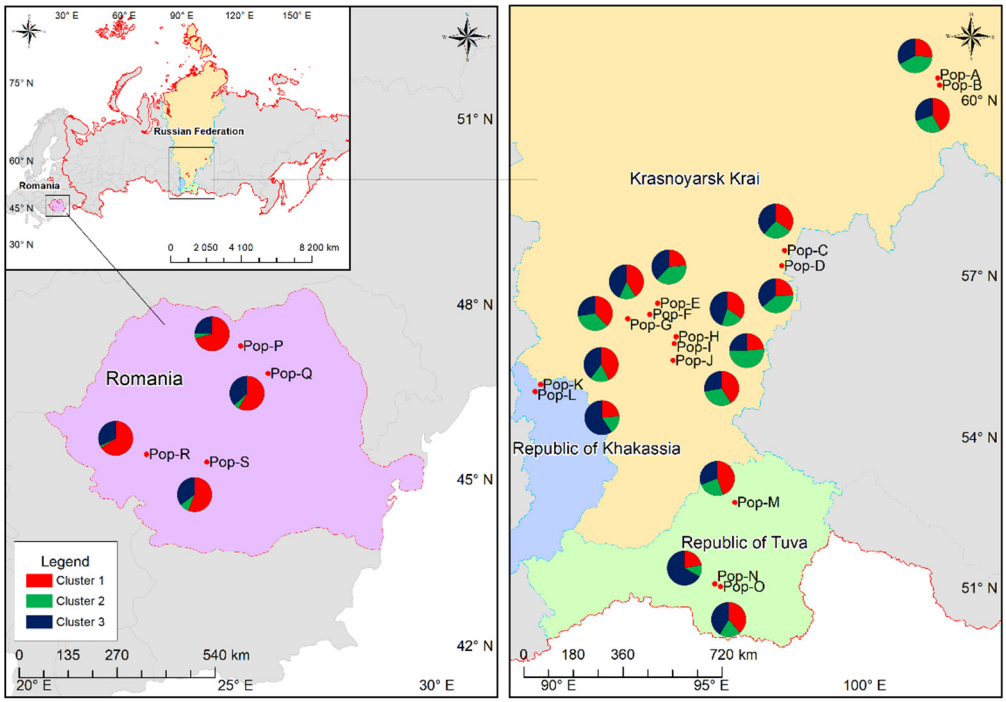


Figure 1. Geographical location of the studied Scots pine populations in Middle Siberia and the Romanian Carpathians (for the abbreviations, see Table 1). Populations of Scots pine are indicated by red dots. The sector maps show the mean cluster membership proportions of the analyzed individuals in each of the 19 Scots pine populations based on the genetic structure at K = 3.

2.2. Molecular Analysis

Genomic DNA was extracted from dried needles with the CTAB method [35]. The quality and concentration of the extracted DNA were measured with a Nanodrop 8000 spec-

trophotometer (Thermo Fisher Scientific, Waltham, MA, USA), then diluted to a concentration of 10–20 ng/µL. Ten chloroplast microsatellite markers were chosen for the genetic analysis: PCP45071, PCP36567, PCP48256, PCP41131, PCP30277, PCP26106, Pt1254, Pt15169, Pt71936, and Pt7268 [28,29]. The cpSSR loci were amplified in two PCR multiplex reactions in a total volume of 10–12 µL using Qiagen Multiplex PCR Kits (Qiagen, Hilden, Germany) under conditions recommended by the manufacturer. PCR amplification was performed in a Corbett thermal cycler (Corbett Research, Mortlake, NSW, Australia) with the following conditions: an initial denaturation of 15 min at 95 °C, then 30 cycles of 15 s at 94 °C, 1 min 30 s at 60 °C (for the set of 6 PCP loci) and 58 °C (for the set of 4 Pt loci), 1 min 30 s at 72 °C, and a final extension of 10 min at 72 °C. Amplified fragments were analyzed on a GenomeLab GeXP Genetic Analyzer (Beckman Coulter, Fullerton, CA, USA) with an internal size standard. Fragment sizing was performed using the GenomeLab GeXP software (Version 10.2, Beckman Coulter, Fullerton, CA, USA).

2.3. Statistical Analysis

POPGENE ver. 1.31 [36] and GenAlEx v. 6.5 [37] were used to estimate the following genetic diversity parameters: the observed number of alleles per locus (*N*a); the mean number of effective alleles (*N*e); Shannon's Information index (*I*); Nei's gene diversity index (*H*); Gene flow (*N*m); haploid genetic diversity (*h*).

Chloroplast DNA haplotypes were determined as a combination of the different microsatellite variants across the ten cpSSR loci. HAPLOTYPE ANALYSIS ver. 1.05 [38] was used to estimate the number of different haplotypes (*A*), number of private haplotypes (*P*), the effective number of haplotypes (N_E), the haplotype diversity (H_{CP}), and the mean genetic distance between haplotypes (D^2sh).

The unweighted pair-group method with arithmetic mean (UPGMA) was used to perform a cluster analysis on the Nei's genetic distances data [39] and STATISTICA software v.8 was used to build a tree diagram [40].

Analysis of the molecular variance (AMOVA) and the Mantel test were performed using GenAlEx v. 6.5 [37]. The statistical significance of the AMOVA and Mantel test were determined for all populations and loci, with the number of permutations set to 999.

Population structure was analyzed using STRUCTURE ver.2.3.4 with a Bayesian clustering approach [41]. Testing ten independent runs with K from 1 to 19, each run had a burn-in period of 50,000 iterations and 100,000 Monte Carlo Markov iterations, assuming no admixture model, correlated allele frequencies, and no prior information on the sampling location. The studied populations were separated into groups by the Structure Harvester program [42], based on ΔK values [43].

3. Results

3.1. Microsatellite Analysis

In total, 49 alleles were observed at ten cpSSR loci across all 190 individuals in the nineteen populations of Scots pine (Table 2). Two loci, PCP45071 and the Pt71936, showed the largest number of alleles, while the locus Pt15169 showed the highest values for *N*e (3.97), *I* (1.53), and h (0.75). The mean *I*, *H*, and *N*m values were 0.94 (0.46–1.53), 0.49 (0.20–0.75), and 3.40 (2.04–6.16), respectively (Table 2).

The mean number of alleles and effective number of alleles were slightly lower in the Siberian populations (*N*a = 2.64; *N*e = 1.95) when compared with the Carpathian (*N*a = 2.73; *N*e = 2.08) (Table 3). The *I* ranged from 0.38 (Pop-N) to 0.88 (Pop-J) in the Siberian populations and from 0.60 (Pop-R) to 0.81 (Pop-Q) in the Carpathian populations. The average genetic diversity (*h*) of the ten analyzed cpSSR loci in the Siberian and the Carpathian populations of Scots pine was 0.42. The results showed that the ten cpSSR loci used in this study had high polymorphism in the Scots pine populations from Middle Siberia and the Romanian Carpathians.

Table 2. Diversity indices for each SSR marker across 190 Scots pine individuals.

ID	*Na*	*Ne*	*I*	*H*	*Nm*
PCP36567	3	1.83	0.78	0.45	3.64
PCP48256	3	1.82	0.69	0.45	2.04
PCP41131	5	1.25	0.46	0.20	3.92
PCP30277	6	2.73	1.27	0.63	2.60
PCP26106	3	1.66	0.71	0.40	3.60
PCP45071	7	2.31	1.08	0.57	3.78
Pt1254	5	1.63	0.79	0.39	2.61
Pt15169	6	3.97	1.53	0.75	2.95
Pt71936	7	3.08	1.39	0.67	2.74
Pt87268	4	1.58	0.71	0.37	6.16
Mean	4.9	2.19	0.94	0.49	3.40
SD	1.60	0.84	0.35	0.17	0.38

Note: Observed number of alleles per locus (*Na*); Mean number of effective alleles (*Ne*); Shannon's Information index (*I*); Nei's gene diversity index (*H*); Gene flow (*Nm*); SD-standard deviation.

Table 3. Genetic diversity of Scots pine populations in Middle Siberia and the Romanian Carpathians based on chloroplast microsatellite loci.

Population	*N*	*Na*	*Ne*	*I*	*h*
Middle Siberia					
Pop-A	10	2.30 (±0.40)	2.02 (±0.35)	0.63 (±0.16)	0.39 (±0.08)
Pop-B	10	2.80 (±0.33)	2.14 (±0.33)	0.78 (±0.12)	0.46 (±0.06)
Pop-C	10	2.70 (±0.26)	1.92 (±0.27)	0.70 (±0.12)	0.40 (±0.06)
Pop-D	10	2.50 (±0.22)	1.85 (±0.16)	0.70 (±0.08)	0.43 (±0.05)
Pop-E	10	2.50 (±0.37)	1.96 (±0.27)	0.66 (±0.15)	0.39 (±0.09)
Pop-F	10	2.80 (±0.33)	1.99 (±0.18)	0.77 (±0.11)	0.45 (±0.06)
Pop-G	10	2.90 (±0.38)	2.06 (±0.29)	0.77 (±0.12)	0.44 (±0.06)
Pop-H	10	2.60 (±0.34)	1.80 (±0.15)	0.65 (±0.13)	0.37 (±0.07)
Pop-I	10	2.70 (±0.21)	2.08 (±0.20)	0.78 (±0.10)	0.47 (±0.06)
Pop-J	10	3.00 (±0.37)	2.35 (±0.30)	0.88 (±0.12)	0.51 (±0.06)
Pop-K	10	2.90 (±0.35)	2.11 (±0.27)	0.81 (±0.11)	0.47 (±0.05)
Pop-L	10	2.30 (±0.21)	1.54 (±0.14)	0.53 (±0.10)	0.31 (±0.06)
Pop-M	10	2.70 (±0.15)	2.06 (±0.15)	0.80 (±0.07)	0.49 (±0.04)
Pop-N	10	1.80 (±0.20)	1.41 (±0.12)	0.38 (±0.10)	0.24 (±0.06)
Pop-O	10	3.10 (±0.28)	2.03 (±0.17)	0.83 (±0.09)	0.47 (±0.05)
Overall Mean		2.64 (±0.29)	1.95 (±0.22)	0.71 (±0.11)	0.42 (±0.06)
Romanian Carpathians					
Pop-P	10	2.80 (±0.36)	2.17 (±0.27)	0.78 (±0.15)	0.45 (±0.08)
Pop-Q	10	3.30 (±0.50)	2.16 (±0.35)	0.81 (±0.17)	0.43 (±0.08)
Pop-R	10	2.30 (±0.42)	1.97 (±0.37)	0.60 (±0.17)	0.35 (±0.09)
Pop-S	10	2.50 (±0.31)	2.00 (±0.19)	0.73 (±0.10)	0.46 (±0.05)
Overall Mean		2.73 (±0.40)	2.08 (±0.30)	0.73 (±0.15)	0.42 (±0.08)

Note: Sample size (*N*); Mean number of alleles per locus (*Na*); Number of effective alleles (*Ne*); Shannon's Information Index (*I*); Haploid genetic diversity (*h*); ±standard errors in parentheses.

3.2. Chloroplast DNA Haplotype Variation

The cpSSR analysis of 190 individuals of Scots pine yielded a total of 158 haplotypes. The majority of the haplotypes (85%) were detected only once (unique haplotypes). The four Carpathian and three Siberian populations of Scots pine (Pop-C/Chunoyar, Pop-G/Zeledeevo and Pop-H/Uyar) were characterized by the highest number of private haplotypes (nine), while the lowest number (five) of private haplotypes was recorded in two Siberian populations (Pop-F/Sukhobuzimskoye_1 and Pop-N/Balgazyn_1 populations) (Table 4).

Table 4. Statistical characteristics of cpSSR haplotypes for 19 Scots pine populations.

Population	*A*	*P*	N_E	H_{CP}	D^2_{sh}
		Middle Siberia			
Pop-A	10	7	10.00	1.00	4.17
Pop-B	10	7	10.00	1.00	5.32
Pop-C	10	9	10.00	1.00	4.38
Pop-D	8	6	7.14	0.96	4.47
Pop-E	10	6	10.00	1.00	5.02
Pop-F	9	5	8.33	0.98	5.43
Pop-G	10	9	10.00	1.00	4.32
Pop-H	10	9	10.00	1.00	3.72
Pop-I	10	8	10.00	1.00	4.41
Pop-J	10	6	10.00	1.00	7.52
Pop-K	10	8	10.00	1.00	5.18
Pop-L	9	6	8.33	0.98	1.90
Pop-M	10	7	10.00	1.00	4.41
Pop-N	7	5	5.56	0.91	0.98
Pop-O	9	7	8.33	0.98	5.96
Mean	9.47	7.00	9.18	0.99	4.48
		Romanian Carpathians			
Pop-P	10	9	10.00	1.00	7.55
Pop-Q	10	9	10.00	1.00	6.59
Pop-R	10	9	10.00	1.00	3.97
Pop-S	9	9	8.33	0.97	5.11
Mean	9.75	9.00	9.58	0.99	5.81

Note: Number of haplotypes (*A*); Number of private haplotypes (*P*); Effective number of haplotypes (N_E); Haplotype diversity (H_{CP}); Mean genetic distance between individuals (D^2sh).

Haplotype H112 is common to six of the studied populations (Pop-A/Vanavara_2, Pop-E/Sukhobuzimskoye_2, Pop-F/Sukhobuzimskoye_1, Pop-H/Uyar, Pop-K/Sarala_2, Pop-N/Balgazyn_1) that were located in taiga, forest-steppe, and Southern Siberian mountain zones in Middle Siberia (Figure 2). The frequency of the H112 haplotype is the highest in the Pop-N/Balgazyn_1 population (30%) (Table S1). Two haplotypes, H64 and H122, are common to three Siberian populations and eleven haplotypes are common to two populations that were located in the same geographical region. Only three common haplotypes (H86, H107, H118) were found in two Siberian (Pop-J and Pop-O) and three Carpathian populations of Scots pine (Pop-P, Pop-Q and Pop-R). High values of haplotype diversity (H_{CP} = 0.91–1) were revealed within all studied populations (Table 4). The effective number of haplotypes varied from 5.56 to 10 and the mean genetic distance between individuals ranged from 0.98 (Pop-N/Balgazyn_1) to 7.55 (Pop-P/Poiana Stampei).

3.3. Population Genetic Structure of Scots Pine

The values of Nei's genetic distance among populations ranged from 0.0032 (Pop-J/Pop-K) to 0.2441 (Pop-B/Pop-P) (Table S2). UPGMA clustering showed that two groups were separated at the population level (Figure 3). The first group consisted of fifteen Middle Siberian populations and the second group was composed of four Carpathian populations.

The hierarchical analysis of molecular variance (AMOVA) showed that the variation among two geographic regions (Middle Siberia and Carpathian Mountains) accounted for 5% of the total variance. The variance among populations within regions was 3% ($p < 0.01$). The AMOVA performed within the Siberian populations of Scots pine showed a lower differentiation among populations (PhiPT = 3%, $p < 0.05$) when compared to the Carpathian populations (PhiPT = 6%; $p < 0.01$) (Table 5).

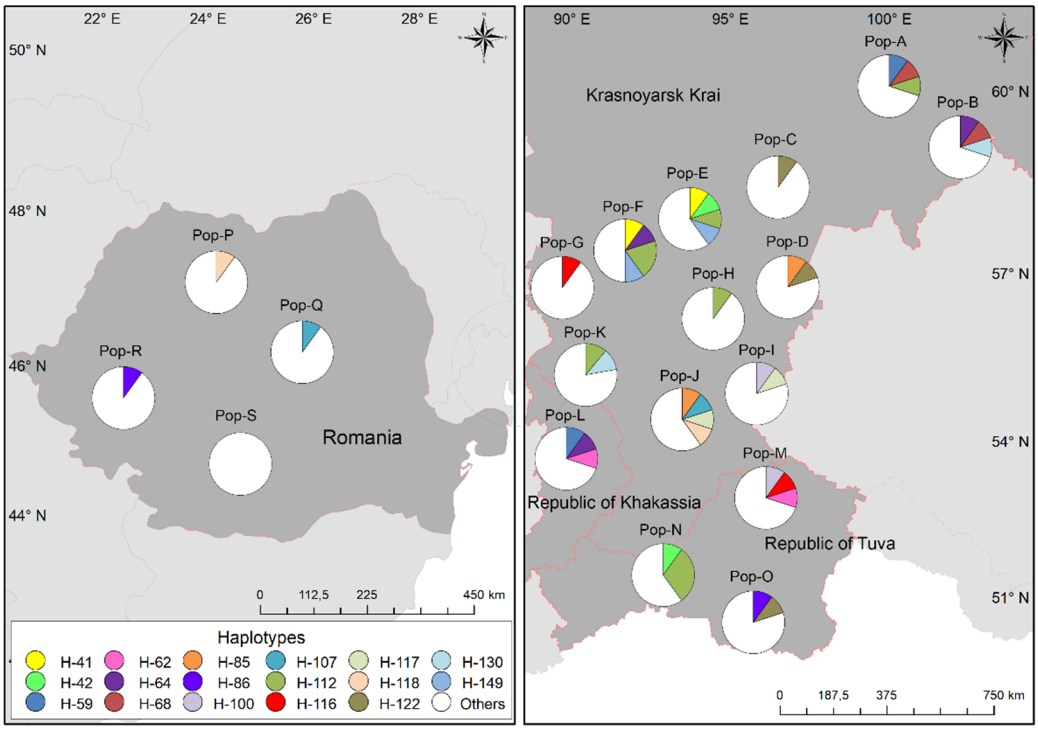


Figure 2. Geographic distribution of the 17 common chloroplast DNA haplotypes in 19 Scots pine populations (for the abbreviations, see Table 1) studied in Middle Siberia and the Romanian Carpathians (Table S1).

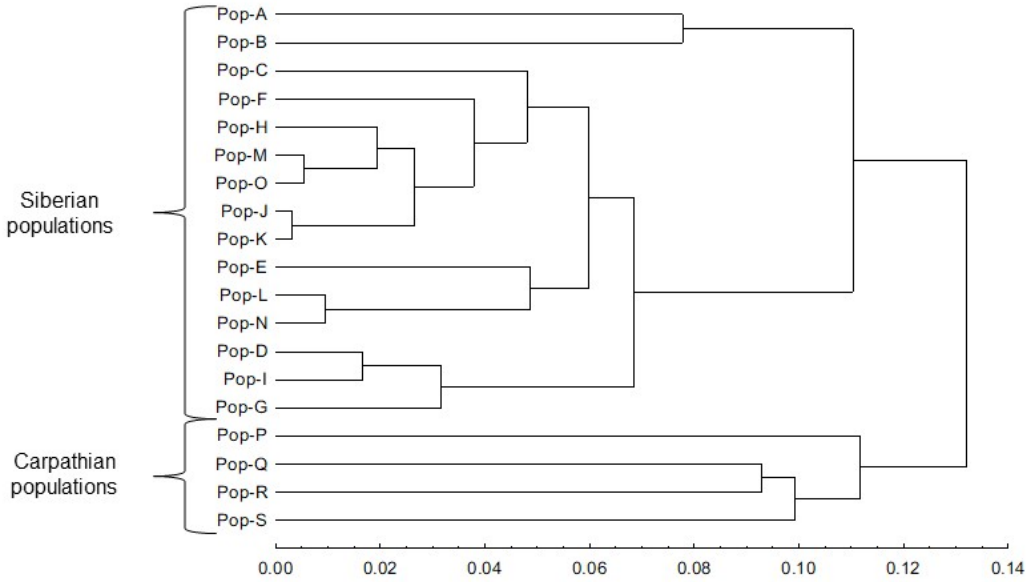


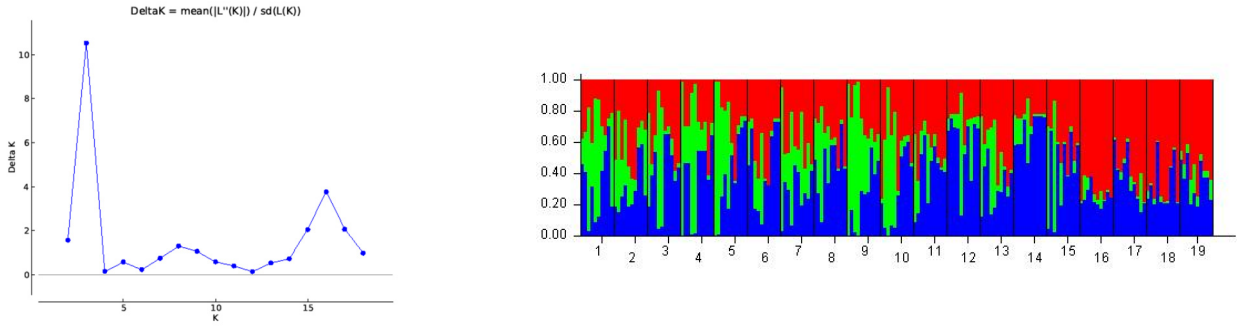
Figure 3. UPGMA dendrogram based on Nei's genetic distances among populations of Scots pine.

The population structure analysis of the Siberian and Carpathian populations of Scots pine showed that ΔK was the highest when K = 3 (Figure 4). All individual trees showed admixture from three genetic clusters. For K = 3, the Siberian populations had a larger membership in cluster 3 (blue color) than the Carpathian populations. The membership in cluster 1 (red color) was higher in the case of the Carpathian populations (Figure 1).

Table 5. Hierarchical analysis of molecular variance (AMOVA).

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	*p*
Among regions	1	10.829	0.121	5	
Among populations	17	54.113	0.085	3	<0.01
Within populations	171	398.900	2.333	92	
Total	189	463.842	2.539	100	
Siberian populations					
Among populations	14	88.880	0.147	3	
Among individuals Within populations	135	659.000	4.881	97	<0.05
Total	149	747.880	5.028	100	
Carpathian populations					
Among populations	3	11.600	0.153	6	
Among individuals Within populations	36	84.200	2.339	94	<0.01
Total	39	95.800	2.492	100	

Note: Degrees of freedom (d.f.); Probability (*p*).



(**A**) (**B**)

Figure 4. Population genetic structure of 19 Scots pine populations in Middle Siberia (Pop 1–15) and the Romanian Carpathians (Pop 16–19). (**A**) Estimation of the best subpopulation numbers based on appropriate K value: the mean ΔK values among the 10 runs reached a maximum at K = 3. (**B**) Structure clustering results obtained for three genetic clusters (Populations are numbered 1–19 as in Table 1).

We further analyzed the correlation between genetic distance and geographic distance for the studied populations using the Mantel test. The results showed a low but significant correlation between genetic differentiation and geographical distance among Siberian populations (R^2 = 0.1746, $p < 0.01$) and there was no significant correlation between genetic distance and geographic distance among Carpathian populations (R^2 = 0.0059, $p > 0.05$).

4. Discussion

The genetic diversity and differentiation of Scots pine populations from different forest zones in Middle Siberia and the Romanian Carpathians were assessed based on the polymorphism of ten cpSSR markers. Our results suggest a high genetic diversity (H_{CP} = 0.91–1.00) in all Scots pine populations and are in line with other regional studies on Scots pine in Europe and Asia [5,21,29]. Nonetheless, in the present study, the four relict pine populations from the Carpathian region were characterized by a relatively higher level of genetic diversity, i.e., the number of alleles and haplotypes, the number of private haplotypes, the effective number of haplotypes, and the mean genetic distance between individuals within the population. The higher level of genetic diversity in the Carpathian populations might be a consequence of the interglacial and postglacial evolutionary history of Scots pine in Southeastern Europe. The existence of glacial refugia in the Carpathian

Mountains and an admixture of phylogenetic lineages may explain a higher level of genetic diversity in this region [7].

In Middle Siberia, the two northernmost populations showed a slightly higher haplotype diversity (H_{CP} = 1.00) than the two southernmost populations (H_{CP} = 0.91 and H_{CP} = 0.98, respectively) (Table 4). A similar trend has also been reported for Scots pine populations from Fennoscandia, which showed a higher haplotype diversity (H_{CP} = 0.807) than those from the south European populations (H_{CP} = 0.750) [4]. Such patterns of genetic diversity may result from peculiarities of post-glacial history of the species, such as the mixing of different refugial lineages [4]. Among the Siberian pine populations, the highest number of private haplotypes was detected in the populations from the core distribution area (Pop-G/Zeledeevo, Pop-H/Uyar and Pop-C/Chunoyar). The process of colonization might trigger new genetic variants for neutral genetic diversity in these populations [4]. The lowest haplotype number (seven) was detected in the Pop-N/Balgazyn_1 population, which refers to the southernmost pine forest of Northern Asia. The Balgazyn pine forest belongs to the category of valuable forests in terms of its species composition, productivity, and genetic quality, as well as performing particularly important protective functions in sharply continental climate [44]. Moreover, it is considered as a Holocene relic and, currently, is of a high conservation priority [45]. Interestingly, another population Pop-O/Balgazyn_2, which refers to the same pine forest, showed a higher haplotype number (nine). Such discrepancy between adjacent populations located at a relatively small geographical distance (21 km) may result from large natural disturbances (forest fires), which occurred in Pop-N population between 1988 and 2014 [44,45]. Local fires may be the cause of the reduction in the haplotype number observed in Pop-N. Decrease of population size, due to fires, logging, pest and diseases, and environmental pollution may lead to a decline in genetic diversity [46,47]. Another explanation would be the relatively small sample size (10 individuals per population).

AMOVA showed a low level of genetic structure (PhiPT = 3%) among the fifteen Siberian populations, even though they are scattered over large distances of more than 1000 km. In contrast, the genetic variation among the four relict Carpathian populations accounted for 6% of the total variance, although the geographic distances among them are much smaller. More significant geographic barriers among the Carpathian populations than among the Siberian ones may have contributed to a higher level of genetic differentiation. However, the low overall genetic differentiation among the Scots pine populations obtained in this study is consistent with previous reports in *P. sylvestris* using chloroplast markers [5,29]. Low values of the PhiPT (0.41–1.7%) were also detected by Semerikov et al. [21] between 38 Scots pine populations from Asia and the European part of Russia. Based on STRUCTURE analysis, three clusters were defined in the Siberian and the Carpathian populations of Scots pine where each individual was comprised of three genetic groups, showing a weak genetic structure between the two geographic regions, which is consistent with the grouping revealed by the UPGMA dendrogram.

There was a weak positive correlation between the genetic and geographic distances for the analyzed populations of Scots pine in Middle Siberia. Pine species are efficient in pollen dispersal, resulting in a low overall population structure and differentiation [8,48]. Furthermore, considering the large geographical areas covered by the investigated populations in Middle Siberia, it seems that their genetic similarity results from a shared phylogeographic history. Additionally, this can also be confirmed by a widespread occurrence of the H112 haplotype across the Siberian populations. Moreover, three common haplotypes were detected in the Carpathian and the Siberian populations of Scots pine. The presence of common haplotypes in two geographical regions located far away from each other may also suggest a common origin of the Siberian and Carpathian pine populations. However, this is highly unlikely; extensive gene flow over large distances via pollen may be the most likely hypothesis.

Our findings, based on chloroplast DNA analysis, indicate that a high genetic diversity of individuals exists in Scots pine populations from Middle Siberia and the Romanian

Carpathians and that, despite large geographic distances and barriers, there is limited genetic differentiation.

5. Conclusions

In the present study, ten cpSSR markers were employed to estimate the genetic diversity within and among nineteen natural populations of Scots pine in Middle Siberia and the Romanian Carpathians. All populations showed high levels of genetic diversity. However, one of the southernmost Siberian populations showed the lowest level of haplotype diversity. Accordingly, the performance of this population should be the focus of long-term study, aimed at the monitoring of population dynamics. Three common haplotypes were found among the Siberian and the Carpathian populations, which can be explained by very efficient long-distance gene flow or common ancestry. A weak genetic structure between the two geographic regions was revealed. Our study may contribute to the development of a strategy of sustainable management of Scots pine genetic resources in Middle Siberia and the Romanian Carpathians.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12121757/s1, Table S1: Frequency of the 17 common haplotypes within Scots pine populations, Table S2: Nei's genetic distances of 19 Scots pine populations.

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Research Article

Comparative analysis of genetic diversity in Norway spruce (*Picea abies*) clonal seed orchards and seed stands

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Abstract

Norway spruce, *Picea abies* (L.) Karst. is the most important conifer species in Romania and the most planted tree species in the Carpathian Mountains. Here we compare the genetic diversity of four Norway spruce clonal seed orchards and two seed stands located in the Eastern Carpathians. A set of highly polymorphic nuclear microsatellite markers was used. The analysis of genotypic identity of ramets for each Norway spruce clone in all seed orchards indicated that nearly all sampled ramets (97%) were genetically identical. The genetic diversity in seed orchards (*He*=0.700) was slightly smaller compared to the seed stands (*He*=0.718). Allelic richness was higher in seed stands (10.874), compared to clonal seed orchards (8.941). The Bayesian analysis indicated a genetic structure with two clusters, one corresponding to the clonal seed orchards and a second one consisting of the two seed stands. Our results provide valuable information for the management of Norway spruce seed orchards in Romania.

Keywords: genetic diversity; microsatellite; Norway spruce; seed orchards; seed stands

Introduction

Seed orchards are the most used method for obtaining forest reproductive materials with superior genetic properties (Funda *et al.*, 2009; Funda and El-Kassaby, 2012). Seed orchards are an important and efficient type of transmission of superior genetic traits to offspring, by creating synthetic varieties (Chaloupková *et al.*, 2019).

Long-term tree improvement implies, among other things, ensuring a balance between expected genetic gain and an appropriate level of genetic diversity, and the most common method of ensuring this is by establishing seed orchards (Tang and Ide, 2001). High genetic diversity in seed orchards may increase resilience and capacity to adapt to changing environments and thus productivity and quality of forest plantations. Genetic diversity among seed orchards crops is significantly influenced by the relatedness of orchard clones, parental fertility variation, and pollen contamination (Geburek, 1997; Ertekin, 2012). At the same time, of great importance is the number of parents involved in cloning the material for the installation of the seed orchard (Kang *et al.*, 2001; Lindgren and Prescher, 2005; Hansen, 2008; Sønstebø *et al.*, 2018).

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In this study, we focused on Norway spruce (*Picea abies* (L.) Karst.), one of the most important forest trees in the boreal and subalpine conifer forests (San-Miguel-Ayanz *et al.*, 2016). Norway spruce occupies approximately 30 million hectare (Jansen *et al.*, 2017; Schiop *et al.*, 2017) and it plays an important role for the society and economy. At present, it is the most common conifer tree species in Romania, occupying approximately 1.488.000 ha and 23.2% of the forest cover (Budeanu *et al.*, 2019). The study of genetic diversity of Norway spruce in Romania is summarized only in a few studies based on allozyme (Curtu *et al.*, 2009; Teodosiu, 2011; Radu *et al.*, 2014) and nuclear microsatellite markers (Mihai *et al.*, 2020) and no genetic analysis was done on seed orchards. In Romania, there have been installed only nine Norway spruce seed orchards that occupy approximately 72.9 ha (Mihai *et al.*, 2019).

Previous studies that aimed at comparing genetic diversity between natural populations and seed orchards have shown a higher genetic diversity in seed orchards than in natural populations (Muona and Harju, 1989; El-Kassaby, 1992; Chaisurisri and El-Kassaby, 1994; Stoehr and El-Kassaby, 1997; Williams *et al.*, 2001) in terms of allelic diversity and heterozygosity. In general, in forest tree species with a high degree of polymorphism, phenotypic selection in the early stages of breeding does not imply a significant reduction in genetic variability, as in *Picea abies* (Bergmann and Ruetz, 1991) or *Picea glauca* (Namroud *et al.*, 2012). However, there are also data that indicate lower genetic diversity in seed orchards compared to natural populations, as an effect of the number of parents selected for cloning (Johnson and Lipow, 2002; Ilinov and Raevsky, 2017), or studies that report a similar genetic diversity (Ruņgis *et al.*, 2019). Given these differences, we aimed to assess the level of genetic diversity in Norway spruce seed orchards and seed stands in the Eastern Carpathians, a region with a widespread distribution of Norway spruce. The specific objectives were: 1) to assess the genetic identity of ramets for Norway spruce clones used in seed orchards and 2) to compare the genetic diversity in clonal seed orchards and seed stands using highly polymorphic DNA markers.

Materials and Methods

Sampling design

Four Norway spruce clonal seed orchards (Paltinoasa – Cso-P, Bodesti – Cso-B, Dalhauti – Cso-D and Alunis – Cso-A) and two seed stands (Cucureasa – Nat-C, Manastirea Casin – Nat-M) have been sampled (Table 1). The seed orchards are located in the Eastern Carpathian region and were established between 1970 and 1981 with a different number of vegetative copies of plus trees (Cso-P - 33, Cso-B - 33, Cso-D – 81 and Cso-A - 197). Most of the plus trees were selected in natural seed stands distributed across the Eastern Carpathian Mountains, only several plus trees used for Cos-D seed orchard originated from the Southern Carpathian Mountains (Table 1). The sampling was done in 2017 and some of the initial clones were not found in the field. At least one individual per clone was sampled in each seed orchard. Two ramets per clone were sampled randomly for most of the clones in all seed orchards to verify the clonal identity.

Table 1. Geographic location of Norway spruce clonal seed orchards and seed stands

Nr. crt.	Abr.	Population	Region of provenance for the source population *	Number of sampled individuals	Number of unique genotypes	Latitude/ Longitude
1	Cso-P	Paltinoasa	A2	50	27	47.571791/ 25.941412
2	Cso-B	Bodesti	A2,G3	90	54	47.042570/ 26.447690
3	Cso-D	Dalhauti	A2,B2,C1	79	69	45.707435/ 27.007750
4	Cso-A	Alunis	A2, G3	190	154	46.325000/ 27.452275
5	Nat-C	Cucureasa	A2	56	56	47.397383/ 25.045132
6	Nat-M	Manastirea Casin	A2	77	77	46.168066/ 26.678455

*Region of provenance (ecological region) according to The National Catalogue of Approved Basic Material for Production of Forest Reproductive Material (Parnuta *et al.*, 2012)

DNA extraction and PCR amplification

DNA was extracted from buds, cambium or leaves using the CTAB (Doyle and Doyle, 1987) or ATMAB (Dumolin *et al.*, 1995) methods.

A number of 12 nSSR nuclear microsatellites (WS00716.F13, WS0022.N15, WS0073.H08, WS00111.K13 and WS0023.B03 (Rungis *et al.*, 2004) Pa_44 and Pa_47 (Fluch *et al.*, 2011), EAC1F04 (Scotti *et al.*, 2002), EATC1E03, EATC1B02, EATC2G05 (Scotti *et al.*, 2002), SpAG2 (Pfeiffer *et al.*, 1997) were used. EAC1F04 was excluded from further analysis because of some ambiguities in its interpretation and due to the presence of a large number of null alleles.

The PCR amplifications were performed using a PCR thermal cycler (Corbertt), in reaction mixtures (15 μL) containing 5 ng of template DNA, 1x Qiagen Multiplex PCR MasterMix 2x, 2μM for each primer and RNase free water. The PCR cycling conditions were as follows; 10 min at 95 °C followed by 30 cycles of 1 min. at 94 °C, 1:30 min. at a primer-specific annealing temperature (53 °C, 55 °C, 58° C and 62 °C), 1 min. at 72 °C and a final elongation step of 30 min at 60 °C.

Amplified PCR products were diluted and were than run on a GemoneLab GeXP Genetic Analyser (Beckman Coulter) using Frag-3 method and Size Standard 400.

Data analysis

Microsatellite markers were tested for genotyping errors due to large allele drop-out, scoring of stutter peaks and non-amplified alleles using MICRO-CHECKER 2.2.0.3 (Van Oosterhout *et al.*, 2004). The software indicated the presence of null alleles at very low frequencies (less than 7%) for two markers (WS00716 and WS00023). No evidence of large allele drop-out or scoring of stutter peaks was found.

The software GenAlEx ver. 6.5 (Peakall and Smouse, 2006, 2012) was used to estimate a standard genetic diversity indices: average number of alleles per locus (*Na*), effective number of allele (*Ne*), observed heterozygosity (*H0*), expected heterozygosity *(He*), number of private alleles (*Np*) and fixation index (*F*). Principal component analysis (*PCoA*) was performed using the same software. Allelic richness (A_R), a measure that is independent of sample size, was estimated with FSTAT 2.9.3 (Goudet, 2001).

A matrix of pairwise genetic differentiation measures between all populations pairs was computed. For genetic differentiation among spruce populations, pairwise F_{ST}'s were computed using ARLEQUIN 3.5.2.2 (Excoffier and Lischer, 2010). The significance of the F_{ST} statistics was tested by 10000 permutations. The graphical representations of all pairwise F_{ST} were done using an Rfunction (*pairFstMatrix.r*) implemented in ARLEQUIN software. Analysis of Molecular Variance (AMOVA) was performed using the same software.

An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was computed with 100 bootstrap replications, based on Nei's (1972) standard genetic distance using the software Populations 1.2.31 (Langella, 1999) and TreeView 1.6.6 (Page, 2003).

The Bayesian clustering method implemented in STRUCTURE software ver. 2.3.3 (Pritchard *et al.*, 2000) was used to genetically assign individuals to clusters. Simulations were run for 50000 steps following a burn-in period of 100000 steps, considering values of K (number of clusters) from one to 7, with 3 replications for each value of K. The analysis was performed using admixture, correlated allele frequencies and no prior information on sampling location. The number of population clusters was estimated using ΔK parameter according to (Evanno *et al.*, 2005) using the STRUCTURE HARVESTER program (Earl and vonHoldt, 2012). The highest value of ΔK statistics was obtained for $K = 2$.

Results and Discussion

Genetic diversity

It was assumed that two ramets originating from the same clone possess identical genotypes. Genotypes of two ramets per clone were compared with each other at three highly polymorphic loci. The multilocus genotypes were identical in nearly 97% of the comparisons. Only in 9 out of the 297 clones (Cso-P - clone 4, Cso-B – clone 163, 169, 265, 267, 272 and 279; Cso-A – clone 270 and 300), the two ramets did not match at two or three loci. This is probably due to the growth of the rootstock to the detriment of scion (Prescher et al., 2007) or because of sampling errors. Only unique genotypes were included for further analyses. Thus, the total sample size was made of 437 individual trees, out of which, 304 and 133, were from seed orchards and seed stands, respectively.

All eleven microsatellite loci showed polymorphism across populations, with the total number of alleles ranging from 33 at the locus WS0023 to four at the locus Pa_47. The mean number of alleles per population was 11.921. Effective number of alleles across all populations was 6.503 ($SD \pm 0.566$), with mean value 5.081 in the seed orchards and 7.945 in natural stands (Table 2).

Table 2. Standard genetic diversity indices

Population		Na	Ne	Ho	He	F	A_R	Np
Cso-P	Mean	8.455	4.505	0.693	0.674	-0.070	8.331	1
Cso-B	Mean	11.182	5.466	0.683	0.682	-0.036	9.253	6
Cso-D	Mean	10.909	5.071	0.734	0.732	-0.028	8.994	0
Cso-A	Mean	12.091	5.280	0.672	0.710	0.022	9.196	3
Nat-C	Mean	13.001	8.088	0.683	0.712	0.014	10.927	9
Nat-M	Mean	13.364	7.763	0.717	0.725	-0.011	10.820	9
Clonal seed orchards	**Mean**	**10.659**	**5.081**	**0.696**	**0.700**	**-0.027**	**8.941**	**10**
Seed stands	**Mean**	**13.182**	**7.925**	**0.700**	**0.718**	**0.001**	**10.874**	**18**
Total	Mean	11.921	6.503	0.698	0.709	-0.013	9.901	28
	SE	0.793	0.566	0.022	0.025	0.024	0.021	-

* *Na* - average number of alleles per locus, *Ne* - effective number of alleles, H_O - observed heterozygosity, *He* - expected heterozygosity, *F* - fixation index, A_R – allelic richness, *Np* – number of private alleles; *SE* – standard error.

One of the roles of seed orchards is to maintain a high level of genetic diversity, which may reflect the genetic diversity of original populations (Ertekin, 2012). Our results show that expected heterozygosity of seed orchards (0.700) is slightly lower than that of natural stands (0.718). This might be because seed orchards are generally derived from a limited number of clones. The mean *He* in natural stands (0.718) were slightly higher than in seed orchards (0.700), which is consistent with other studies in Norway (Sønstebø *et al.*, 2018) and Latvia (Ruņgis *et al.*, 2019). Furthermore, the mean *He* was lower than it was previously

reported for Norway spruce core stands in Slovenia (0.935) (Westergren *et al.*, 2018) and Czech Republic (0.780) (Máchová *et al.*, 2018).

Allelic richness is one of the most important genetic diversity parameters, particularly when analysing populations of different sample size. This parameter is of importance when elaborating genetic conservation strategies (Foulley and Ollivier, 2006). In our study, larger differences were observed for allelic richness (A_R), which varied between 8.331 and 11.071. Mean A_R over all samples was 10.619. The allelic richness in seed orchards (8.941) was higher than previously reported values for this species using genomic SSRs markers in seed orchards (5.990) (Sønstebø *et al.*, 2018). Although it has been reported that allelic richness increases with increasing number of parents (Sønstebø *et al.*, 2018), allelic richness in Cso-A (9.196), the seed orchard with the highest number of clones, was similar to the other seed orchards. Moreover, Cso-P, which has the lowest number of clones, has the lowest value for A_R compared to the other three seed orchards. Compared to the seed orchard with the highest number of clones (Cso-A), in Cso-P the value of A_R is with 9.4% lower. On the other hand, the highest level of allelic richness was observed in the two natural stands.

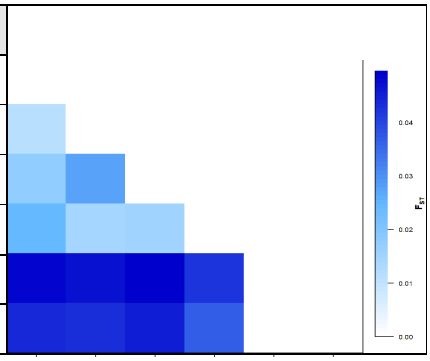
The fixation index (F) ranged from -0.070 (Cso-P) to 0.022 (Cso-A). The total number of private alleles (Np) was 28, out of which 10 alleles in seed orchards and 18 alleles in the natural stands. Overall, the mean values of the genetic diversity parameters were slightly higher in the natural Norway spruce populations compared to the clonal seed orchards.

Genetic differentiation among populations

The genetic divergence among all Norway spruce populations was measured using F_{ST}. Pairwise Wright's F_{ST} showed the lowest genetic differentiation between Nat-C and Nat-M (F_{ST} = 0.0004), and the highest genetic differentiation was detected between Cso-D and Nat-C (F_{ST} = 0.0496). The genetic differentiation among seed orchards was relatively low (Table 3).

Table 3. Matrix of pairwise Wright's F_{ST}

	Cso-P	Cso-B	Cso-D	Cso-A	Nat-C	Nat-M
Cso-P	0.0000					
Cso-B	0.0109	0.0000				
Cso-D	0.0173	0.0277	0.0000			
Cso-A	0.0244	0.0147	0.0154	0.0000		
Nat-C	0.0486	0.0467	0.0496	0.0424	0.0000	
Nat-M	0.0439	0.0428	0.0450	0.0366	0.0004	0.0000



PCoA analysis showed that the first principal coordinate separated clonal seed orchards from the seed stands. The second principal coordinate separated population Cso-D from other clonal seed orchards (Cso-A, Cso-P and Cso-B). This may be a consequence of using plus trees from three region of provenance (C2 – region from the Southern Carpathians, A2 and B2 – region from the Eastern Carpathians) in the clonal seed orchard Cos-D. However, even if plus trees from the same regions of provenance were used for the installation of the Cos-A and Cos-B seed orchards, the observed differences can be due to the number of selected clones (the number of clones in population Cos-A is larger than the number of clones from Cos-B population). A balance between the expected genetic gain and the assumed but reduced loss of genetic diversity is necessary. Also, for advanced generations of seed orchards, the breeding strategy must provide the infusion of new genotypes in the breeding program, in order to avoid the risk of reducing genetic diversity (Funda and El-Kassaby, 2012).

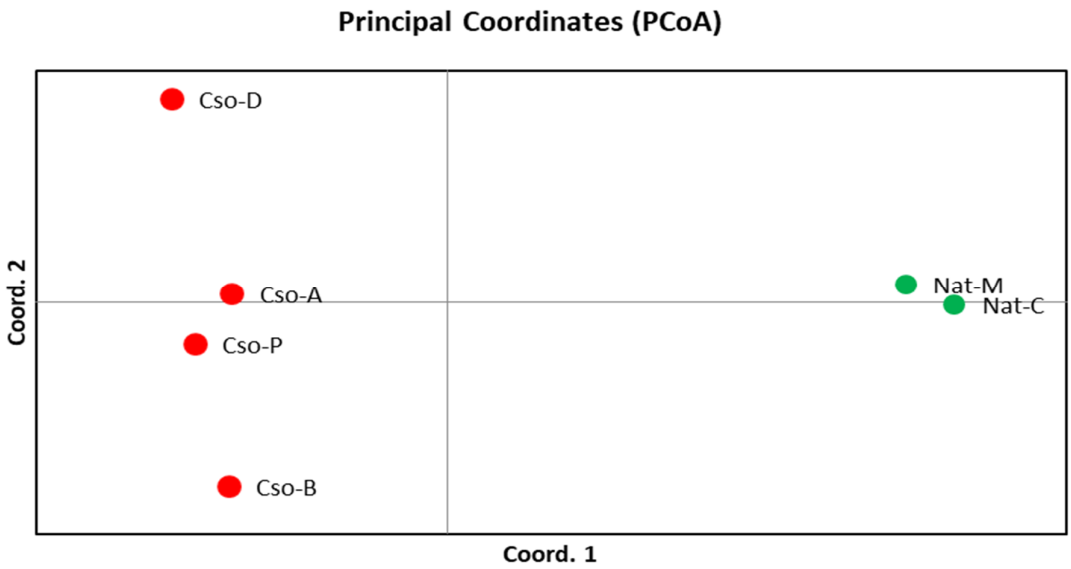


Figure 1. Principal component analysis of six Norway spruce populations. Axes 1 and 2 explain 57.28 % and 19.95 % of the genetic variation detected

Relationships among all six Norway spruce population were further illustrated by a dendrogram, using UPGMA based on Nei's (1972) standard genetic distances (Figure 2 - B). The dendrogram showed a clear separation (bootstrapping value for the pair was 85) between natural Norway spruce stands and clonal seed orchards. Nat-C and Nat-M were grouped together although the geographical distance among them was considerable.

Population genetic structure

Using the Bayesian analysis (Figure 2 - A) we found that the best inferred number of genetic clusters is two (K = 2). All seed orchards showed a higher membership in the red genetic cluster. However, there were many admixed individuals and even individual clones with a higher membership value in the second genetic cluster (with green colour). As in the UPGMA dendrogram, the two seed stands are closely related to each other, showing a high membership in the second genetic cluster. This separation is also valid for K = 3 (Figure 2 - A).

The four seed orchards consists of vegetative copies of plus trees selected in several seed stands located across the Eastern Carpathian region, including the two stands sampled in this study. This can explain the observation of individual clones with high membership values in the genetic cluster that is specific for the two natural stands. The similarity between the two natural stands might be due to extensive gene flow between Norway spruce forests along the Eastern Carpathians. Little genetic differentiation was found among natural Norway spruce populations across the Romanian Carpathians (Radu *et al.*, 2014).

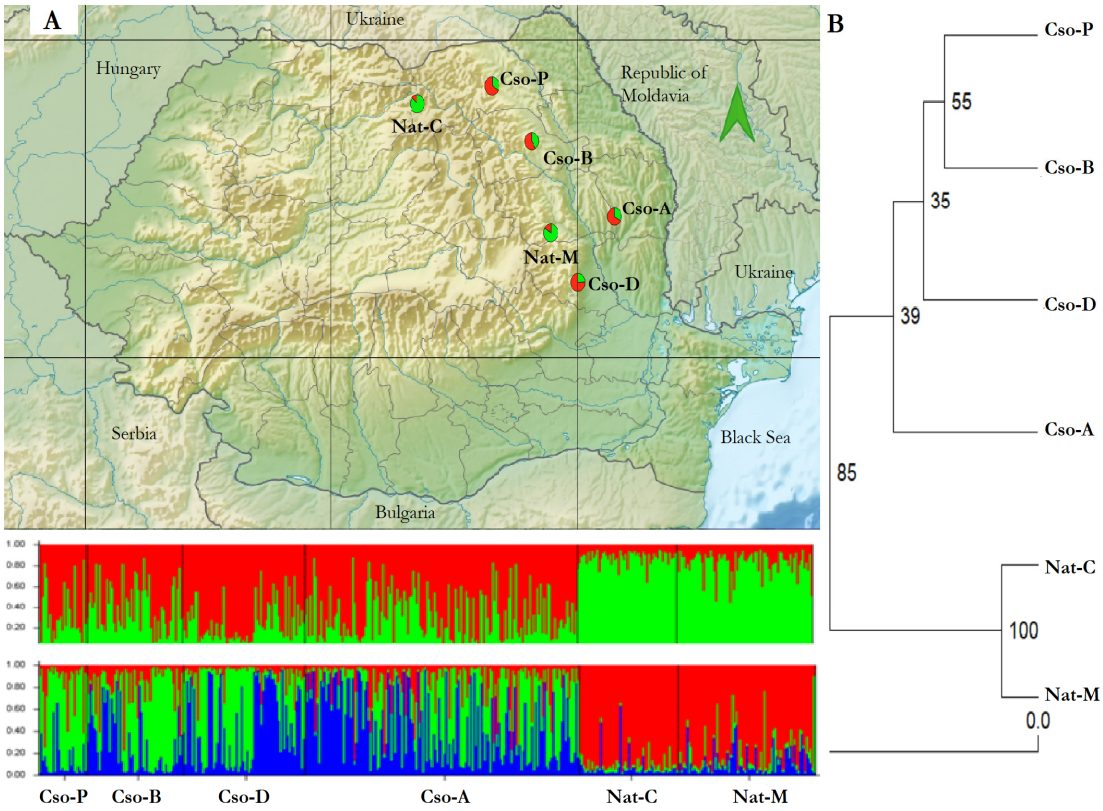


Figure 2. Map of Norway spruce populations. Pie charts represent the average inferred ancestry of individuals for each cluster identified by STRUCTURE for *K*=2. STRUCTURE results for two and three distinct genetic clusters (A). UPGMA dendrogram constructed using Nei's genetic distance (B)

Table 4. Analysis of molecular variance (AMOVA) for Norway spruce populations using 11 microsatellite loci

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*p*
Among populations	5	89.477	0.10541 Va	3.02	0.0224
Among individuals within populations	431	1484.095	0.06322 Vb	1.81	<0.001
Within individuals	437	1449.50	3.31693 Vc	95.16	<0.001
Total	873	3023.072	3.48557	-	-
Among groups (first group seed orchards, second group seed stands)	1	50.963	0.10819 Va	3.06	<0.001
Among populations within group	4	38.514	0.04700 Vb	1.33	0.0127
Among individuals within populations	431	1484.095	0.06322 Vc	1.79	<0.001
Within individuals	437	1449.500	3.31693 Vd	93.82	0.0615
Total	873	3023.072	3.53535	-	-

* *d.f.*- degrees of freedom; *Va, Vb, Vc, Vd*- associate covariance components; *p* – significance level.

Two different AMOVA analyses were conducted. The first analysis included all populations and the second one considered the two different groups established by STRUCTURE (first group for seed orchards and second group for natural stands). Most of the genetic variation between the six Norway spruce populations can be explained by intraindividual variation 95.16 % ($p < 0.001$) (Table 3). When the genetic variance was partitioned into two distinct groups, a small but significant ($p < 0.001$) amount of genetic variation (3.06 % of the total) was the result of differences between groups (Table 4).

Conclusions

The genotypic identity of the putative ramets of the same clone was certified for Norway spruce clonal seed orchards based on highly polymorphic DNA markers. Mismatches were very rare and may be explained by the growth of the rootstock to the detriment of scion or sampling errors. Slightly higher values for genetic diversity parameters were found in seed stands compared to clonal seed orchards. As expected, the degree of genetic admixture was higher in the four clonal seed orchards than in the two studied seed stands. Our molecular analysis provides valuable information for the management of Norway spruce seed orchards in Romania.

Authors' Contributions

Conceptualization - EC, ALC; Data curation - EC, ALC, NS, GM, MT; Formal analysis - EC, MT;; Investigation - EC, ALC, NS, GM, MT; Methodology - EC, MT; Project administration - ALC; Resources - ALC; Software - EC, MT; Supervision – ALC, NS, GM; Validation - NS, GM; Visualization - EC, ALC, NS, GM, MT; Writing - original draft- EC; Writing - review and editing – EC, ALC, NS, GM, MT. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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Growth and Adaptive Capacity of Douglas Fir Genetic Resources from Western Romania under Climate Change

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Abstract: The most recent climate change scenarios show that Southern and Eastern Europe will be affected by a significant increase in temperature and drought frequency by the end of the 21st century. Romania has already recorded very high temperatures and long periods of drought over recent decades, the most affected regions being the south, west and east of the country. Considering that successful forest management requires suitable species and high-quality reproductive material for reforestation, the aim of this study was to evaluate and compare the growth and drought response of Douglas fir (*Pseudotsuga menziessi* var. *menziesii*) and Norway spruce (*Picea abies*) in two stands installed at the end of the 19th century in western Romania. The growth traits, wood characteristics and drought parameters (resistance, recovery, resilience and relative resilience) of Douglas fir and Norway spruce trees have been analyzed and compared. The climate–growth relationship was determined using growth response functions over the period 1938–2017. Additionally, to simulate the potential impact of climate change on Douglas fir in this region, the RCP4.5 scenario was used over two periods: 2041–2070 and 2071–2100. The results reveal that Douglas fir has an exceptional growth capacity, overcoming the Norway spruce since the early ages in both site conditions. The highest growth performances were seen in the low-productivity site. From analyzing the responses to drought events, considerable differences were found between species. The results highlight the high resistance and relative resilience to extreme droughts of Douglas fir compared to Norway spruce. However, autumn–winter temperatures play an important role in the adaptation of Douglas fir to site conditions in Romania. The use of appropriate provenances of Douglas fir in mixed stands with native broadleaved species may be an option for climatically exposed sites, thus increasing the value of these stands.

Keywords: *Pseudotsuga menziessi*; climatic suitability; standardized precipitation evapotranspiration index; generalized linear mixed models; ring width index; forest genetic resources; climate scenarios; Southeastern Europe

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1. Introduction

The Douglas fir (*Pseudotsuga menziessi*) is one of the most important forest tree species in North America. Since the middle of the 19th century, it has been successfully introduced into many regions with a temperate climate in both hemispheres [1]. In Europe, Douglas fir was introduced in 1827 and currently covers the largest area outside its natural range, being present in 35 countries [2]. In Romania, the first Douglas fir plantations were established in 1887 in eastern Romania (Moldavia) and one year later, in 1888, in the western part of the country (Banat) [3,4]. Although it is one of the most important non-native tree species,

it covers only 0.40% of the total forest area in Europe (approximately 823,534 ha) [2] and 0.12% (7300 ha) in Romania (NFI 2016).

Douglas fir has a wide distribution area in North America, where it is represented by two geographically distinct varieties: coastal (*P. menziessi* var. *menziesii* (Mirbel) Franco) and interior (*P. menziessi* var. *glauca* (Beissn.) Franco). The coastal variety of Douglas fir is the most widespread in Europe because it was more suitable to climate conditions than the interior variety. The costal variety ranges along the North American Pacific coast (California, Oregon, Washington, and British Columbia) from the sea level to about 1500 m and is adapted to an oceanic climate characterized by mild, wet winters and cool, relatively dry summers, and long frost-free growing seasons. Compared to the climatic conditions in Europe, most of the precipitation occurs in winter, with the annual precipitation rates ranging from 760 to 3000 mm [1,5].

Results from long-term provenance trials in the natural range indicate high adaptive genetic variation among Douglas fir populations and clinal variation patterns in growth, phenology, and cold hardiness [6–9]. Douglas fir can be referred to as an "adaptive specialist" because its populations are closely adapted to their ecological niches [10,11]. The coastal Douglas fir populations reach an increased height, diameter, and volume compared with the interior variety, and they are more tolerant to needle diseases but less tolerant to fall frost, winter cold, and drought. Winter temperatures and frost date are the major factors involved in the adaptation of the coastal Douglas fir, whereas summer drought is of less importance [12]. Furthermore, there is a large variability for wood characteristics within the natural range of the species. The wood of the northern populations is considered more valuable (denser and stronger) than that of the southern ones [13,14].

Since the 20th century, a large number of Douglas fir provenance trials have been established at the national and European levels, indicating a substantial level of interest for the species. Thus, after the Second World War, it became a major tree species used in reforestation in Western Europe. In most European countries, it showed higher productivity compared with the native conifers and good resistance against fungal pathogens and low numbers of pests and diseases [15,16].

Subsequently, the results of International Union of Forest Research Organizations (IUFRO) experiments on Douglas fir provenances in Europe show its sensitivity to winter drought and frosts and the importance of the geographic origin of forest reproductive material [17,18]. Thus, the interior populations (particularly in British Columbia) are the most resistant to early and winter frosts but susceptible to late frosts [19,20]. Coastal populations, with late flushing, are the most resistant to late frost but rather sensitive to early and winter frosts [15]. Further, Breidenstein [21], in a synthesis of the results from 108 Douglas fir provenance tests established by IUFRO (1967) in 15 European countries, has shown that low-elevation coastal and Cascade provenances from Washington State underwent the most rapid growth over most sites and broad adaptability, although mortality was high on several colder sites [22]. Some northern Oregon provenances from west of the Cascades as well as a few southwestern British Columbia sources also had high productivity across much of Europe. Only in continental climates (e.g., in Sweden, Finland, and the Czech Republic), the interior variety outperforms the coastal one. The highest mortality rates were found for the southern coastal Oregon provenances on the coldest sites and the interior of British Columbia provenances on the mildest sites. The success in reforestation work is mostly dependent on the genetic selection of the appropriate forest reproductive material for planting sites.

In Romania, Douglas fir breeding activities started in the 1970s and focused on assessing the genetic variability in provenance trials [23], establishing first-generation seed orchards [24], and preserving valuable stands as genetic resources [25]. Unfortunately, the breeding program was abandoned in the last two decades, and recommendations on the use of appropriate provenances in Romania still need scientific validation. There is also a lack of knowledge regarding the growth potential of Douglas fir in Romania, relationships

between growth and climatic variables, the selection of suitable seed sources, and species response to drought and rapid climate change.

Climate change scenarios show that Southern and Eastern Europe will be affected by a significant increase in temperature and drought frequency by the end of the 21st century [26]. Over recent decades, Romania has experienced record temperatures [27], decreasing wind speed [28,29] and snow depth [30] changes in the frequency of hydrometeors [31], as well as drought periods, which were associated with global climate change [32–35] or changes in atmospheric circulation [36–39]. Climate change will certainly have detrimental effects on forest ecosystems in these regions [40–43]. Norway spruce, one of the most planted conifer species outside the natural distribution area, showed a high sensitivity to decreases in water availability and weak adaptive capacity at lower elevations [44,45]. However, recent studies showed that Douglas fir might be an alternative species because some provenances are well adapted to drought and warmer conditions [46–48]. Therefore, interest in using Douglas fir at lower altitudes or in response to climate change has increased in Europe in recent years.

The aim of this study was to evaluate the growth response and adaptive capacity of Douglas fir genetic resources from western Romania in the context of climate change. These populations were established at the end of the 19th century and are mixed stands with Douglas fir, Norway spruce, and broadleaved species. They are among the oldest Douglas fir plantations in Romania and were designated as forest genetic resources for Douglas fir to be included in the National Catalog of Forest Genetic Resources [49]. Based on the assumption that global warming will reduce the growth and adaptive capacity of Norway spruce at lower elevations in Southeastern Europe, the objectives were to (1) comparatively assess the growth and adaptive capacity of Douglas fir and Norway spruce, (2) evaluate the Douglas fir response to drought, (3) determine climate–growth relationships, (4) evaluate the Douglas fir suitability to future climate conditions, and (5) provide recommendations for the use of Douglas fir.

Considering that sustainable forest management requires productive species and high-quality reproductive material for reforestation, the need to identify valuable seed sources has emerged. Furthermore, knowing the provenance of seed sources or plant material is particularly important for the success of reforestation in response to climate change. This study aimed to provide further information and offer solutions for the sustainable management of forest ecosystems and forest adaptation strategies to climate change.

2. Materials and Methods

2.1. Study Sites

This study was conducted in two of the most valuable forest genetic resources (FGR) of Douglas fir located in the Ana Lugojana Forest District (Banat region). These stands are 105 and 130 years old, respectively, and are mixed stands with Douglas fir, Norway spruce, and broadleaved species. The two stands are located in highly contrasting site conditions: FGR 1 in high-productivity site conditions for European beech at an altitude of 880 m (45°35′ N, 22°15′ E), whereas FGR 2 is in low-productivity site conditions at an altitude of 460 m (45°35′ N, 22°25′ E). The characteristics of the two stands are presented in Table 1.

Table 1. The sites and stands characteristics of the Douglas fir forest genetic resources.

Stand Characteristics	FGR 1	FGR 2
Species composition	80% Douglas fir; 20% Norway spruce	30% European beech; 50% Douglas fir; 10% Norway spruce; 10% other species
Age	105	130
Class of production	I	I

Table 1. *Cont.*

Stand Characteristics	FGR 1	FGR 2
Soil	Eutricambosoil mollic	Eutricambosoil lithic
Site conditions	High productivity	Low productivity
Vegetation layer	European beech layer	European beech layer
Altitude	880 m	450 m
Climatic province (by Köppen–Geiger)	Cfb-warm and temperate climate with slight Mediterranean influences	Cfb-warm and temperate climate with slight Mediterranean influences

2.2. Field Measurements and Analyses

In each stand, 20 dominant or (co-)dominant trees per species of 105 and 130 years old, respectively, were measured for total height and diameter at 1.30 m (DBH) and cored at breast height using 5 mm increment borers (Haglof, Sweden). Cores were prepared using standard dendrochronological methods [50]. Then, each core sample was scanned at 1200 dpi, using an Epson Expression 10,000 XL, and the wood characteristics were measured using the Ligno Vision software package to the nearest 0.001 mm. The assessed characteristics were: ring width (RW), earlywood width (EW), latewood width (LW), and latewood percentage (LWP), as an indication of wood quality.

For each study site, a master series was constructed and cross-dated using COFECHA [51] to avoid dating errors due to missing or false rings, which could be present in an increment radial core. Only dendrochronological series that presented intercorrelation values >0.328 ($p < 0.01$) were included in final tree ring data. All tree-ring time series were standardized to a mean value of one to obtain a dimensionless ring-width index (RWI), thus preserving a large portion of low-frequency variability due to the influence of climatic events [52,53]. The negative exponential regression in the R package (dplR) [54] was applied for each raw measurement series because it is deterministic, meaning that it follows a model of tree growth. The final data set consisted of data from 38 Douglas fir trees and 32 Norway spruce trees. The analyzed period was 1938–2017 for each tree-ring series.

2.3. Climate Data

Climatic data series for the study sites were extracted from the CRU TS (Climatic Research Unit gridded Time Series) dataset v.4.04, over the period 1901–2017. CRU TS is a widely used dataset made by interpolating the monthly climate anomalies from weather station measurements at a spatial resolution of 0.5° × 0.5°, covering all continents except Antarctica [55].

High-resolution gridded climate data over Romania (1961–2020) were derived from daily precipitation and temperature grids over the period 1961–2020. The dataset consists of a spatial resolution of 1 km × 1 km and was made using state-of-the-art interpolation techniques for an improved reproduction of the spatial climatic variability (e.g., [56,57]).

Climate projections were derived from the RoCliB dataset [58,59], which includes air temperature and precipitation data from 10 general circulation models dynamically downscaled by several regional climate models and bias corrected (adjusted) over Romania for the 1971–2100 period. We used the 10-model ensemble data for the scenarios RCP 4.5 and RCP 8.5 for the periods 2041–2070 and 1971–2100.

The following climatic variables of FGR location were calculated: mean annual temperature (MAT); mean temperature of the growing season (April to September) (MT_{VEG}); mean temperatures for January (MT_{JAN}) and July (MT_{JUL}) (i.e., the coldest and warmest months, respectively); mean temperature from October to December of the previous year ($MT_{OCT\text{-}DEC}$); mean temperature from October of the previous year to March of the current year ($MT_{OCT\text{-}MAR}$); mean temperature from January to March of the current year ($MT_{JAN\text{-}MAR}$); sum annual precipitation (SAP); sum precipitation during the growing sea-

son (SP_{VEG}); sum precipitation of the coldest (SP_{JAN}) and warmest (SP_{JUL}) months; sum precipitation from October to December of the previous year ($SP_{OCT-DEC}$); sum precipitation from January to March of the current year ($SP_{JAN-MAR}$); and sum precipitation from October of the previous year to March of the current year ($SP_{OCT-MAR}$).

According to the Köppen and Geiger climate classification [60], the site's climate is warm and temperate (Cfb), with slight Mediterranean influences being representative of vegetation conditions in the west of the country. The climate is characterized by mild winters and a richer rainfall regime. Comparative climatic data for Douglas fir populations from western Romania and for the natural distribution range are presented in Table 2.

Table 2. Comparative climatic data for Douglas fir forest genetic resources from western Romania and at the natural distribution range in Pacific Northwest.

Site	MAT (°C)	MTJAN (°C)	MTJAN–MAR (°C)	MTVEG (°C)	SAP (mm)	MPJAN (mm)	SPJAN–MAR (mm)	SPVEG (mm)	Frost-Free Days
Western Romania	8.97	−2.56	0.01	15.62	754	43	183	477	248
Pacific Northwest	3.5 to 14.4	–2.0 to 3.0	–1.5 to 5	7.4 to 23	760–3000	15 to 524	43 to 1233	90 to 750	195–260

MAT—the mean annual temperature; MTJAN—the mean temperature of the coldest month (January); MTJAN–MAR—the mean temperature from January to March of the current year; MTVEG—the mean temperature of the growing season; SAP—the sum annual precipitation; SPJAN—the sum precipitation of the coldest month (January); SPVEG—the sum precipitation during the growing season.

2.4. Determination of Drought Events and Drought Response Parameters

As an indicator for meteorological droughts, we calculated the Standardized Precipitation Evapotranspiration Index (SPEI) [61], which is based on precipitation and the potential evapotranspiration (PET) over the period 1901–2019. The PET was calculated according to the Thornthwaite equation [62]. Given that an extreme drought event must last for a minimum of 2 to 3 months, we calculated the SPEI at a time scale of 3 months (SPEI-3), with the R package "SPEI" [63]. This approach allowed us to detect both seasonal and annual variations of drought events during the analyzed period. The drought years were classified as follows: SPEI ≤ -2 extreme drought year; SPEI between -1.99 and -1.50: severe drought year; SPEI between -1.49 and -1.0: moderate drought year; SPEI between -1.0 and + 1.0: normal precipitation year.

The species response to drought events was evaluated using four drought parameters [64]: resistance (Res), recovery (Rec), resilience (Rsl), and relative resilience (rRsl). Resistance (Res) was calculated as the ratio between ring width during drought (Dr) and before the drought event (preDr)—Res = Dr/preDr—and indicates how much the radial growth decreased during drought (Res = 1 means high tolerance; Res < 1 means low tolerance).

Recovery (Rec) was calculated as the ratio between the ring width after the drought event (postDr) and during drought (Rc = postDr/Dr) and indicates the revitalization capacity after a drought period.

Resilience (Rsl) represents the ratio of the ring width after drought (postDr) and pre-drought (preDr)—Rsl = postDr/preDr—and describes the species capacity to reach pre-drought increment after a drought event (Rsl = 1 means full restoration; Rs < 1 means long-term growth reductions). Relative resilience (rRsl) was calculated by rRsl = (postDr-Dr)/preDr. Pre- and post-drought ring widths were calculated as average values for a three-year period before or after the drought year.

2.5. Data Analysis

Analysis of variance were performed using the Generalized Linear Mixed Models (GLM) procedure (SPSS v19). The total amount of variation was divided into tree species, years, FGR (sites), and the species site interaction. Apart from the FGR, which was considered fixed, all effects were considered random. The assumptions of the model were checked by a Shapiro and Wilk test for normality and by Levene's test for homogeneity.

ANOVA was performed as described in the following mixed model:

$$Y_{ijk} = \mu + S_i + R_j + Y_l + BF_{ij} + e_{ijlk}$$

where: X_{ijk} = performance of kth tree in ith species in jth RGF; μ = overall mean; S_i = effect of ith species; R_j = effect of jth RGF; Y_l = effect of lst year; SY_{il} = interaction of ith species and lst year; e_{ijlk} = random error associated with ijlkth trees.

The relationships between climatic variables of the plantation sites and ring-width indices and latewood percentage of Douglas fir and Norway spruce were evaluated through response function analysis. The quadratic models based on both temperature and precipitation as predictor variables were applied in the SPSS program (stepwise selection method), considering them more suitable [64–66]. We used seven temperature and seven precipitation variables, and the best models were chosen based on the R^2 coefficient. Response functions analysis was performed for the period 1938–2017 using the formula of Wang:

$$Y_{ij} = \beta_0 + \beta_1 T_{nj} + \beta_2 P_{nj} + \beta_3 T_{nj}^2 + \beta_4 P_{nj}^2 + e_j$$

where Y_{ij} is the observation of the population i at the site j; βs are the intercept and regression coefficients; T_{nj} and P_{nj} are the temperature and precipitation variables, respectively, at the site j; and e_j is the residual.

To assess the potential distribution area of Douglas fir in Romania, the species climate envelope was developed based on climate predictors that reflect species ecological requirements [67]. The predictors included the yearly (annual) precipitation amount, the sum of precipitation of the coldest month and the mean annual temperature for the period 1961–2020. The same ecological indicators were also used for modelling the species suitability to future environmental conditions in Romania under the RCP 4.5 and RCP 8.5 scenarios by 2100—for the intervals 2041–2070 and 2071–2100.

3. Results

3.1. Variation in Growth and Wood Characteristics

The analysis of variance for each FGR and trait are presented in Table 3. There were significant differences for all traits ($p < 0.001$) within and between species for each site (FGR) and among sites as well. There was also a highly significant year effect for traits and species x year interaction in both study sites.

Table 3. Analysis of variance of growth and wood traits for the studied FGRs.

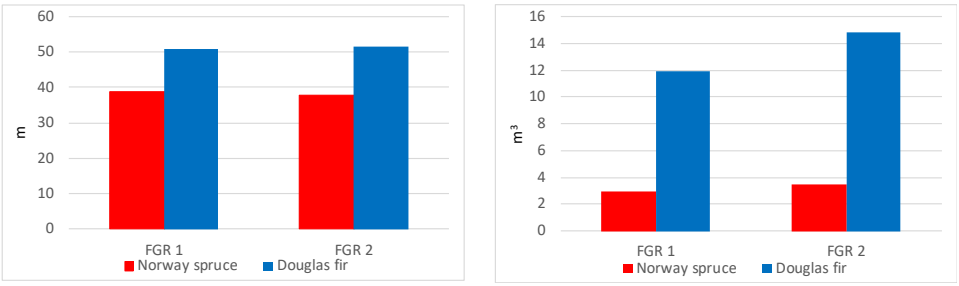
	Source of Variation	DF	Variance (s^2)				
			Total Height (m)	Diam. 1.30 m (cm)	Volume/ Tree (m^3)	Ring Width (mm)	Latewood %
FGR 1	Douglas fir (DU)	18	5.917 ***	102.894 ***	9.030 ***	34.859 ***	2104.688 ***
	Norway spruce (NS)	15	9.772 ***	29.857 ***	0.622 ***	13.413 ***	2489.699 ***
	Species (S)	1	1282.276 ***	12613.321 ***	694.107 ***	841.898 ***	5330.840 ***
	Year (Y)	79	-	-	-	1.322 ***	388.354 ***
	Interaction S x Y	79	-	-	-	0.618 ***	297.571 ***
	Error	2640	7.669	69.695	5.208	0.080	164.581
FGR 2	Douglas fir (DU)	18	14.618 ***	238.937 ***	24.728 ***	27.718 ***	1592.266 ***
	Norway spruce (NS)	15	7.947 ***	52.250 ***	1.007 ***	13.223***	2137.951 ***
	Species (S)	1	1686.527 ***	15754.121 ***	1260.260 ***	451.099	228.388 ***
	Year (Y)	79	-	-	-	2.371 ***	375.961 ***
	Interaction S x Y	79	-	-	-	0.543 ***	387.809 ***
	Error	2560	12.160	181.358	19.589	0.098	182.471

Table 3. *Cont.*

	Source of Variation	DF	Variance (s^2)				
			Total Height (m)	Diam. 1.30 m (cm)	Volume/ Tree (m^3)	Ring Width (mm)	Latewood %
FGR 1 and FGR 2	Douglas fir (DU)	1	4.995	974.623 *	127.097 *	383.776 ***	55.155 ***
	Norway spruce (NS)	1	12.005	220.500 *	1.620	121.424 ***	30.932 ***
	Species (S)	1	2953.924 ***	28115.320 ***	1898.461 ***	121.424 ***	149.833
	Year (Y) for DU	79	-	-	-	1.144 ***	335.557
	Interaction DU x Y	79	-	-	-	0.721 ***	311.100
	Year (Y) for NS	79	-	-	-	2.429 ***	408.470 ***
	Interaction NS x Y	79	-	-	-	0.495 ***	384.963 ***
	Year (Y)	79	-	-	-	2.911 ***	424.742 ***
	Interaction S x Y	79	-	-	-	0.742 ***	320.916 ***
	Error	5360	9.839	138.784	13.842	0.092	208.275

The level of significance is represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In the case of Douglas fir, the total height ranged from 45.70 m to 54.70 m in FGR 1 and 41.80 m to 57.60 m in FGR 2. DBH ranged from 70.38 cm to 100.34 cm in RGF 1 and 54.78 cm to 129.62 cm in FGR 2. The average of volume/tree was 11.9 m^3 in FGR 1 and 15.6 m^3 in FGR 2. The best growth performances were obtained by both conifer species in FGR 2, located on low-productivity site conditions for European beech. Overall, Douglas fir exceeded the Norway spruce for all growth characteristics, with percentages on average between 24 and 27% for total height, 56% for DBH, and between 75 and 77% for volume per tree (Figure 1).



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

Figure 1. Total height (**a**) and volume/tree (**b**) of Douglas fir (DU) and Norway spruce (NS) in forest genetics resources (FGR) located in western Romania.

The ring width and latewood percentage varied significantly across species, site, and year. The ring width for Douglas fir ranged from 0.14 mm to 8.21 mm along all years and sites, whereas for Norway spruce, it ranged from 0.11 mm to 6.20 mm. Averaged by site and species, the average ring width for Douglas fir ranged from 2.74 mm in FGR 1 to 2.02 mm in FGR 2 and from 1.64 mm in FGR 1 to 1.20 mm in FGR 2 for Norway spruce. The overall average values per species were 2.39 mm for Douglas fir and 1.42 mm for Norway spruce.

The average latewood percentage ranged from 6 to 96% for Douglas fir and from 7 to 43% for Norway spruce. The overall average values per species were 53% for Douglas fir and 43% for Norway spruce. The average ring widths and latewood percentage were about 41% and 19% higher, respectively, for Douglas fir than those of Norway spruce for the period of 1938–2017. In both study sites, ring width decreased with increasing age, but growing patterns differed between species (Figures 2 and 3). Thus, at the same age, 105 and 130 years, respectively, the ring width of Norway spruce was 50% lower in FGR 1 and 31% in FGR 2 compared with Douglas fir.

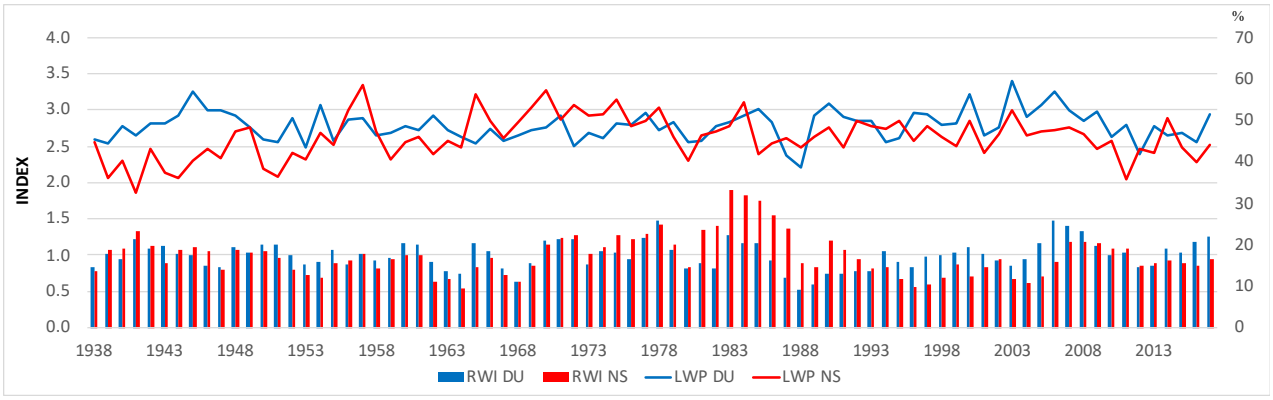


Figure 2. Variation of the ring width index (RWI) and latewood percentage (LWI) of Douglas fir (DU) and Norway spruce (NS) in FGR 1.

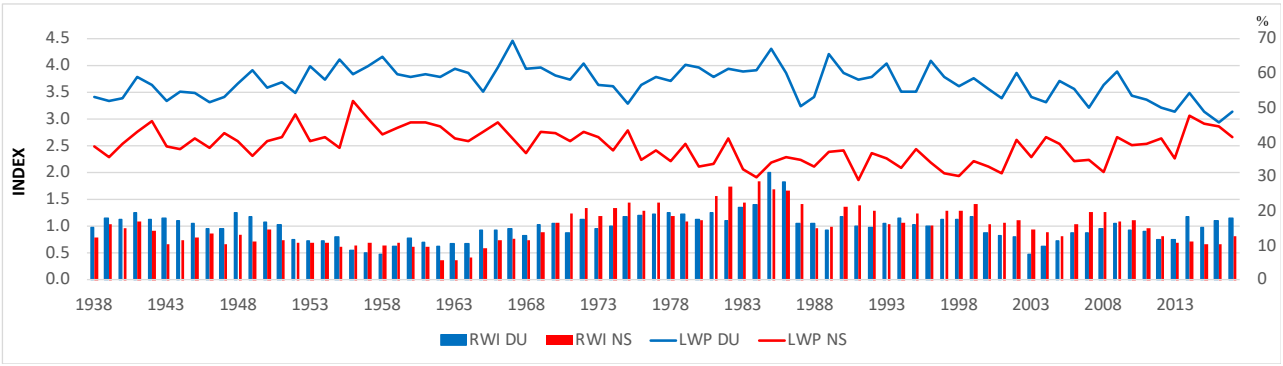


Figure 3. Variation of the ring width index (RWI) and latewood percentage (LWP) of Douglas fir (DU) and Norway spruce (NS) in FGR 2.

3.2. Climate Variation and Identification of Drought Years

The two FGRs are located very close to each other and, therefore, a single climate database was used. The gridded climate data analysis revealed considerable changes in climate conditions of the FGR sites over the analyzed period (Figure 4). MAT varied between 6.8 °C in 1940 and 10.9 °C in 2014. SAP ranged from 467 mm in 2000 to 1055 mm in 2010, whereas SP_{VEG} varied from 284 mm in 2000 to 686 mm in 2010. The most significant changes occurred over the last two decades; MAT increased by 0.9 °C, TM_{VEG} by 1.0 °C, and $TM_{JAN\text{-}MAR}$ by 1.1 °C. Surprisingly, in the last 20 years, the precipitation amount did not decrease; in fact, there was a slight increase—SAP by 30 mm, SP_{VEG} by 17 mm, and $SP_{OCT\text{-}MAR}$ by 14 mm—whereas MP_{JAN} remained constant.

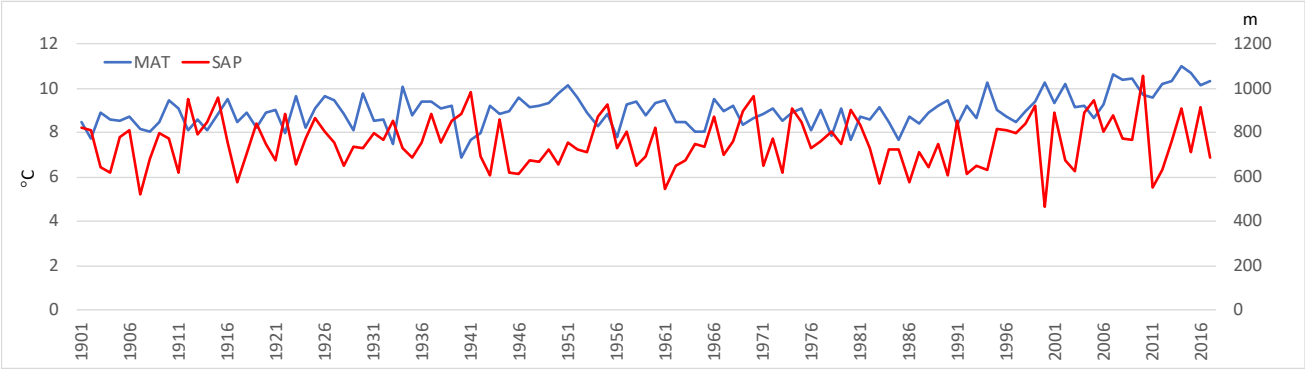


Figure 4. Variation of the mean annual temperature (MAT) and sum annual precipitation (SAP) in 1901–2017 period.

Based on SPEI values, we identified the years of severe and extreme drought for the period 1901–2017. Figure 5 presents the frequency of drought events in these sites. There were six extreme drought years (1958, 1968, 1986, 2000, 2003, and 2012) in this period, whereas the years of severe drought were a total of 32. Most of the drought events (13 out of 38 extreme and severe drought years) occurred in the period 2000–2017. Further, during this period, the highest number of consecutive years of drought was recorded (2006–2009 and 2011–2015). Other such drought periods were 1961–1963 and 1972–1975.

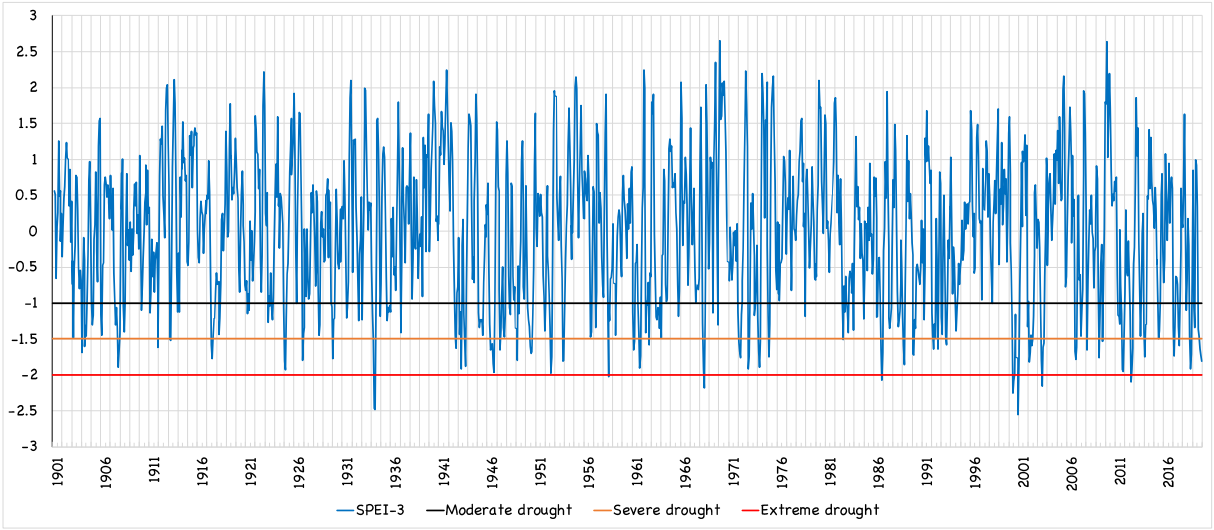


Figure 5. Variation of standardized precipitation evapotranspiration index (SPEI) across the analyzed period.

The most significant drought event occurred in 2000, when two negative peaks were observed. The 2000 drought had the highest intensity and longest duration over the last century. In that year, eight months of drought were observed, of which six extreme droughts included the growing season and fall, until the end of December. The same intensity of drought was observed in 1934 only.

Furthermore, the years characterized by a long duration of drought but lower intensity were 2003 (five months of drought, of which four were of extreme/severe drought), 2011 (seven months of drought, of which three were of extreme/severe drought), and 2012 (five months of drought, of which three were of extreme/severe drought).

3.3. Species Response to Drought

The analysis of variance revealed significant differences between species for all drought parameters in FGR 2, located on a low-productivity site (Table 4). The drought reaction parameters of the two species differed significantly depending on the extreme drought years in both FGRs. Significant differences between study sites regarding the species drought reaction were found for Norway spruce only. Within-species differences were non-significant for both species and FGR. The variation of drought parameters for all extreme drought years on species and FGR is presented in Figure 6.

Table 4. Analysis of variance of Douglas fir and Norway spruce drought parameters for years of extreme drought.

Trial	Source of Variation	DF	Variance (s^2)			
			Resistance	Recovery	Resilience	Relative Resilience
FGR 1	Douglas fir (DU)	18	0.064	0.252	0.106	0.152
	Norway spruce (NS)	15	0.084	0.083	0.360	0.131
	Between species	1	0.027	0.056	0.001	0.035
	Extreme drought year for DU	5	0.658 ***	3.130 ***	1.289 ***	1.706 ***
	Extreme drought year for NS	5	0.120	1.652 ***	1.414 **	1.208 ***
FGR 2	Douglas fir (DU)	18	0.045	0.241	0.082	0.132
	Norway spruce (NS)	15	0.090	0.116	0.118	0.089
	Between species	1	0.595 **	1.454 *	0.147	0.156
	Extreme drought year for DU	5	0.638 ***	3.303 ***	0.865 ***	1.947 ***
	Extreme drought year for NS	5	0.305 **	1.109 ***	1.977 ***	1.187 ***
FGR 1 and FGR 2	Douglas fir (DU)	1	0.076	0.029	0.442	0.143
	Norway spruce (NS)	1	0.423 *	1.221 **	0.067	0.835 *
	Between Species	1	0.183	1.055 *	0.072	0.025
	Between sites for DU	1	0.076	0.029	0.442	0.143
	Between sites for NS	1	0.423 *	1.221 **	0.067	0.835 *
	Extreme drought year for DU	5	0.420 ***	5.353 ***	1.589 ***	3.282 ***
	Extreme drought year for NS	5	0.164 *	2.669 ***	2.688 ***	2.278 ***

The level of significance is represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In FGR 2, differences in species reaction were obtained for all extreme drought years, except the year 2000 (Table 5). In FGR 1, only the extreme droughts during 2000 and 2003 produced a significant interspecific variation for recovery, resilience, and relative resilience.

Table 5. Differences between Douglas fir and Norway spruce for the drought parameters during the years of extreme drought.

FGR	Drought Parameters	Variance (s^2)					
		1958	1968	1986	2000	2003	2012
FGR 1	Resistance	0.036	0.198 *	0.042	0.284	0.044	0.014
	Recovery	0.020	0.120	0.008	1.373 *	0.840 *	0.510
	Resilience	0.026	0.389	0.024	1.105	1.355 *	0.224
	Rel. resilience	0.001	0.034	0.002	2.504 *	0.893 *	0.122
FGR 2	Resistance	0.552 *	0.284	0.107	0.001	0.972 ***	0.004
	Recovery	1.791 *	0.782 **	0.112 *	0.238	3.773 **	1.903 ***
	Resilience	0.041	2.609 **	0.017	0.224	0.005	1.326 **
	Rel. resilience	0.893 *	1.159 **	0.209 *	0.204	0.845 **	1.183 **

The level of significance is represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

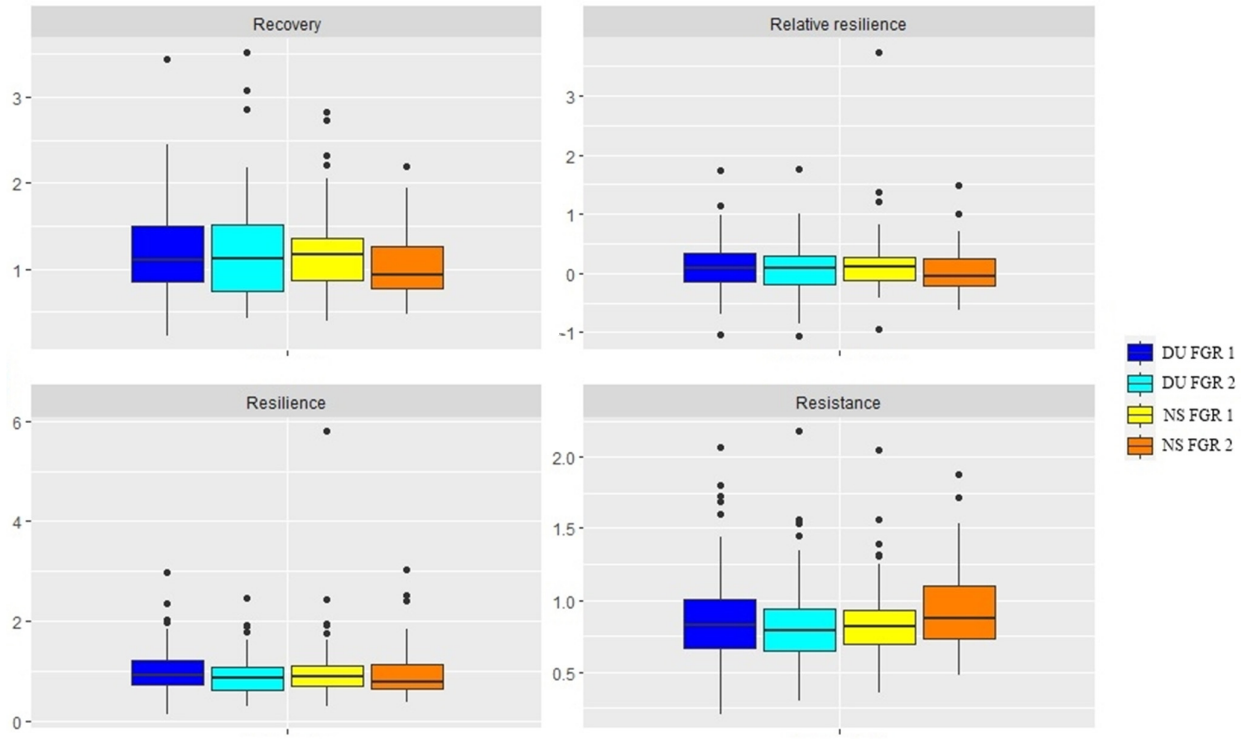


Figure 6. Variation of drought parameters for Douglas fir (DU) and Norway spruce (NS) in forest genetic resources (FGR 1 and FGR 2).

In FGR 1, Douglas fir exceeded Norway spruce regarding resistance capacity during extreme drought in 2000 and resistance, recovery, resilience, and relative resilience during extreme droughts in 2003 and 2012. In FGR 2, Douglas fir displayed a higher recovery during the extreme drought in 2003 and higher resistance, recovery, resilience, and relative resilience during the extreme drought in 2012. It should be noted that we used a three-year period before and after the extreme event to calculate drought parameters. For the year 2000, the analyzed period after the drought included the years 2002 (with moderate drought) and 2003 (with extreme drought).

Norway spruce was slightly better than Douglas fir in terms of recovery and resilience capacity during the extreme event in 2000 in both sites, and resistance and resilience in FGR 2 during the summer drought in 2003. Norway spruce also showed a higher capacity of adapting to the spring drought in 1968 and autumn drought in 1986 than Douglas fir.

Establishing the ranking of drought parameters in both study sites for all years of extreme drought, Norway spruce showed a higher resilience to extreme drought events, whereas Douglas fir showed higher resistance and relative resilience. Both tree species recorded similar recovery capacities (Figure 7). Correlations among growth traits and drought parameters demonstrated that Douglas fir had the highest resistance to drought and the widest growth rings (Table 6).

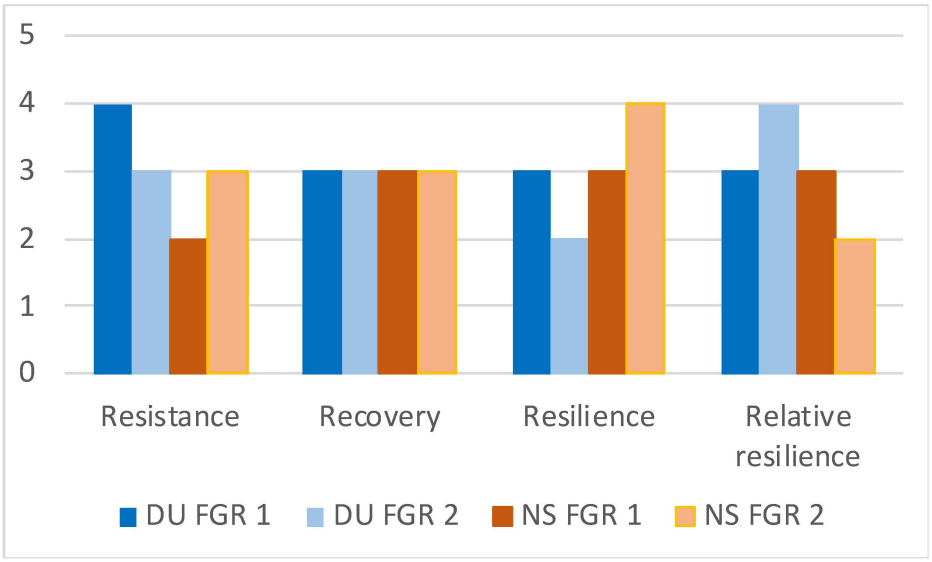


Figure 7. The ranking of drought parameters for Douglas fir (DU) and Norway spruce (NS) in forest genetic resources (FGR 1 and FGR 2).

Table 6. Phenotypic correlations between wood characters and drought parameters of Douglas fir (DU) and Norway spruce (NS).

Species	Trait	RW	LW	EW
DU	Resistance	0.594*	0.769 **	0.524
	Recovery	−0.888 ***	−0.692 *	−0.643 *
	Resilience	−0.476	−0.105	−0.224
	Rel. resilience	−0.811 **	−0.594 *	−0.580*
NS	Resistance	−0.046	−0.269	0.151
	Recovery	−0.790 **	−0.173	−0.476
	Resilience	−0.776 **	−0.363	−0.462
	Rel. resilience	−0.797 **	−0.159	−0.483

RW—ring width; LW—late wood width; EW—early wood width; The level of significance is represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4. Growth Response Functions

The climate–growth response relationships varied between conifer species and study sites (Table 7). The main climate variables linked to tree radial growth of Douglas fir located on the high-productivity site (FGR 1) were $MT_{OCT-MAR}$ and SP_{VEG}, whereas for FGR 2 on the low-productivity site, these variables were MT_{JUL}, $MT_{OCT-MAR}$, and $SP_{OCT-MAR}$. The main climatic drivers explaining radial growth of Norway spruce were MT_{JUL} and $SP_{OCT-MAR}$ in the first study site and MT_{VEG} and $SP_{JAN-MAR}$ in the second study site.

Douglas fir appears to be less sensitive to precipitation than to temperature. The autumn and winter temperatures were positively correlated with Douglas fir growth, but the July temperatures negatively influenced both species. A negative relationship between radial growth and MT_{VEG} for Norway spruce was also found. Increasing precipitation in the growing season and in the autumn–winter interval positively influenced Douglas fir radial growth, but this influence was negative on Norway spruce.

The growth–climate relationships were weak. R^2 ranged between 0.10 and 0.12 for Douglas fir and between 0.06 and 0.10 for Norway spruce, which suggest that only a small portion of the radial growth variation can be explained by climatic factors.

Similarly, a modest response was found for latewood percentage. Thus, significant correlations between climate and LWP of Douglas fir were found in FGR 2, whereas for Norway spruce in FGR 1, MT_{JUL}, MT_{VEG}, and $SP_{OCT-MAR}$ negatively influenced the LWP.

Table 7. Climatic response models for radial growth (RW) and latewood percentage (LWP) of Douglas fir and Norway spruce.

Species	FGR	Growth Response Model	Signf.	R^2
Douglas fir	FGR 1	$RW = 0.864 + 0.008\ MT^2_{OCT\text{-}MAR} + 0.001\ SP_{VEG}$	*	0.12
Douglas fir	FGR 2	$RW = 1.686 - 0.002\ MT^2_{JUL} + 0.005\ MT^2_{OCT\text{-}MAR} + 0.001\ SP_{OCT\text{-}MAR}$	*	0.10
Norway spruce	FGR 1	$RW = 1.527 - 0.001\ MT^2_{JUL} - 0.0001\ SP^2_{OCT\text{-}MAR}$	*	0.06
Norway spruce	FGR 2	$RW = 2.813 - 0.003\ MT^2_{VEG} - 0.001\ SP^2_{IAN\text{-}MAR}$	*	0.10
Douglas fir	FGR 2	$LWP = 74.941 - 0.044\ TM^2_{JUL} - 0.001\ SP^2_{OCT\text{-}MAR}$	***	0.23
Norway spruce	FGR 1	$LWP = 56.419 - 0.038\ MT^2_{VEG} - 0.014\ SP_{OCT\text{-}MAR}$	*	0.07

MT_{JUL}—the mean temperature for July; MT_{VEG}—the mean temperature of the growing season; $MT_{OCT\text{-}MAR}$—the mean temperature from October of the previous year to March of the current year; $SP_{OCT\text{-}MAR}$—the sum precipitation from October of the previous year to March of the current year; $SP_{JAN\text{-}MAR}$—the sum precipitation from January to March of the current year. The level of significance is represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.5. Potential Impact of Climate Change Projections

The regions suitable for growing Douglas fir in Romania based on climate data over 1961–2020 are shown in Figure 8. For climatic envelope modeling, we used climatic variables, considering them to be the most important in the context of adaptation to climate change and because it is well known that Douglas fir grows on different soil types that are moderately acidic.

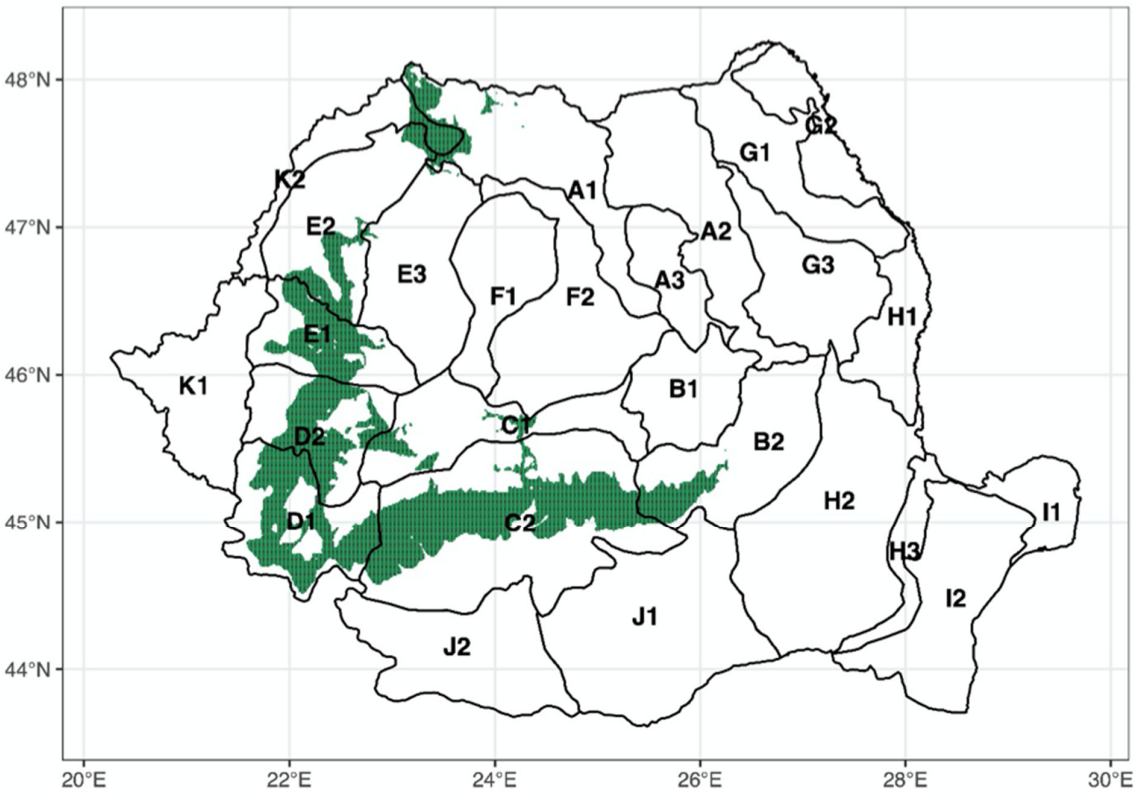


Figure 8. Suitability areas for the Douglas fir in Romania according to the high-resolution climatological data over 1961–2020. The polygons denote the regions of provenance (as in [49]): A1: Eastern Carpathians, western cline; A2: Eastern Carpathians, eastern cline; A3: Giurgeu–Ciuc

depression; B1: Brasov depression; B2: Curvature Carpathians, outer cline; C1: Southern Carpathians, northern cline; C2: Southern Carpathians, southern cline; D1: Mehedinti/Cerna/Semenic Mountains; D2: Tarcu/Poiana Rusca Mountains; E1: Zarand/Metaliferi Mountains; E2: Western Apuseni Mountains; E3: Eastern Apuseni Mountains; F1: Transylvania Plain; F2: Transylvania Plateau; G1: Suceva/Siret/Iasi Hills; G2: Jijia Plain; G3: Barlad Plateau; H1: Covur Plateau; H2: Siret and Baragan Plains; H3: Danube water holes; I1: Danube Delta; I2: Dobrogea Plateau; J1: Bucharest Plain; J2: Oltenia Plain; K1: Timis and Arad Plain; K2: Cris/Carei/Somes Plain.

The projection suitability maps under RCP4.5 and the RCP8.5 scenarios over the periods 2041–2070 and 2071–2100 are presented in Figure 9. The maps show that the species suitability will increase along altitudes and in other Carpathians regions with moderate climates. Our results suggest that this species may be cultivated on a larger scale than has been considered so far.

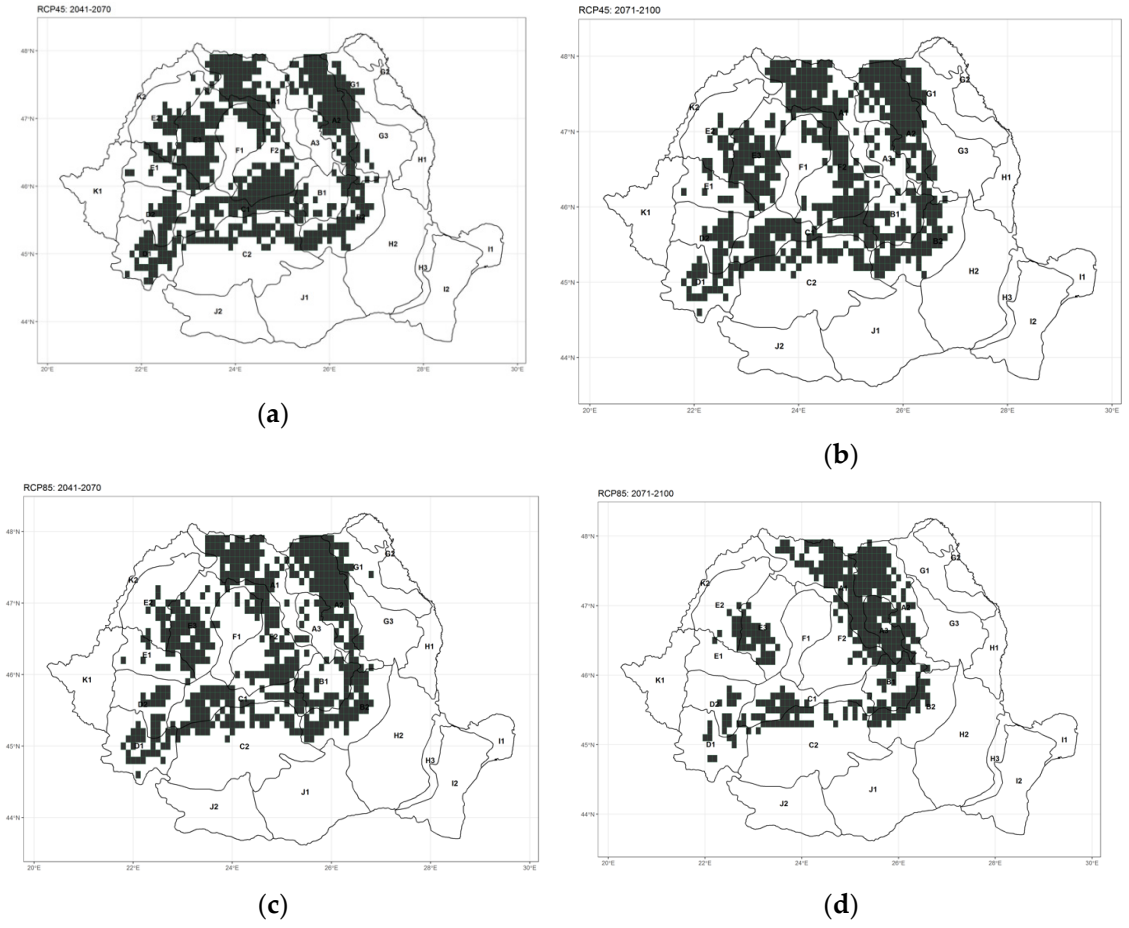


Figure 9. Projected suitability areas for the Douglas fir in Romania under the RCP4.5 (**a**,**b**) and RCP8.5 (**c**,**d**) scenarios, over the periods 2041–2070 (**a**,**c**) and 2071–2100 (**b**,**d**). The polygons denote various regions of provenance (as defined by [49]). A1: Eastern Carpathians, western cline; A2: Eastern Carpathians, eastern cline; A3: Giurgeu–Ciuc depression; B1: Brasov depression; B2: Curvature Carpathians, outer cline; C1: Southern Carpathians, northern cline; C2: Southern Carpathians, southern cline; D1: Mehedinti/Cerna/Semenic Mountains; D2: Tarcu/Poiana Rusca Mountains; E1: Zarand/Metaliferi Mountains; E2: Western Apuseni Mountains; E3: Eastern Apuseni Mountains; F1: Transylvania Plain; F2: Transylvania Plateau; G1: Suceva/Siret/Iasi Hills; G2: Jijia Plain; G3: Barlad Plateau; H1: Covur Plateau; H2: Siret and Baragan Plains; H3: Danube water holes; I1: Danube Delta; I2: Dobrogea Plateau; J1: Bucharest Plain; J2: Oltenia Plain; K1: Timis and Arad Plain; K2: Cris/Carei/Somes Plain.

4. Discussion

In this study, we analyzed the growth and adaptive capacity to climate change of Douglas fir compared to Norway spruce in two forest genetic resources, which are among the few oldest plantations with Douglas fir in Romania.

Substantial differences in growth traits and wood characteristics of the two stands were found. Douglas fir exceeded Norway spruce for all studied characteristics in both locations, but the best growth performances were obtained on the low-productivity site. The average volume per tree, ring width, and latewood percentage were approximately 76%, 41%, and 19%, respectively, higher for Douglas fir than for Norway spruce.

The analysis of climate data showed significant changes in climate conditions in the last century in western Romania, indicating that the growing conditions for forest tree species have changed. Significant variations occurred in the last two decades, when the mean annual temperature increased by 0.9 °C, mean temperature in January by 1.3 °C, and mean temperature during the growing period by 1.0 °C. It is surprising that the precipitation amount did not decrease; in fact, a slight increase was observed.

The growth response functions revealed that the climatic variables of the study sites were a major driver of the growth performance of the two conifer species, but the climate variables depend on species. There was a significant negative response to the temperature in July and a positive influence of previous autumn–winter temperature for Douglas fir. Provenance studies show that winter air temperatures are of the utmost importance for populations' adaptation, limiting Douglas fir growth in Europe [15,21]. Our study confirms that autumn–winter temperatures are the most important factor in determining the tree-ring width in Douglas fir. Furthermore, increasing precipitation during the growing season or in the previous autumn–winter period had a positive effect on trees' radial growth but negatively influenced latewood percentage. These growth–climate relationships are similar to those observed in a number of regional studies in North America [68–70].

For Norway spruce growing outside its natural range in the study locations, the July temperatures and temperatures during the growing season were the limiting factors in determining tree growth. The observed significant negative influence of SPOCT-MAR and SPIAN-MAR point to Norway spruce being less sensitive to precipitation during the period of vegetative rest compared to Douglas fir. The latewood percentage was sensitive to increasing the July and growing season temperatures and autumn–winter precipitation for both species.

The growth–climate relationships were, in general, weak. According to Fritts [52], the strength of the climate–growth relationship depends on how strongly growth is limited by the climate of the study site. At Ana Lugojana Forest District, the mean values of SAP for the 1901–2017 period was 754 mm, and MAT was 8.97 °C, which indicates that Norway spruce is placed in a climatic suboptimum and that decreased precipitation and increased temperatures can have negative effects on its growth and survival. The coastal Douglas fir is a variety adapted to highly different site conditions; in western Oregon and Washington, it occurs from sea level to over 1700 m. Its adaptation is a consequence of trade-offs between selection for traits to avoid exposure to frost and traits that confer high vigor in mild environments [71].

The climate of Ana Lugojana Forest District is at the lower limit of its climatic optimum concerning SAP, winter temperature, and frost-free days. This may explain the exceptional growth performance of Douglas fir in the two study sites from western Romania. The difference between the climate within the native distribution range and western Romania consists of the precipitation distribution over the year. Although precipitation is nearly evenly distributed over the year in Romania, precipitation at the place of origin has a pronounced maximum in winter and a minimum in summer [1].

Considerable differences in response to drought events were found between the two tree species. The species reaction depended on the timing and duration of the drought event. Generally, Douglas fir had higher resistance, recovery, resilience, and relative resilience to the summer droughts from 2000, 2003, and 2012 than Norway spruce, which showed a

higher tolerance to spring drought in 1968 and autumn drought in 1986 than Douglas fir. Regarding the ranking of drought parameters for both sites and all extreme drought years, Norway spruce displayed a higher resilience to extreme droughts, whereas Douglas fir showed a higher resistance and relative resilience. Both species recorded a similar recovery capacity.

Norway spruce is particularly vulnerable to drought [72–75]. In contrast, Douglas fir is more drought resistant than Norway spruce [2,47,48]. Rising temperatures and decreasing precipitation in the near future may increase mortality risk for Norway spruce and other native coniferous species, particularly at low elevation sites [44,76–78]. Douglas fir is a fast-growing tree and can be a potential species for biomass production. In Europe, Douglas fir produces high-quality timber, which generally equals or exceeds the value of indigenous softwoods species. Thus, the Douglas fir dry wood density is on average 0.45 t/m^{-3} [2], while of Norway spruce is on average 0.40 t/m^{-3} (wood-database.com/Norway spruce). One of the reasons for this potential is that under the global warming scenario, the likelihood of cold damage to low-elevation sources will decrease with time. Recent studies have shown that planting Douglas fir with broadleaf species had a positive effect on the survival of this species [2,78]. In this context, the selection of suitable Douglas fir forest reproductive material is essential because it affects the growth, frost sensitivity, and tolerance to diseases [15,22,71,79].

Our results based on growth response functions and climate models by 2100 suggest that Douglas fir has a high growth potential in many Carpathians regions, not only in the west of Romania as has been considered so far. Our results confirm that climate change can increase Douglas fir productivity at higher elevations as a consequence of improving growth conditions. Further, the developed models used for the assessment of Douglas fir suitability under future climate in Romania showed good survivability.

5. Conclusions

Selecting suitable tree species and provenances adapted to ongoing climate change is of great significance in forest management. The cultivation of Douglas fir, a non-native tree species, has often been associated with higher risk and uncertainty. The developed models used for the assessment of Douglas fir suitability under the predicted future climate in Romania showed good survivability in many Carpathian regions. Douglas fir has an exceptional growth capacity, overcoming the Norway spruce in both high- and low-productivity sites, currently as well as in the past. Considerable differences were found in drought tolerance as well, with our results demonstrating the high resistance and relative resilience to extreme droughts of Douglas fir compared to Norway spruce. However, autumn–winter temperatures play an important role in the adaptation of Douglas fir to site conditions in Romania.

The use of appropriate provenances of Douglas fir in mixed stands with native broadleaved species may be an option for climatically exposed sites, thus increasing the value of these stands. Therefore, the conservation of the most valuable genetic resources of Douglas fir should have priority because these stands can be potential seed sources for ecosystem restoration. This study reveals the importance of improving our knowledge about the growth, ecology, and adaptive capacity of this non-native species in the context of climate change.

Author Contributions: Conceptualization: G.M., A.-L.C. and M.-V.B.; methodology: G.M. and A.-L.C., E.C. and M.-V.B.; field measurements and dendrochronological analyzes: A.-M.A.; climate data analysis and processing: I.-A.N. and M.-V.B.; maps: I.-A.N. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Variation of Cones Production in a Silver Fir (*Abies alba* Mill.) Clonal Seed Orchard

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Abstract: The current study is the first to describe fertility dynamics in a Silver fir (*Abies alba* Mill.) seed orchard and among the few reporting related information from a mature seed orchard. The research aim was to evaluate the female cone production in order to provide support to future management measures. Observations were conducted in a clonal seed orchard from the Eastern Carpathians over six years; all the ramets of 44 clones and the zero-inflated data were analyzed with generalized lineal mixed effects models in a Bayesian framework. The results indicated a higher influence of the year and probably of the sensitivity to climate, and less variability between clones, in both the Poisson and zero-inflated components of cones production. The repeatability of mean annual clone production suggests moderate continuity of cone crops in the production rank of individual clones, while the estimates of heritability were under a moderate genetic control. The values of heritability were influenced by the reporting scale (latent vs. original data scale); therefore, caution in the analysis of non-Gaussian data and in comparisons of heritability between seed orchards is required. The variation of the female cone production was higher than expected for a seed orchard, but with marked variation across years, similar to other related indicators, patterns specific to mature, and productive commercial seed orchards. Several management options to be applied in the future were also discussed.

Keywords: cone production variation; zero-inflated; Markov chain Monte Carlo (MCMC); seed orchard management; South-Eastern Europe

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1. Introduction

Seed orchards represent the close link between breeding activity and forestry practice, genetically improved seeds being used to produce seedlings for artificial forest regeneration, and perhaps, are the most widely used production populations [1]. They maintain almost as much genetic diversity as natural populations and represent a qualitative and quantitative improvement in comparison with the seed stands [2]. The main goal of obtaining genetically improved seeds, which is pursued when establishing clonal seed orchards, can be achieved if several essential conditions are met: the isolation of seed orchard from sources of foreign, genetically inferior pollen, equally and simultaneous blooming of clones, and a very low level occurrence of self-pollination [3]. The degree to which the clones pass on their superiority and genetic diversity to the seeds determines how genetically efficient a seed orchard is. Practical experience and many studies since the 1970s indicated that the transfer is limited [4].

Fertility represents the reproductive potential of a clone, quantitatively estimated in plant species by flowers, pollen, fruits, and seeds [5]. Fertility variation plays an important role in breeding and conservation [2] and its understanding is crucial for the management of seed orchards, as well as for predicting gene diversity, particularly in terms of seed

production yield [6,7]. Cones and seeds are the primary sources of income; the fertility of female parents might be prioritized over that of male parents, as the latter is more challenging to quantify than that of seeds [2].

Different factors influence the individual fertility variation: age, size, individual genotype, stand condition, and environment [8]. In a seed orchard, the genetic composition of the produced seeds is also shaped by pollen contamination and flowering time synchronization, counts and weights of clones and ramets in spatial pattern, environment, and climate [7]. Climate (temperature and precipitation) plays a complex role and significant year-to-year variation was reported [9,10]. Higher variation of fertility was found in natural and managed stands compared to the clonal averages from seed orchards, in seed orchards of low age and in years of low production, respectively [8]. Many studies highlighted the small number of clones, which account for a large proportion of the seed orchard crop [11–15]. This was dubbed as the 20:80 rule [16], according to which 20% of the parents produce 80% of the seed-cone crop. However, when coupled with observed fertility variations due to the orchard's developmental stage, with environmental conditions or management practices, a more realistic view would assume that the genetic composition of any orchard's crop is unique. In estimating the gene diversity of seed orchard crops, the use of multiple fertility-related tools (i.e., variance partitioning of contributing factors and repeatability, assessment of parental balance, and indicators of fertility variation) is advised [17].

In clonal seed orchards, multiple sources of variability contribute to overestimations of fertility: the presence of scale effects, avoidance of variability between ramets within clones and reporting clone values only, and the influence of the annual characteristics (e.g., climate) [7]. The skewed distribution of phenotypes generates differences among the predicted and observed traits, and in genetic parameters (overestimation of heritability) [18]. Tree fertility, as a skewed trait [7], sometimes following a zero-inflated distribution, and requires adequate data treatment [19–21]. In tree genetics, the common approach in fertility analysis is data transformation [15,22], but this could lead to known potential issues [23,24]. In other organisms, genetic measures associated to zero-inflated traits were reported (heritability and evolvability) in a Bayesian framework [25,26], which offer some advantages in comparison to the frequentist approaches, such as flexibility, incorporation of uncertainty [27], accurate estimates of variance and genetic parameters, or providing highest posterior density intervals (HPD) for the genetic measures [28].

The Silver fir (*Abies alba* Mill.) is an important component of the European forests, the most significant in terms of both economic importance and ecological relevance among the various fir species naturally occurring in Europe, and a fundamental species to maintain biodiversity in forest ecosystems [29–31]. Due to climate change, it is estimated that the Silver fir geographic spread would decline [32], with the peripheral populations from the eastern limit of its distribution having a great potential to reduce the adverse consequences of climate warming [33–35]. In Romania, Silver fir is one of the most important conifer species and occupies 4.3% of forest area [36], secondly ranked after the Norway spruce. The Silver fir range is discontinuous and the maximum area is located in the Romanian Eastern and South-Eastern Carpathians, in pure or mixed stands with spruce and beech. The interest in the culture of Silver fir has increased in recent years, hence the need to supply the Romanian and foreign requests with increasing amounts of seeds. The seed orchards are convenient options to ensure this requirement, but also for the ex situ conservation of valuable gene pools. In Romania, the setting up of the seed orchards dates back to 1958, the first being established in 1961–1962 for Grayish oak *(Quercus pedunculiflora* K. Koch.). From 375 ha in 1975, the rate of seed orchards establishment increased in 1990 to 1004.2 ha. In Silver fir, the breeding program started in 1972, by identifying valuable regional natural populations and the phenotypic selection of more than 600 plus trees, installed in 11 seed orchards, with a total area of 92.1 ha [37]. Currently, ten Silver fir seed orchard with a total area of 84.9 ha are included in the National Catalog of Basic Materials, from which seven

are classified as "provenance seed orchard" (*Pc.P*) and three as seed orchards with the origin of clones in the same "region of provenances" (*PC.R*).

Despite the importance of the species, with the exception of some general reports [38], data about the Silver fir seed orchard fertility is lacking. We present in this study an analysis of the female cone production of 44 clones over six years. The objectives of this study were: (i) to estimate the variability of clones cone production in a zero-inflated modeling framework, (ii) to determine the female fertility variations based on multi-and single year observed cones, and (iii) to discuss the practical implications for seeds production and seed orchard management.

2. Materials and Methods

2.1. Silver Fir Clonal Seed Orchard

The studied Silver fir clonal seed orchard is located in the north-eastern part of Romania (47°03′29″ latitude north and 26°26′06″ longitude east, close to Bodeşti, Neamţ county), at an altitude between 479–512 m and is administrated by Gârcina Forest District, Neamț National Forest Administration (RNP-Romsilva). The clonal seed orchard PS-BR-NT81 was installed in 1981, by multiplying via grafting 44 plus trees, selected from natural Silver fir forest stands from four provenance regions (Eastern Carpathians-A2, South-Eastern Carpathians, the external wedge-B2, north of Southern Carpathians-C1 and Banat Mountains, Ţarcu-Poiana Ruscă-D2) (Table 1). The orchard has an area of 5 ha, with a completely random design and a planting scheme of 6 × 6 m. The number of ramets per clone varies from 1 to 32, with an average of 14. Among the methods used to estimate the clonal contribution in seed orchards, Woods [39] mention different approaches to estimate the parental contribution, the simplest being the visual estimation of the number of cones on each ramet before harvest, while the most complex is the estimation of seed viability for each clone. To evaluate the maternal contribution, the visual assessment of cone production was used [39] with the aid of Nikon binoculars. For six years (2013, 2015, 2018, and 2020–2022), the female cone production of all the ramets (100% sampling) in the seed orchard was counted during July–August.

Table 1. Clone origin in the Silver fir (*Abies alba* Mill.) seed orchard.

Provenance (Provenance Region)	Clone IDs	Number of Clones	Latitude N	Longitude E	Altitude (m)	Mean Annual Temperature (°C)	Mean Annual Precipitations (mm)
Avrig (C1)	3.1–3.18	7	45°39′	24°29′	750	7.5	694
Rusca Montană (D2)	3.55–3.72	4	45°39′	24°29′	1000	6.6	878
Sinaia (B2)	4.17–4.36	7	45°19′	25°32′	1150	4.8	783
Văratec (A2)	158–181	26	47°08′	26°15′	600	6.9	606

2.2. Data Analysis

2.2.1. Genetic Variation

The genetic variation was estimated by fitting zero-inflated Poisson generalized linear mixed effects models (ZIP GLMM) to both multi- and single-year data. All the models contained the clone identity (n = 44) as random effects, while the multi-year models additionally included the year (n = 6) and the interaction between clone and year. The data included a large number of zeros, and we tested it for the presence of dispersion and excess of zeros [40–44]; the results confirmed the presence of zero-inflation (Supplementary Material S2).

The count of cones Y of the ramet k in the clone j in the year i was modeled using the Poisson distribution:

$$Y_{ijk} \sim \text{ZIP}(\lambda_{ikj}, \pi_{ijk}) \quad (1)$$

The two separates, a linear predictor for the conditional mean and another for the excess of zeros were:

$$\log\left(\lambda_{ijk}\right) = \beta_0 + O_i + C_j + O_{cj} \quad (2)$$

$$\text{logit}\left(\pi_{ijk}\right) = \frac{\log\left(\pi_{ijk}\right)}{\left(1-\pi_{ijk}\right)} = \beta_0^{(\pi)} + O_i^{(\pi)} + C_j^{(\pi)} + OC_{ij}^{(\pi)} \quad (3)$$

In the equations, β_0 is the overall fitted mean, O_i is the random effect in the ith observation year (i = 1...6 ~ NID (0, σ_o^2), C_j is the random effect in the jth clone (j = 1...44 ~ NID (0, σ_c^2)), and OC_{ij} is the random effect of the interaction between year and clone (NID (0, σ_{oc}^2)). The single-year models are similar, with the exception of the presences related to the observation year (i.e., O_i and OC_{ij}). The models were fitted within a Bayesian framework (package MCMCglmm, version 2.32) [45]. In the Bayesian analysis, the prior influences model results [46] and, for zero-inflated models, there are different reports on the prior influence on the heritability estimates [47–49]; our prior analysis is presented in Supplementary Material S3.

Each model was run for 7,505,000 iterations, with the first 5000 iterations discarded (burning) and every 2500 iterations stored (thinning), resulting in size of effective samples larger than 1000 (close to 3000) and autocorrelations <0.1. The overall settings were chosen to ensure the models passing of the Heidelberger and Welch's convergence diagnostic and the stationarity tests (functions heidel.diag and autocorr.diag of coda package) [50] (Supplementary Material S3).

The results of MCMCglmm are in unobserved latent scale and, for interpretation, the latent scale posterior distributions of the parameters were back-transformed to observed data scale with the package QGglmm (version 0.7.4) [51].

Repeatability of mean clone cones production (i.e., the broad-sense heritability) was analyzed with correlation-based and GLMM-based methods [24]. In multi-year data, we calculated the Pearson product moment and Spearman's rank correlation coefficients to test stability and relative order across years, respectively [52]. The model based of multi-year repeatability was calculated as:

$$H^2 = \frac{\sigma_c^2}{\sigma_c^2 + \frac{\sigma_{oc}^2}{oc} + \frac{\sigma_e^2}{or}} \quad (4)$$

In single year analysis, it was:

$$H^2 = \frac{\sigma_c^2}{\sigma_c^2 + \frac{\sigma_e^2}{r}} \quad (5)$$

In both the formulas, σ_c^2 is the variance between clones, σ_{oc}^2 is the variance of the interaction between clone and year and σ_e^2 is the error variance; o, c, r are the numbers of clones, years, and the mean number of ramets per clone, respectively. As the heritability can have values only between 0 and 1 and some of the lower bounds of its 95% credible intervals presented values close to 0, we considered that if the posterior values follow a normal distribution (e.g., mean close to median), then the heritability is not zero.

2.2.2. Female Fertility Variation

The parental balance was assessed by constructing cone yield curves [11], which plot the percentage of clones participating to cone production. The premise of calculating cumulative yield curves or effective population numbers based on cone counts starts from the correspondence between reproductive success (e.g., seed cones count) and reproductive energy (counts of filled seeds per cone) [2]. First, we calculated the cumulative percentage of cone production per clone, then sorted these from high to low, and plotting the cumulative

contribution against the proportion of clones [53]. The female fertility was described by the 'sibling coefficient Ψ' [17,54]:

$$\Psi_{(f)} = N \sum_{i=1}^{N} p_i^2 = {CV_{(f)}}^2 + 1 \quad (6)$$

where N is the number of clones, p_i is the proportional contribution of clone i, and CV_f is the coefficient of variation of the clonal proportion of cones production. Female fertility variation—along with male fertility variation Ψ_m and the component of the clone fertility variation Ψ—is an adimensional measure that relates parents to their progeny and expresses the probability that successful gametes, commonly known as "sibs," would come from the same parent. Its values cannot be below 1; values Ψ = 1 means that the individuals have the same fertility, while Ψ = 2 means a twice chance that two individuals would share a parent, compared to the above equal parental fertility. For seed orchards, Kang et al. [9] suggested that Ψ = 2.

In an idealized population, of which individuals produce the same number of offspring as the real population, the sibling coefficient can be used to calculate the effective number of female parents ($N_{p(f)}$) [8]:

$$N_{p(f)} = \frac{N}{\Psi_{(f)}} = \frac{N}{{CV_{(f)}}^2 + 1} \quad (7)$$

The relative effective number of female parents permits comparisons among the census and status number in a seed orchard [8,52]:

$$N_{r\%} = \frac{N_{p(f)}}{N} \cdot 100 \quad (8)$$

The genetic diversity [55,56] can be calculated as:

$$GD = 1 - \frac{0.5}{N_{p(f)}} \quad (9)$$

All the statistical analyses were conducted in R (version 4.2.1, Vienna, Austria) [57].

3. Results

3.1. Genetic Variation

Among the 3811 counted ramets in the observational years between 2013 and 2022, the cone production varied between 0 and 350 (Table 2). Although the number of the ramets remained almost constant, a total of 848 (22.25%) have no cones (i.e., zero fertility, Supplementary Material S2, Figure S2.1). The raw average yearly cone production was 41.10 ± 46.44 SD, with extremes in 2021 (7.12 ± 15.10 SD) and 2018 (79.58 ± 61.41 SD) (Table 2). In pooled clone data, the top values were between 58.00 and 64.64 (clones 182, 176, 173), while the lowermost were between 4.70 and 17.75 (clones 3.18, 168, 3.17) (Supplementary Material S1, Table S1.1).

Table 2. Statistics of cones production across years.

Parameter/Year	2013	2015	2018	2020	2021	2022	Pooled
Alive ramets (n)	636	636	636	636	636	631	-
Fertile ramets (%)	75.3	95.12	91.5	94.3	42.6	67.5	77.7
Mean	28.0	67.92	79.6	47.5	7.1	16.2	41.1
Std. deviation	35.9	41.18	61.4	36.3	15.1	21.3	46.4
Median	14.5	67.5	70.0	42.0	0.0	7.0	25.0
Range	0–206	0–200	0–350	0–215	0–115	0–110	0–350

In the multi-year model of cones production, most of the variance is explained by year, both in the Poisson (13.2%, credible interval -CI hereafter- 3.7–33) and zero-inflated component—(37.9%, CI 18.1–61.9). The clone-related values (clone and clone x year interaction) are small in Poisson (1.4%, CI 0.6–2.8) and 1.3% (CI 0.6–2.1), but increased in the zero-inflated part (8.7%, CI 3.2–17.7 and 10.2%, CI 5.0–17.1) (Figure 1). In single-year models, no clear pattern due to clone was observed, just overall larger values in the zero-inflated part compared with Poisson, and wider 95% posterior intervals (Table 3).

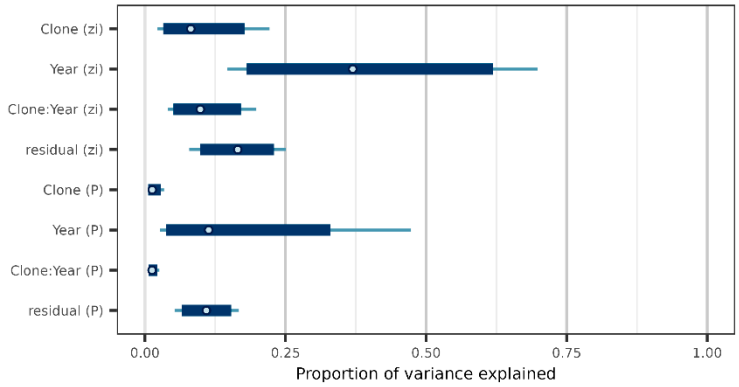

Figure 1. Variance explained by random effects in the multi-year model, Poisson (P), and zero-inflated (zi) components. Posterior means (points), 99%, and 95% credible intervals (lines, respective blocks) are in latent scale.

Table 3. Proportion of variance explained by random effects and heritability in single-year models. Estimates are reported as posterior mode (95% credible interval, proportion of variance from latent scale, heritability in both latent ($H^2_{l(P)}$), and data scale ($H^2_{(zi)}$, $H^2_{(P)}$), for Poisson (P) and zero-inflated (zi) component of the model.

Parameter/Year	2013	2015	2018	2020	2021	2022
$Clone_{(zi)}$	33.2 (17.9–50.4)	18.9 (0.1–55.6)	56.8 (36.9–74.6)	20.2 (0.1–52.6)	26.4 (13.1–42.7)	24.6 (10.1–42.8)
$residual_{(zi)}$	27.3 (20.1–34.2)	55.4 (30.3–69.9)	25.9 (15.5–38)	46 (27.3–58.7)	31.3 (23.8–38.3)	37 (27.9–45)
$Clone_{(P)}$	10.2 (5–18.3)	5.5 (2.2–10.6)	2.6 (1–5.4)	6.2 (2.6–11.7)	5.7 (1.1–13.4)	6 (2.3–12)
$residual_{(P)}$	29.3 (21.2–37.4)	20.2 (10.9–26.3)	14.6 (8.5–21.7)	27.6 (16.3–36)	36.7 (27.4–46.2)	32.4 (24–40.2)
$H^2_{(zi)}$	0.127 (0.07, 0.237)	0 (0, 0.049)	0.088 (0.033, 0.188)	0 (0, 0.065)	0.147 (0.07, 0.229)	0.106 (0.037, 0.191)
$H^2_{l(P)}$	0.83 (0.723, 0.914)	0.797 (0.663, 0.891)	0.733 (0.548, 0.846)	0.783 (0.616, 0.866)	0.698 (0.323, 0.868)	0.724 (0.541, 0.877)
$H^2_{(P)}$	0.607 (0.543, 0.633)	0.662 (0.536, 0.751)	0.616 (0.46, 0.715)	0.614 (0.486, 0.698)	0.443 (0.188, 0.594)	0.549 (0.353, 0.628)

The Pearson product moment correlation coefficients of cone production between the years were positive, significant, and higher between the years of good production, while moderate between others; a remark is on the weak, significant correlation between the 'good' and the 'poor' years (Table 4). The pattern is similar in Spearman's rank correlation coefficients. No significance was found in negative correlations (2020–2021 and 2015–2022) and in correlations between a specific year (2022) and any others (Table 4). The results support the continuity of cone crops, especially in production rank of individual clones.

The broad-sense heritability of clonal cone production from pooled data was 0.569, with a large 95% CI (0.369, 0.709) (Figure 2). In the zero-inflated component, the values of heritability were very small (0.043 [0.009, 0.113]), tending to zero in good years (e.g., 2015, 2020), but were normally distributed both in pooled and yearly data and, therefore, were

different than zero (Table 3, Supplementary Material S4). Across the monitoring period, the lower heritability value was recorded in the poor production year and tended to be similar in the good years. Marked differences were between the latent scale and data scale heritabilities of the Poisson component of the cone production model (Table 3).

Table 4. Pearson product moment and Spearman's rank correlation coefficients (above and below the diagonal) among clone mean cone production across years.

Year	2013	2015	2018	2020	2021	2022
2013	1	0.473 ***	0.462 *	0.297 *	0.276	0.001
2015	0.535 ***	1	0.797 ***	0.560 ***	0.343 *	−0.003
2018	0.492 **	0.811 ***	1	0.588 ***	0.162	0.077
2020	0.346 *	0.599 ***	0.596 ***	1	−0.069	0.163
2021	0.280	0.329 *	0.159	−0.049	1	0.136
2022	0.151	0.28	0.302	0.262	0.295	1.000

Statistical significance: ***—$p < 0.001$, **—$p < 0.01$, *—$p < 0.05$.

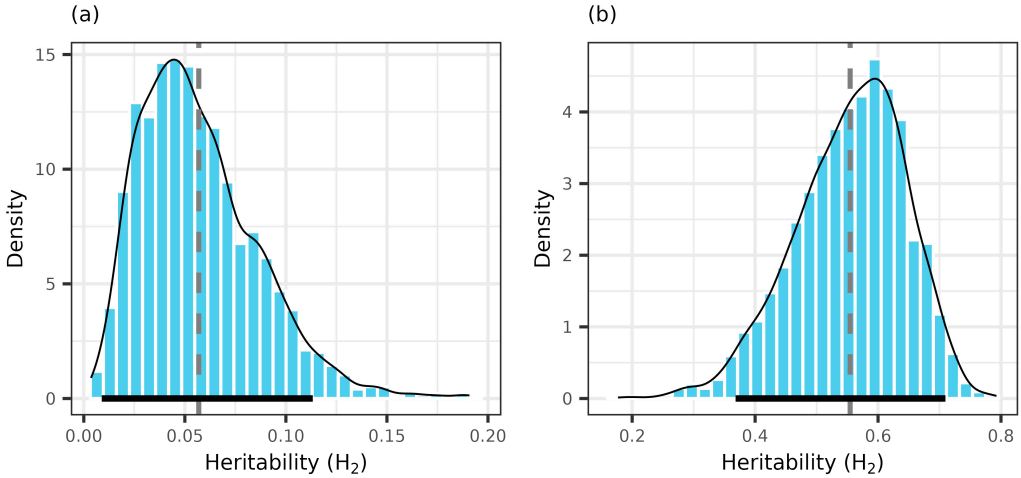

Figure 2. Posterior MCMC samples (bars), kernel density estimation (thin solid line), posterior mean (dotted line), and 95% credible intervals (thick solid line) of heritability in zero-inflated (**a**) and Poisson (**b**) components of cone crops. Estimates are in data scale.

3.2. Female Fertility Variation and Genetic Diversity

Yearly cumulative curves of cones crop (Figure 3) showed deviations from the ideal case of a seed orchard with equal contribution, grouping the years in two groups of higher production (2015: closest to the expectation line, 2018 and 2020), and of poor production (2013 and 2021: furthest to the expectation line, and 2022). We found little evidence of the "20:80 rule"; the top 20% parents produced 50.5% of production in the poor year (2021) and 64.58 in the best (2015).

There was also individual clonal variation regarding the presence in the 20% list of parents. No clones were present in this 'top' list across all the monitoring years, and just a few were included in more than half of the observed period (the same three and five clones appeared in four and three years, respectively). More constancy was noticeable in the 'bottom', poor clones, where 1–2 clones were present persistently in all/most of the years (5–6 years). The two clone groups, one whose production exceeds the cumulative average of observation years, different as provenance (182, 173, 166, 176, 175, 178—local, Văratec; 3.1, 3.2. 3.16—Avrig; 4.29, 4.32—Sinaia), and of low production, below the multi-year average or without cones in some observation years (161, 167, 4.28) (Supplementary Material S1, Table S1.1).

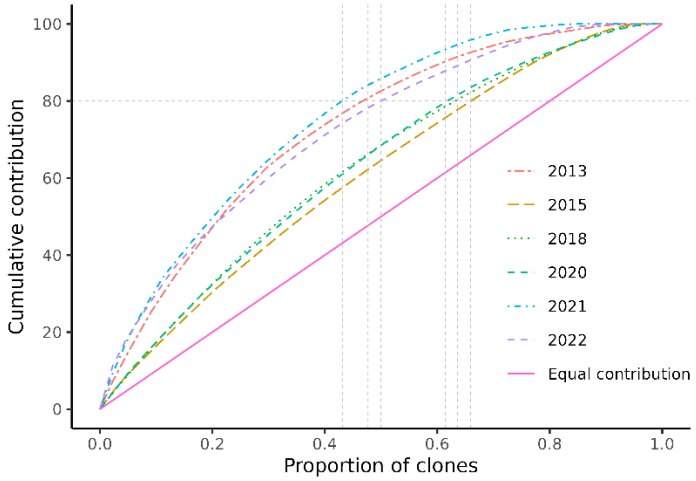

Figure 3. Cone crop parental balance curve over six years, based on the entire orchard clones (n = 44). The straight line represents an ideal orchard with equal contribution among individuals, while the dotted horizontal line is the 20:80 rule.

The average female fertility coefficient (Ψ_f) has a value of 2.28 in pooled data, and inversely depicted the cones production across years, with extremes of 1.37 (2015, 'top') and 5.49 (2021, 'poor') (Table 5 and Supplementary Material S1, Table S1.2). Similarly, the effective number of female parents ($N_{p(f)}$) was smaller in 2021 (8.19) and the largest in 2015 (32.90). The relative effective number (N_r) of female parents was between 19% and 75%, in 2021 and 2015, respectively. The lower genetic diversity in cone crops was associated with the poor year (0.939), which was slightly different than in the good years (0.985) (Table 5).

Table 5. Clonal coefficient of variation (*CV*), female fertility coefficient (Ψ_f), effective number of female parents ($N_{p(f)}$), relative effective number of female parents ($N_{r\%}$), and gene diversity (*GD*) in the Silver fir clonal seed orchard.

Parameter/Year	2013	2015	2018	2020	2021	2022	Pooled
CV	128.24	60.63	77.16	76.44	211.96	131.06	113.00
$\Psi_{(f)}$	2.64	1.37	1.60	1.58	5.49	2.72	2.28
N_p	17.02	32.90	28.21	28.40	8.19	16.56	19.76
$N_{r\%}$	37.82	73.12	62.68	63.12	18.20	36.80	43.92
GD	0.971	0.985	0.982	0.982	0.939	0.970	0.975

4. Discussion

Reproductive success is the confirmation that the genetic diversity of future generations will be spread [3]. It is common in tree's fertility studies to present results based on seeds produced in good years [7]; however, for an effective selection of clones in a seed orchard, conclusions based on observations of mature grafts and cumulative cone output over many years are required [58]. The present study is the first describing the clonal genetics and female fertility variation in a mature Silver fir seed orchard, based on a full sampling (100% of ramets observed), over a longer monitoring period.

The presented inter-annual variation is recorded in a mature seed orchard, relative to other findings, which consider the annual variation observed more often in young seed orchards [15,59]. The largest yearly-based proportion of variance, both in Poisson and zero-inflated component, and the lower clone-by-year interaction, as compared to the genetic variation attributable to clone, is still quite high but in agreement with other results [7,60]. Generally, this could be attributed to climate as it has been reported that a warm and dry May contributes to good seed production of Silver fir seed stands [61–63] and also, in our case, the extent of observation period, which encompasses more years than other studies

and thus opens the possibility to catch the extreme production years, or an increase in the reactions of clone to yearly climate variations as a seed orchard ages [64].

We found small clone-by-year interaction in both the multi-year models, which could suggest stability in the pattern of clone production ranking [2,15]. The low between-clone variation, not frequent in other studies [8], was reported in *Pinus sylvestris* [7]. Even that though the within-clone variation, associated with response to environmental conditions, is expected to stabilize with age [15] the mature seed orchard of our study still largely responds to the yearly, climatic conditions.

The repeatability in mean clone production was consistent across methods, with the highest heritabilities and correlations among the good years and a second lower value for the year not correlated with the others. We still found a moderate genetic control among clones variation with the estimated values of heritability in the Poisson component being higher than coniferous seed orchards of similar age (H_2 = 0.38, 30 years *Pinus sylvestris* seed orchard) [65]. The overall values are similar to other species, e.g., *Pinus nigra* [10], with values between 0.61–0.71, higher than in *Pinus halepensis* [66], and lower than in *Pinus radiata* [2]-H_2 = 0.91 or in *Pinus patula* (H_2 = 0.80) [15] (see the further comments section). The small genetic control found in the zero-component of fertility (data in latent scale) indicates a small, but existing overall probability (4%) that some of the clones will not be fertile, which could increase almost four times in the years of low production.

One common characteristic of seed orchards is the large variation of number of ramets between clones [67], which impact the clone-level statistics [7]. If we also add to this the (present) case of a full, multi-year inventory, the possibility of observing ramets with no cones and with a skewed distribution is real and must be considered when deciding the methods of data processing. The usual methods of transformation of non-gaussian data, with the aim of stabilizing the variance, conduct results which are not in the original data scale, but in latent scale. Even though, in theory, these variance estimates are not changing, in practice this is not true, especially in the case of fertility data (e.g., different counts), which could result in 25% differences when using log and squared root transformations [24]. As one of the components of repeatability (or broad-sense heritability), the residual variance, is related to both the link function used and to details of the computation statistical software (e.g., the approach of overdispersion and additive vs. multiplicative), the recommendations to use proper models in analysis should be followed [24,68]. Our results indicated over-estimates in latent scale compared to original data scale larger than mentioned, observed variation being between 16% and 36%, the latter in what we described as a 'poor fertility year' (Table 3). Considering the different possible sources of variation, the observation method and sampling, and the statistical treatment of data, the comparisons of heritability estimations across seed orchards should be made with caution; for interpretations close to real data, the above recommendations, to present the results also back-transformed to original data scale are necessary [51]. This was suggested some time before the mentioned studies, and points to the fact that the in practice clonal variability may be smaller than in published data [7].

Fertility variation presents importance in the management of forest genetic resources in different areas like seed production programs and gene conservation [5] and is also a management tool for an efficient seed crop management and in preventing the potential diminishment of genetic diversity [56].

The cumulative contribution curves or the parental balancing curves [11,53] showed a clonal contribution less than expected (i.e., equal contribution). In seed crops from seed orchards, the greatest gene diversity is only achievable when all parents equally contribute to the gamete gene pool (e.g., the H-W equilibrium). This hardly happens and frequently a small percentage of orchard parents provides an excessively large quantity of seed crops [17]. In the present analysis, the cone crop clonal involvement was relatively low, but consistent with other conifer results (e.g., 15); the top 20% of clones contributed between 51–65% of production. According to the current analysis, in top 20% only 3–5 clones ranked highly for their output in more than half of the monitoring period, an additional plus

argument for extended periods of observation, which capture different performance years and, thus, more reliable average results.

In the present study, the pooled values of $\Psi_{(f)}$ are slightly higher than expected in seed orchards, but there was marked year-to-year variation, lower than expected in good production years and more than double in poor years. Similar variations of clone fertility over productivity years were reported in young seed orchards [8]. A possible source of variation in female fertility remains the unbalanced number of ramets per clone, as in our case, which will contribute to a larger coefficient; the mentioned reference value $\Psi = 2$ could be used in order to pass this [7]. The effective number of female parents ($N_{p(f)}$), derived from effective population sizes, is used to assess the level of gene diversity in the population and refers to the number of individuals which, in a hypothetical population, would produce the same number of siblings (relatives) as the actual population [5,52]. Our results indicated a value over the years of $N_{p(f)}$ less than half the number of clones (Table 5) and yearly values which mirror the fertility variation and the cumulative curves. Similarly, the largest deviations from the equal fertility, expected when $N_{p(f)}$ equals the number of clones in the seed orchard [4], were found in poor years. The relative number of female parents $N_{r\%}$ in pooled data of 44% and annual extremes between 18% and 73% (Table 5) is different than the pattern reported in similar studies, but observed in young seed orchards, where the fertility changes with ageing [52]. The expected gene diversity of seed crops (GD) were in accordance with the other fertility indicators.

The scope of the seed orchards is to provide high genetic quality crops, under a balance of genetic gain and gene diversity [56]. From a seed orchard manager perspective, the female fertility will be prioritized because its aims are centered on the seeds as an income source, which can be quantitatively better estimated than the male, i.e., the precise number of successful female gametes of a ramet is known at harvest [2]. Although by counting the number of cones the female fertility was not fully estimated, it being necessary to estimate the number of seeds and their viability, our approach is useful in making informed decisions regarding the cone harvest and seed orchard management.

For commercial use, the values of clone-related variances present direct practical implications: single-years values depict the gene diversity received by customers using single-year seeds, while the multi-year value portray the gene diversity of the corresponding forest area regenerated using multi-year seeds from the seed orchard [7]. Thus, in order to accurately estimate the predicted genetic composition and the genetic gain of reforestation seed lots, the clonal differential contribution to the gamete gene pool is crucial [52]. Compared to earlier studies reports, the variance in female fertility is less in the studied seed orchard. Still, other factors can influence the genetic variation in cone production, including root-stock effect, crown-size, flowering synchronization and individual fecundity [16,69,70]. Also, climatic factors have a great influence in the variation of the clone production. According to marginality indices [71], the seed orchard is located in marginal site conditions for Silver fir. Recent studies have since highlighted that the climate has changed in Romania over the last few decades [72], which has certainly had a negative effect on the reproductive capacity of Silver fir in this seed orchard as the cone production gradually decreased from 2015 to 2022.

The use of information from year-to-year variation in cone crop can be useful for forestry practice. According to the lower between-clone variance and the rather substantial interaction with years, additional measures need to be added to fecundity as criteria for selecting clones. In our case it is unlikely that a few clones will have a dominant influence on the genetic diversity of the seed crop, as indicates the same magnitude of variance between clones, as between ramets within clones [7], and the parental balance of cumulative curves. Even though that the heritability estimates suggest that observations we made in the seed orchard are useful, for the future selection of clones to be developed in second-generation orchards, the fertility-based conclusions will be improved with other traits results [15]. Multiple techniques are available to manage the fertility variation: genetic thinning of individuals of low genetic values and fertility capabilities, toping and pruning, irrigation,

and fertilization or intensification of flowering with plant growth regulators [2]. In our seed orchard, the estimates of the relative effective number were low and some measures to improve the genetic diversity would be required, such as genetic thinning of low fertility individuals and top pruning, which are already tested and successful methods in other Silver fir seed orchards (authors data) [52,56,70].

Our analysis presents some limitations. All the results of female fertility estimations are based on visual estimations, a method prone to errors, which could have been present even in our case, where all the trees were relatively small (about 7 m height), the cones were visible, and their count was relatively easy. In such estimations, the literature indicates possible underestimates in cones counts by a factor between 4 and 10 [7]. We based our conclusions on a set of assumptions, which are not valid in the real world, e.g., no possibility of pollen contamination, no influence of male fecundity, no clones inbreeding (e.g., the 44 plus trees acting as mothers and selected from different stands have no common genetic found), and equal gamete contributions from all the clones [2,7]. As these have implications in defining and applying further goals, these will be considered when defining the final management measures [15].

5. Conclusions

The studied Silver fir seed orchard have been established with 44 clones originating from plus trees selected in natural seed stands from four provenance regions; hence, the hypothesis that the genetic base is quite broad. Although the variation was observed between the clones, the influence of the provenances is insignificant, which means that maintaining the level of high genetic diversity is achieved. Clonal variation in cone production across years indicate that the good cone crop years, closer to the ideal situations (equal contribution of all clones) is the year with the average number of cones per tree as evenly distributed as possible, while for the years with high total production but wide variation between ramets, equal seed harvest is recommended. Considering that the storage of Silver fir seeds is possible for a period of 8 years [73], mixing seeds from consecutive years could apply, while in years with poor fructification the cone harvest will be dropped. Our results complete the fertility data of Silver fir and can be further integrated in breeding the tasks regarding the selection of the most adapted Silver fir clones to climate change, for conservation as a forest genetic resource, and to advance to the next generation of seed orchards.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14010017/s1, Supplementary Material S1. Summary of clone fertility, Supplementary Material S2. Dispersion and zero-inflation in cones distribution [19,40–43], Supplementary Material S3. Prior analysis and model diagnostics [46–49], Supplementary Material S4. Posterior distributions of clone variance and heritability, annual data.

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Genetic legacy of southern Middle Siberian mountain and foothill populations of Scots pine (*Pinus sylvestris* L.): Diversity and differentiation

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Scots pine (*Pinus sylvestris* L.) is a widespread and economically important tree species in Eurasia. Southern Siberian, Scots pine mountain and foothills forests are especially valuable natural objects that help to stabilize environmental conditions. Due to the associated impact of natural and anthropogenic factors, the habitats of the species in southern Siberia are constantly shrinking. In this regard, the study of genetic diversity and structure of Scots pine forests in southern Siberia is very relevant. Here, we studied the genetic diversity and structure of eight Scots pine populations located in southern Middle Siberia (Russia). A high level of genetic diversity (H_E=0.518) was detected in the studied populations. No recent bottleneck effect, isolation by distance or isolation-by-environment were detected. Most genetic diversity was found within populations, while only 7% of genetic diversity occurred among populations. Both STRUCTURE analysis and UPGMA clustering showed two genetic groups. Two populations from the Minusink basin and a population from the Western Sayan Mts. formed the first group and the second group was composed of the other populations from Kuznetsk Alatau Mts., Central Tuva basin and Todzha basin. Our findings suggest that the studied Scots pine populations originate from different gene pools. The pattern of genetic diversity revealed by our study may be useful for the elaboration of conservation measures of genetic resources of Scots pine in southern Middle Siberia.

KEYWORDS

Scots pine, *Pinus sylvestris* L., nuclear microsatellite markers (nSSR), genetic diversity, genetic structure, Middle Siberia

1. Introduction

Genetic diversity is recognized as one of three basic components of biodiversity, i.e., the genes, species and ecosystems (Hoban et al., 2020). It plays an important role in species adaption to changing climate, habitats, and biotic interactions (Spielman et al., 2004). Furthermore, it helps to maintain ecosystem functions, stability and services (Hoban et al., 2020). The

characterization of genetic diversity pattern within species and among populations is a fundamental requirement for the establishment of programs aimed at biodiversity preservation (Belletti et al., 2017).

Scots pine (*Pinus sylvestris* L.) is one of the most widespread conifers in the world, which has great economic and ecological importance (Floran et al., 2011). Within its vast distribution, Scots pine grows in various soil and climatic conditions. It forms over 20 geographical races and about 100 forms and varieties (Ekart et al., 2014). A significant part of its range is located on the territory of Siberia where permafrost and forest fires determine the northern and southern boundaries of the species' area of distribution. At the same time, the impact of fires has not only negative, but also positive consequences; it ensures the removal of ground cover that prevents the germination of pine seeds, as well as the mineralization of sandy and gravelly soils (Sannikov, 1992; Pimenov, 2015). In the south of Siberia, pine forests are classified as especially valuable natural objects that stabilize environmental conditions. The maximum spectrum of adaptive variations for Scots pine is observed in this part of the region. Relict morpho-and genotypes of Scots pine can still be preserved in the south of Siberia (Pimenov, 2015). In Asia, Scots pine growing in the south of 52°N was identified as a steppe Scots pine variety (*Pinus sylvestris* var. *mongholica* Litv.) (The Plant List, 2023; World Flora Online, 2023), which most often known as Syn.: *P. s. ssp. Kulundensis* (Pravdin, 1964). *P. s.* var. *mongholica* is exceptionally resistant to adverse conditions and, growing on the sandy shores of salt lakes, can tolerate slight salinization of the soil (Zvereva, 2017). In the southern border of the species` distribution range in Siberia Scots pine forms island-like and ribbon-like forests and due to the structure and location features, they can serve as important objects for studying such processes as isolation, selection, genetic drift, mutation and inbreeding (Novikova and Zhamyansuren, 2012).

In recent decades, due to the combined effect of natural and anthropogenic factors, the dieback of coniferous forests in the south of Siberia has become catastrophic (Bazhina, 2010; Pavlov et al., 2011). For instance, by the end of the last century, due to root rot damage, as well as wind damage, forest fire, sanitary cuttings, Minusinsk pine forests (Minusinsk basin) suffered on an area of 28 thousand hectares (Tatarintsev et al., 2015). Over the past decade, in Khamar-Daban mountain range (the south of Lake Baikal) 40% of Siberian stone pine (*Pinus sibirica* Du Tour) forests has died because of bacterial infection caused by *Erwinia* sp. and *Pseudomonas* sp. and damages caused by *Dendrolimus sibiricus* Tschetw (Stavnikov, 2013; Voronin et al., 2013). Furthermore, since 1970s, there has been an intensive dieback of Siberian fir (*Abies sibirica* Ledeb.) in the mountains of Southern Siberia (Khamar Daban Mts., Western and Eastern Sayan Mts.) (Tretyakova et al., 2008). In this regard, the study of genetic diversity and structure of coniferous forests in the south of Siberia is very relevant.

Population genetic studies of Scots pine based on isoenzyme, chloroplast DNA markers show the evidence of genetic heterogeneity of pine populations in the south of Siberia (Sannikov and Petrova, 2012; Ekart et al., 2014; Semerikov et al., 2014). This is mainly due to the island type of the species' range in the steppes of Southern Transbaikalia, Northern Mongolia and its mountainous island type in the mountains of Southern Siberia and the Amur region (Sannikov and Petrova, 2012).

In this study, we used nuclear SSR markers to explore the genetic diversity and structure of Scots pine populations in southern Middle Siberia. Specifically, we aimed to: (a) assess the patterns of genetic diversity within and among populations, (b) test whether genetic differentiation is related to climatic variables.

2. Materials and methods

2.1. Plant material

Initially, 210 Scots pine individuals were sampled from eight populations located in southern Middle Siberia (Russia) on a vast territory covering the mountain and foothill forests of the Western Sayan Mts., Kuznetsk Alatau Mts. and forest-steppe and steppe landscapes of the Minusinsk basin, Todzha basin and Central Tuva basin (Table 1; Figure 1). Due to amplification failures, the number of studied individuals was reduced to 169.

2.2. DNA extraction and microsatellite analysis

Total genomic DNA was isolated according to the CTAB method (Doyle and Doyle, 1990). The quality and quantity of the extracted DNA was measured with a Nanodrop 8,000 spectrophotometer (Thermo Fisher Scientific, United States), then adjusted to a concentration of 10 ng/μL. Seven nuclear microsatellite primers were selected: Psyl16, Psyl42, Psyl44, Psyl57 (Sebastiani et al., 2012); PtTX2146 (Elsik et al., 2000); lw_isotig04306, lw_isotig07383 (Fang et al., 2014). All primers were combined into two multiplex sets: set 1 consisted of Psyl44, Psyl57 and lw_isotig04306; set 2 comprised of Psyl16, Psyl42, PtTX2146, and lw_isotig07383. Reverse primers were labelled with a fluorescent dye. Polymerase chain reaction (PCR) was performed in a 10 μL reaction volume using Qiagen Multiplex PCR Kits (Qiagen, Germany) under the manufacturer's instructions. Amplification was performed in a Corbett thermal cycler (Corbett Research, Australia) with the following cycling parameters: an initial denaturation at 95°C for 5 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 90 s, extension at 72°C for 50 s and a final extension at 72°C for 7 min. Then, the amplified fragments were run on a GenomeLab GeXP genetic analyzer (Beckman Coulter, Fullerton, CA) with an internal size standard. Genotyping was

TABLE 1 Geographic location of eight Scots pine populations in southern Middle Siberia.

Pop ID	Locality	N	Latitude/ Longitude	Altitude (m)
SAR-1	Kuznetsk Alatau Mts.	29	55.00/89.40	550–560
SAR-2	Kuznetsk Alatau Mts.	26	54.87/89.22	500–520
PER-1	Minusinsk basin	20	53.28/92.00	294–295
PER-2	Minusinsk basin	20	53.28/91.97	286–288
KER	Western Sayan Mts.	20	52.12/92.23	707–710
TOD	Todzha basin	18	52.72/95.74	860–980
BAL-1	Central Tuva basin	22	51.08/95.09	970–1,060
BAL-2	Central Tuva basin	24	51.02/95.28	880–890

Sample size (N).

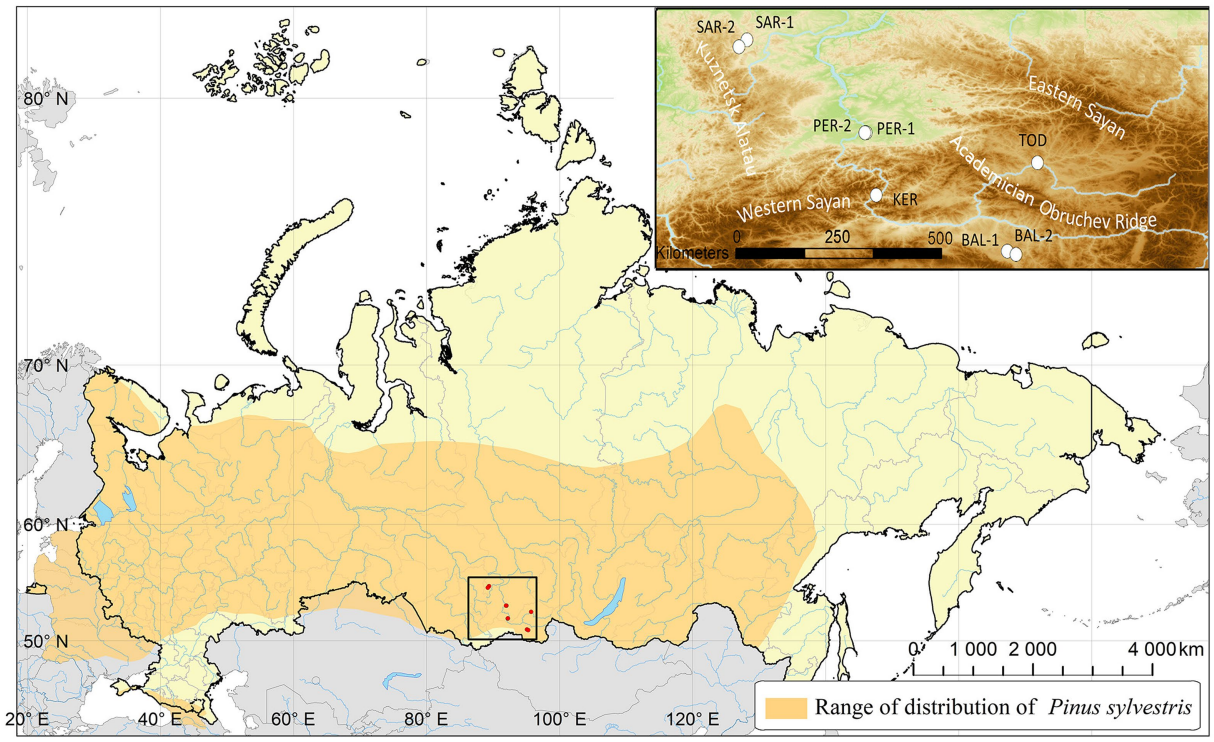

FIGURE 1
Location of the studied Scots pine populations in southern Middle Siberia.

performed with the GenomeLab GeXP software (Version 10.2, Beckman Coulter, Fullerton, CA).

2.3. Data analysis

Genetic diversity per locus and population was estimated by using GenAlEx v. 6.5 software (Peakall and Smouse, 2006). The number of alleles (N_A); number of effective alleles (N_E); inbreeding coefficient of an individual relative to the subpopulation (F_{IS}); inbreeding coefficient of an individual relative to the total population (F_{IT}); genetic differentiation coefficient (F_{ST}); observed heterozygosity (H_O); expected heterozygosity (H_E) were calculated. The allelic richness (A_R) was computed in R (R Core Team, 2013) using the "hierfstat" package (Goudet, 2005). Presence of null alleles and possible genotyping errors was checked using Micro-Checker software (Van Oosterhout et al., 2006). No evidence of null alleles or genotyping errors was found in the populations.

BOTTLENECK software v.1.2.02 (Piry et al., 1999) was used to test for recent population bottlenecks on the basis of the infinite allele model (IAM). Statistical significance was determined by two-tailed Wilcoxon signed-rank test with 1,000 iterations.

Analysis of molecular variance (AMOVA) was performed using the software GenAlEx v. 6.5. The significance of differences was tested by 999 permutations. The unweighted pair-group method with arithmetic mean (UPGMA) was applied to perform cluster analysis on the Nei's genetic distances data (Nei, 1978) and a Principal Coordinates Analysis (PCoA) was conducted using the "FactoMineR" package in R (Lê et al., 2008) to compare genetic differentiation among populations.

A Bayesian clustering approach implemented in STRUCTURE v.2.3.4 was used to estimate the number of clusters in the nSSR dataset (Pritchard et al., 2000). The analysis was performed with an admixture model with correlated allele frequencies and a LOCPRIOR setup. K value was set to 1–10 with a burn-in period of 100,000 iterations followed by 500,000 Monte Carlo Markov repetitions. Twenty repetitions were set for each run. The number of clusters was estimated using ΔK parameter according to Evanno et al. (2005) using the STRUCTURE HARVESTER program (Earl and Vonholdt, 2012). To average the results of the replicated runs, the CLUMPP software v.1.1.2. was used (Jakobsson and Rosenberg, 2007).

SAMOVA software v.2.0 (Dupanloup et al., 2002) was used to identify groups of populations that are maximally differentiated from each other. Runs were conducted with the number of groups set from two to seven, performing 100 independent simulated annealing processes. The maximum F_{CT} value was chosen as the indicator of the best grouping.

Followed by the detection of genetic clusters, Genetic Landscape Shape Interpolation analysis was carried out, as implemented in Alleles in Space (Miller, 2005), to produce a surface plot that shows major genetic discontinuities, indicating probable contact areas between the detected genetic clusters. On the surface plot, positive peaks indicate areas with high genetic discontinuities (high genetic distances) and negative peaks represent of areas with genetic similarities (low genetic distances).

To detect the presence of isolation by distance (IBD), the correlation between geographical distances and genetic distances between population pairs was tested with Mantel test (Mantel, 1967). The test was performed with the "adegenet" R package with 1,000 bootstrap replicates (Jombart, 2008).

To test whether climatic variation contributed to the patterns of genetic differentiation, i.e., to test the isolation-by-environment (IBE) hypothesis (Wang and Bradburd, 2014), three different approaches were taken. First, in addition to the genetic and geographic distances calculated for IBD, Euclidean climatic distances were calculated from recent (c. 1950–2000) climate data using 19 bioclimatic variables (Supplementary Table S1) which were extracted from the global climate layer data using a grid size of 30 arc-seconds and downloaded from the WorldClim v.1.4 database.[1] After, genetic, geographic, and the climatic distances were used in Mantel, partial-Mantel and MMRR (Multiple Matrix Regression with Randomization) regression analyses. The partial-Mantel test was conducted using the "vegan" package (Oksanen et al., 2022), while the MMRR carried out using the custom script of Wang (2013). The MMRR R script is deposited in the Dryad Data Repository under DOI:10.5061/dryad.kt71r.

3. Results

Using seven nuclear microsatellite loci, we identified a total of 49 alleles in the 169 individuals (Supplementary Table S2). The average number of alleles per locus (N_A) was seven. F_{IS} ranged from-0.055 (Psyl42) to 0.249 (Psyl44). The values of genetic differentiation (F_{ST}) varied in the range between 0.028 (lw_isotig07383) to 0.111 (Psyl42).

The mean number of alleles present per population varied from 3.571 (BAL-2) to 5.143 (PER-1 and SAR-1) (Table 2). Effective number of alleles (N_E) ranged between 2.191 in KER population and 2.910 in PER-2 population, with an average of 2.517 per population. Shannon Information Index (I) varied from 0.891 (BAL-1) to 1.118 (PER-2) population. The BAL-2 population had the lowest values for allelic richness ($A_R = 3.571$) and PER-1 population had the highest value ($A_R = 4.764$). The values of expected heterozygosity (H_E) ranged from 0.472 (BAL-1) to 0.565 (PER-2). The inbreeding coefficient (F_{IS}) values were between-0.161 and 0.229, but in general placed around zero in most of the populations. The value of the number of migrants per generation was high ($Nm = 5.690$) indicates high gene flow between populations. The ratio of observed and expected heterozygosity was balanced (mean H_O: $H_E = 0.510$: 0.518).

The population stability analysis revealed no evidence for recent bottlenecks in the studied populations.

We performed AMOVA among and within Scots pine populations (Supplementary Table S3) and the results showed that the genetic variation among populations was 7%, whereas most of genetic variation occurred within populations (93%, $p < 0.001$).

The matrix of pairwise F_{ST} values (Figure 2A) revealed that the highest differentiation apparent between KER, PER-1, PER-2 and SAR-1, SAR-2 and BAL-1 populations, while the lowest between KER, PER-1, PER-2 populations. Similarly, the UPGMA clustering indicated two groups. The first group consisted of PER-1, PER-2 and KER populations and the second group was composed of the five remaining Scots pine populations (Figure 2B).

The Bayesian STRUCTURE analysis revealed two Scots pine gene pools in the south of Middle Siberia (Figure 3), based on the Mean L(K) (±SD) and ΔK values. Group I included PER-1, PER-2 and KER populations, whereas Group II included all remaining populations.

Principal Coordinates Analysis (PCoA) based on the F_{ST} values identified two major groups (Supplementary Figure S1) at Dim. 1 vs. Dim. 2, explaining jointly 74.01% of the total variation for the nSSR markers. One group included populations from the Minusinsk basin (PER-1 and PER-2) and the Western Sayan Mts. (KER). The second group contained five populations from the Kuznetsk Alatau Mts. (SAR-1 and SAR-2), the Central Tuva basin (BAL-1 and BAL-2) and the Todzha Basin (TOD). The second and third axes (Dim. 2 vs. Dim. 3) explained much less, only 31.69% of the total variation and grouped SAR-1 and SAR-2 with PER-1 and PER-2 populations.

Genetic Landscape Spatial Interpolation has detected a significant barrier to gene flow in the form of a genetic discontinuity in the contact zone between the genetic lineages in this region (Figure 4). The estimation of the contribution of genotypes in each population showed that the PER-1, PER-2 and KER populations contained a higher proportion of genotypes originated from the Minusinsk basin, compared to other samples from the Kuznetsk Alatau Mts. (SAR-1 and SAR-2), the Central Tuva basin (BAL-1 and BAL-2) and the Todzha basin (TOD).

Spatial analysis of molecular variance (SAMOVA) produced values of F_{CT} ranging from 0.0305 ($K = 3$) to a maximal value of 0.03336 ($K = 6$), which indicated number 6 to be the preferred number of genetically homogenous clusters for the whole dataset (Supplementary Table S4). The six defined clusters contained the following populations: (I) PER-1 and PER-2; (II) BAL-1; (III) BAL-2; (IV) SAR-1 and SAR-2; (V) KER; (VI) TOD.

We further analyzed the correlation between genetic distance and geographic distance for the studied populations using the Mantel test. The results showed no correlation between genetic differentiation and geographical distance among Scots pine populations ($R^2 = 0.043$, $p = 0.163$). In addition, none of the matrix regression approaches (Mantel, partial-Mantel, and MMRR) to investigate IBE, were able to find significant relationships between the genetic, geographic and climatic distances (Supplementary Table S5).

4. Discussion

Forest fragmentation is constantly increasing in southern Siberia due to intensive logging, livestock grazing, as well as drought-and fire-related forest declines, and has been detected for many forest species (Wirth et al., 1999; González de Andrés et al., 2022). Fragmentation of forest landscapes causes isolation, and poses genetic and ecological threats to populations (Gustafson et al., 2010; Khansaritoreh et al., 2018; Erasmi et al., 2021). Conservation and rational use of Siberian forest genetic resources is one of the most important environmental and economic tasks. The effectiveness of solving this problem depends on the degree of knowledge of forest genetic resources. Unfortunately, despite some success, Siberian forest genetic resources have not been studied enough (Tarakanov and Krutovsky, 2016). Here, we assessed the genetic diversity and structure of eight southern Siberian Scots pine populations.

1 http://www.worldclim.org/

10.3389/ffgc.2023.1152850

TABLE 2 Genetic statistics averaged across seven microsatellite loci for each Scots pine population.

Population		N_A	N_E	I	A_R	H_O	H_E	F_{IS}	Bottleneck
KER	Mean	4.143	2.191	0.909	3.861	0.443	0.474	0.066	0.563
		(±0.595)	(±0.317)	(±0.174)	(±0.588)	(±0.087)	(±0.089)	(±0.043)	
PER-1	Mean	5.143	2.655	1.094	4.764	0.543	0.535	−0.013	0.688
		(±0.800)	(±0.444)	(±0.208)	(±0.783)	(±0.108)	(±0.099)	(±0.065)	
PER-2	Mean	4.857	2.910	1.118	4.510	0.421	0.565	0.229	0.578
		(±0.670)	(±0.587)	(±0.202)	(±0.686)	(±0.086)	(±0.094)	(±0.079)	
SAR-1	Mean	5.143	2.740	1.069	4.255	0.557	0.534	−0.011	0.688
		(±0.705)	(±0.458)	(±0.203)	(±0.565)	(±0.122)	(±0.105)	(±0.058)	
SAR-2	Mean	4.571	2.579	1.037	4.179	0.495	0.522	0.049	0.078
		(±0.685)	(±0.452)	(±0.199)	(±0.635)	(±0.098)	(±0.098)	(±0.051)	
BAL-1	Mean	4.286	2.209	0.891	3.768	0.552	0.472	−0.161	0.688
		(±0.474)	(±0.362)	(±0.164)	(±0.488)	(±0.096)	(±0.083)	(±0.045)	
TOD	Mean	4.571	2.632	1.061	4.352	0.571	0.538	−0.056	0.813
		(±0.528)	(±0.398)	(±0.188)	(±0.559)	(±0.105)	(±0.096)	(±0.038)	
BAL-2	Mean	3.571	2.217	0.918	3.571	0.500	0.506	0.075	0.078
		(±0.429)	(±0.272)	(±0.126)	(±0.463)	(±0.092)	(±0.063)	(±0.119)	
Total mean		4.536	2.517	1.012	4.158	0.510	0.518	0.022	
		(±0.216)	(±0.143)	(±0.062)	(±0.596)	(±0.034)	(±0.031)	(±0.027)	

N_A, number of alleles; N_E, number of effective alleles; I, Shannon Information Index; A_R, allelic richness; Ho, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index; ±, standard deviation.

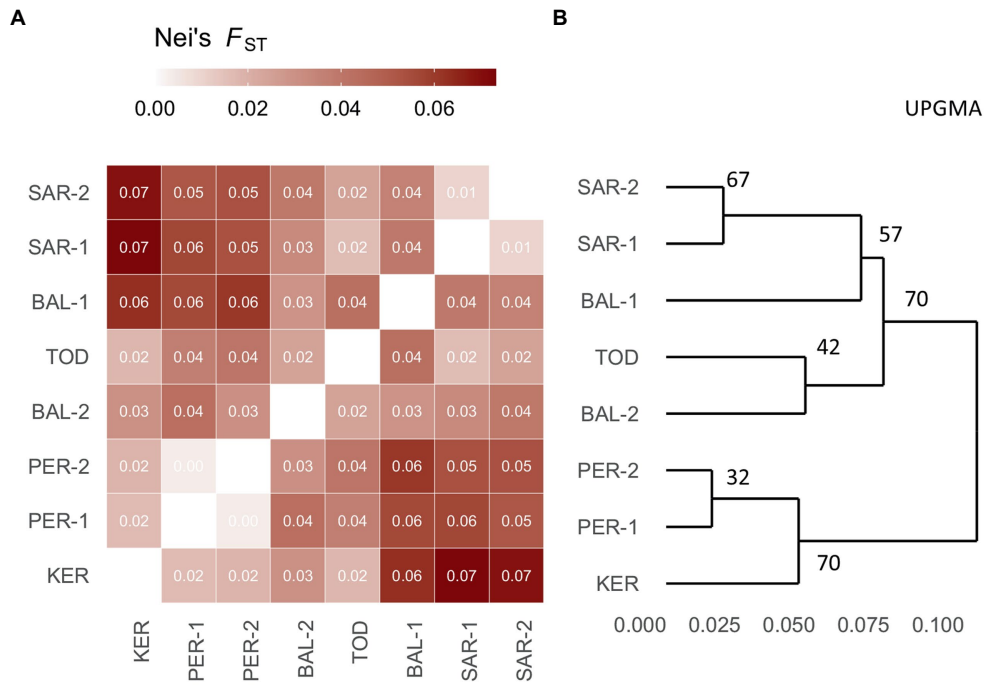

FIGURE 2
Heatmap of pairwise *Fst* (Nei, 1978) values **(A)** and bootstrapped UPGMA tree **(B)** of the eight Scots pine populations investigated.

Our results showed that, despite the detectable effects of fragmentation, the level of genetic diversity in Scots pine populations in southern Middle Siberia is high (H_E = 0.518) and is similar to the other Scots pine populations in Middle Siberia (H_E = 0.514) (Sheller et al., 2023). At the same time, our estimates are lower than those observed in other studies of Scots pine from Italy (H_E = 0.81) (Scalfi et al., 2009), Romania and Hungary (H_E = 0.55) (Bernhardsson et al., 2016), Turkey (H_E = 0.772) (Bilgen and Nuray, 2017), and Lithuania

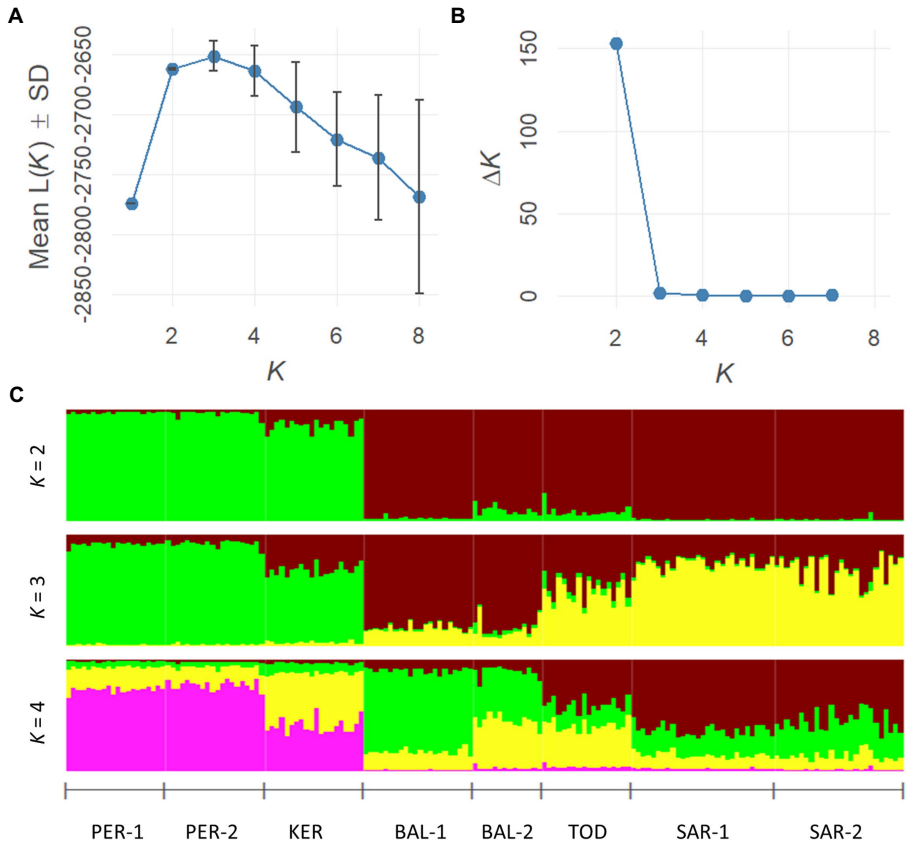

FIGURE 3
Estimation of the best subpopulation numbers based on Mean L(*K*) (±SD) and Δ*K* values **(A,B)**. Genetic structural plot of eight Scots pine populations (acronyms are as in Table 1) **(C)**.

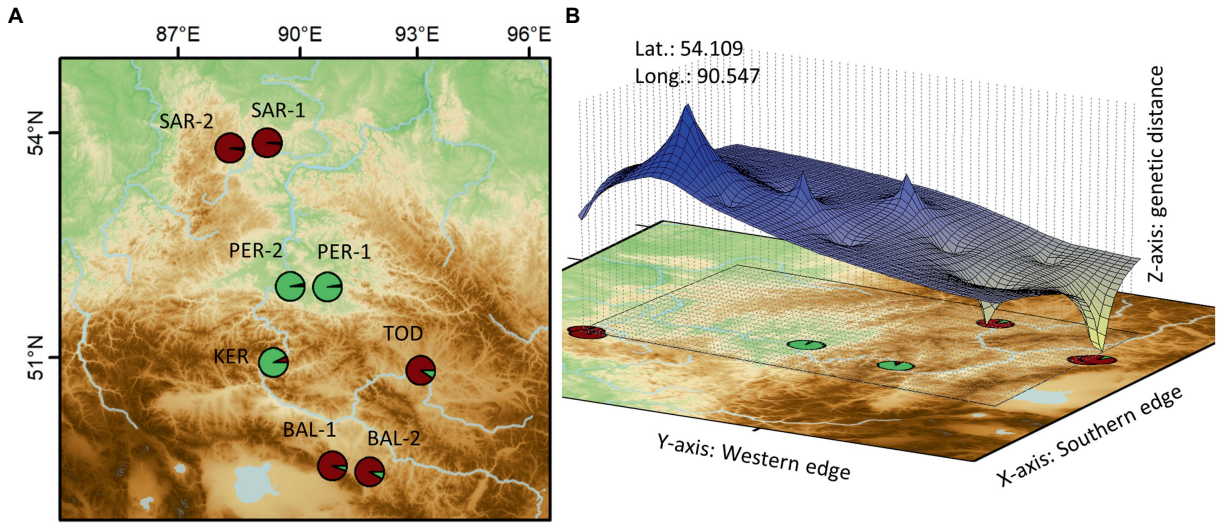

FIGURE 4
Spatial extent of the detected genetic clusters **(A)** and the genetic discontinuity revealed by the Genetic Landscape Shape Interpolation analysis **(B)** (for the acronyms, see Table 1).

(H_E = 0.59) (Kavaliauskas et al., 2022). The highest level of genetic diversity and allelic richness were detected in PER-2 and PER-1 populations (H_E = 0.565 and A_R = 4.764 respectively) located in the Minusinsk basin. PER-1 and PER-2 populations represent geographically isolated pine forests, which are unique in their origin and ecological functions (Polyakova, 2008; Tatarintsev et al., 2015).

The Minusinsk basin populations are distributed on the territory of the National Park "Sushenshky Forest," which is specially protected natural area of national importance. The extrazonal ribbon-like Scots pine forests are particularly valuable natural objects of the National Park "Sushenshky Forest" (Pate, 2020). The obtained results indicate the need to maintain the genetic variability of these valuable Scots pine forests in the Minusinsk basin. In the study by Ekart et al., 2014 the highest values of heterozygosity (*Ho* and H_E) were also found in Scots pine population from the Minusinsk basin.

Gene flow is one of the most important factors that maintains genetic diversity and improves population fitness and/or adaptive potential and decrease extinction probability especially in small populations (Berthaud et al., 2001; O'Connell et al., 2007). We detected high number of migrants (*N*m) indicating substantial gene flow among the studied populations, also the ration of expected and observed heterozygosity was balanced, indicating neither inbreeding nor isolation-breaking effects. This hypothesis was further supported by the inbreeding coefficient, with an overall mean of $F_{IS} = 0.022$, which generally indicated random mating of individuals.

However, toward the south the pattern of genetic diversity changes, and considerably lower levels of genetic diversity were found. Expected heterozygosity and allelic richness in two southernmost populations, BAL-1 and BAL-2, were $H_E = 0.472$ and $A_R = 3.571$, respectively. These populations are located in the Central Tuva basin and belong to Balgazyn relict pine forest. It is possible that the adverse effects of the extremely continental climate of Central Asia and the anthropogenic factors in the area of the Balgazyn pine forest, which is steadily shrinking, contributed to the decreased diversity. For instance, due to fires, natural forests decreased by four times in the last 25 years (1988–2014) (Kuular et al., 2015). Decrease of population size, due to fires and logging, diseases and environmental pollution leads to the reduction of genetic diversity (Ellstrand and Elam, 1993). In the study by Sheller et al. (2021) the lowest level of chloroplast DNA haplotype diversity was also detected in one of the Balgazyn Scots pine populations, confirming the need the conservation of these genetic resources.

Our estimate on total genetic variation occurring among populations showed a 7% applying AMOVA, which is considerably high, by taking into account the relatively short geographical distances among some of the populations (from 3 to 593 km). In similar microsatellite studies, but on a larger geographical scales, Shuvaev et al. (2022) found a F_{ST} value of only 0.026 in Scots pine populations in Krasnoyarsk region (Middle Siberia) and Sheller et al. (2023) found a F_{ST} value of 0.097 among distant Scots pine populations in Russia. Based on allozyme analysis, Sannikov and Petrova (2012) revealed that the genetic differentiation of Scots pine populations in the southern part of the range (south of 52°–53° latitude) in Central and Eastern Siberia is 2–4 times higher than in the contiguous forest zone. By decomposing our F_{ST} value among populations, based on pairwise estimates, majority of differentiation was found between populations located in the Kuznetsk Alatau Mts. (SAR-1 and SAR-2), the Minusinsk basin (PER-1 and PER-2), and the Western Sayan (KER).

The results obtained through the utilization of regression approaches (Mantel, partial-Mantel, MMRR) did not reveal a significant association between genetic differentiation and either geographic or climatic distances. This finding suggests that genetic differentiation may be attributed to past demographic events, rather than the specific environmental conditions of the region where the population resides.

Our Bayesian and the spatial clustering approaches (STRUCTURE and SAMOVA) were consistent with the F_{ST} estimates. The STRUCTURE analysis divided the studied populations into two main groups ($K = 2$), with PER-1, PER-2 and KER forming one group and the remaining five populations forming another group. The Minusinsk basin and the Western Sayan populations (PER-1, PER-2 and KER) wedged among the members of the other genetic group. However, toward the south they showed mixing, albeit at an extremely low proportion. This was evident also on our pairwise F_{ST} estimates, because lower F_{ST} values were typical here than toward the north, in the direction of the West Siberian Plain. We assume that this genetic differentiation across the landscape provided evidence of a contact zone of distinct genetic lineages, and a sharp boundary limiting gene flow, of a wedged population group. The genetic specificity of many population groups in marginal parts of the species' distribution range was also observed in Iberia, Transcaucasia, Asia Minor and Eastern Siberia (Prus-Glowacki and Stephan, 1994; Sinclair et al., 1999; Sannikov et al., 2005; Cheddadi et al., 2006; Naydenov et al., 2007; Pihäjärvi et al., 2008; Dering et al., 2017, 2021; Sheller et al., 2023). At the same time, a significant homogeneity of populations was revealed within certain regions of Scandinavia, Central, Western and Eastern Europe, Siberia (Goncharenko et al., 1993; Zhelev et al., 1994; Sinclair et al., 1999; Robledo-Arnuncio et al., 2005; Cheddadi et al., 2006; Pihäjärvi et al., 2008; Semerikov et al., 2018; Sheller et al., 2021, 2023).

It should be noted, as an interesting fact, that SAMOVA indicated six different groups, and dissected the population groups in the same order as F_{ST} decreases. First separated the Minusinsk basin and the Western Sayan populations (PER-1, PER-2, and KER), from the remaining ones, and so on. This shows that the primary barrier to restriction of gene flow is located here. Our Genetic Landscape Spatial Interpolation concurs with this, having identified a significant barrier to gene flow in the form of a genetic discontinuity in the contact zone between the genetic lineages in this region.

However, this pattern changes as we move toward the south. It is known, that an increase in F_{ST} indicates a decrease in number of migrants (*N*m) and vice versa (Whitlock and McCauley, 1999). In line with this, we observed that there was less differentiation to the south, potentially indicating increased gene flow. Historical and contemporary gene flow between genetic lineages, of the same species, has been described in many species (McDermott and McDonald, 1993; Ottenburghs, 2020), and it has been known to cause introgression (i.e., admixture), through hybridization and backcrossing (Petit and Excoffier, 2009). However, in this case increased genetic diversity is expected (Pazouki et al., 2016; Dering et al., 2017). In contrast, our observations revealed a discrepancy in the southern populations. Despite the notable degree of differentiation, the level of genetic diversity was found to be low, for example in BAL-1 and BAL-2 (described above). It is plausible that these populations are relict, since the legacy of long-term isolation is characterized by low genetic diversity. Such relict populations generally harbor low levels of allelic and haplotypic variation, have limited adaptive potential for range expansion and increased risk of extinction (Rinaldi et al., 2019; Urbaniak et al., 2019; Méndez-Cea et al., 2023). However, to rule out misclassification of these southern populations, further investigations targeting past demography, gene flow and introgression are required.

5. Conclusion

In our study, we assessed the genetic diversity and population structure of eight Scots pine populations in southern Middle Siberia using seven nuclear SSR markers. The study has revealed genetic heterogeneity of Scots pine populations near the southern boundaries of the species distribution in Middle Siberia. Despite fragmentation, the studied populations preserved high genetic diversity. The highest level of genetic diversity and allelic richness was detected in two populations from the Minusinsk basin while the lowest level of genetic diversity and allelic richness was found in two southernmost populations, which belong to a relict Balgazyn pine forest in the Central Tuva basin. However, to confirm a relictary status of Balgazyn pine forest additional studies should be carried out. Two clustering methods showed that the Minusinsk basin and the Western Sayan populations formed a distinct genetic group. The pattern of genetic diversity suggests a different origin of the studied Scots pine populations. However, further investigation is needed to study the evolutionary history of Scots pine populations in southern Siberia.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MS and AC designed the experiment. EC performed laboratory work. MS and ET analyzed the data. MS wrote the manuscript. ET reviewed and edited the manuscript. SK, AT, NK, AI, TS, NM, and PM collected the samples. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1152850/full#supplementary-material

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Article

Genetic Characterisation and Core Collection Construction of European Larch (*Larix decidua* Mill.) from Seed Orchards in Romania

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Abstract: European larch (*Larix decidua* L.) is an important forest tree species, with a large range and genetic variability; however, little is known about the characterisation of its genetic structure and diversity based on molecular markers in seed orchards, and no core collection—an improved tool for managing germplasm—has been developed for this species to date. In the present study, we employed nSSR molecular markers to characterise the genetic diversity and structure of five seed orchards and to construct a core collection for further use in breeding programmes. The results indicate that the values of heterozygosity in the seed orchards were slightly higher than the averages obtained in natural populations. The seed orchards displayed heterozygote deficiency, similar to other studies in *Larix decidua* or other *Larix* spp., which can be associated with a strong selective pressure on populations in a highly fragmented area. The presence of clones of autochthonous origin increased the allelic richness in the seed orchards where they were found. The degree of differentiation between individuals within the seed orchards was similar to that of populations originating from the Tyrolean Alps and Southern Carpathians (11.03% vs. 13% in the present study). The assignment, which was based on clustering, did not always match with the passport data (i.e., provenance), and we found that clones originating from stands from the northern Romanian Carpathians are distinct from the stands of the southern region, and most probably originated from a centre other than the Tyrolean Alps. The final extracted core collection (50 entries, 28% sampling effort) increases the rate of allele preservation, incorporates every allele from the entire collection, and provides candidate resources for the enhanced breeding of larch. The genetic characterisation of these germplasms will be essential for future breeding tasks, as well as for the preservation of valuable genotypes or populations.

Keywords: genetic structure; planted populations; native populations; germplasm

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1. Introduction

Genetic diversity is the foundation of forest sustainability and plays a key role in the adaptation of forest tree populations to climate change [1,2]. Among the "ex situ" methods for preserving forest trees as genetic resources or the germplasm outside native habitats, seed orchards (i.e., seedling or clonal) are a common example [3]. An evaluation of the genetic diversity of seed orchards can provide reference information for germplasm conservation, the selection of elite germplasms, and the parental selection for crosses.

The management and use of a germplasm collection requires significant resources for appropriate evaluation, particularly due to duplicate and redundant accessions [4–8]. To overcome these challenges, the "core collection" concept, devised at the beginning of the 1980s, further expanded the construction principles and methods [9,10] proposed to maintain a maximum level of genetic diversity in a relatively small number of samples [4].

Initially, geographic origin (e.g., passport data) and phenotypic traits were employed to establish core collections. These characteristics were later combined with molecular genetic markers, as they provide an increased reliability in capturing genetic diversity without the influence of environmental factors, as in the case of phenotypic traits [5,11]. Molecular markers such as simple sequence repeats (SSRs), are useful for revealing population structure and are often used as tools in core collection development, with the advantage of presenting a higher polymorphism level, which results in the occurrence of population-specific alleles [6]. In population studies of genetics, it is generally accepted that more loci ensure the increased reliability of the estimates of genetic parameters, although highly polymorphic loci may provide a similar efficiency due to a larger number of loci that are less polymorphic [12]. Various methods have been proposed to construct core collections based on molecular genetic markers, including the maximisation strategy (M-method) [13], which amplifies the number of alleles, or the advanced stochastic local search algorithm, both of which are highly suitable [14]. Most studies have constructed core collections of annual or perennial fruit tree species [9], in addition to several forest tree species, including Norway spruce (*Picea abies* L. Karst) [11], Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) [15], and black locust (*Robinia pseudoacacia*) [5].

European larch (*Larix decidua*) is found not only in its natural habitat at high altitudes in the Alps, Carpathians, and Sudetes, but also at low altitudes in the Polish lowlands [15]. As a consequence of this species introduction to areas outside its native distribution area, often without tracking the original source of the seeds and seedlings [16], the current distribution area is the result of anthropogenic influences, with as yet unknown implications for the alteration of the native gene pool. Studies on the genetic structure of *Larix decidua* in Europe based on molecular markers, however, have indicated a higher degree of genetic variation and low differentiation among populations from the natural range distribution [17], with the percentage of planted specimens limited to only 2–3%, thereby contributing minimal effects on the genetic structure of natural populations [18]. In the Carpathians, the native populations appear to be genetically different than others that have been introduced (and unknown) [19,20], and the strict protection regime and lower accessibility limit their use in afforestation programmes. Thus, *Larix decidua* plays an important role in the seed orchards of the species as a source of genetically improved seeds and a means of conserving intraspecific genetic diversity [21].

In Romania, the total area occupied by larch, both naturally occurring and cultivated, comprises only 0.3% of the forested terrain [22]. Natural larch populations are located in five main areas: Ceahlău, Ciucaș, Bucegi, Lotru, and Apuseni [23], with a maximum presence in the Bucegi Mountains. Beginning in the middle of the 19th century, many stands of these trees were planted in Transylvania and the southeastern region of Romania using reproductive materials of Alpine origin [24], with the Austrian Tyrol region being a principal source of seeds transferred to the Carpathians [25,26]. The undocumented, human-mediated impact of non-native reproductive material has resulted in negative long-term consequences or effects in terms of the trees' ability to adapt to a changing environment, particularly in fragmented populations [25–27]. In Romania, the genetic improvement of larch began in 1963 with the selection of seed stands and the establishment of 26 seed orchards (with a total surface area of 134 ha) through the phenotypic selection of more than 500 trees, from both natural populations and artificial stands [26]. Currently, 20 larch seed orchards (119.2 ha) remain in operation.

Despite the importance of *Larix decidua*, there is still limited information on the genetic diversity of this species, particularly in seed orchards, and no activity to date has been devoted to developing core collections or collecting information on the broad genetic base of the germplasm for future research. Thus, the present study aimed to evaluate the degree of genetic diversity in five seed orchards in Romania, in both autochthonous samples and those of unknown origin, and to create a core collection for further use in breeding programmes. With the use of nuclear DNA microsatellite markers (nSSRs), an attempt was made to verify the following hypotheses: (i) Are there major differences in the degree

of genetic diversity within the seed orchards based on the origin of the trees (i.e., native or unknown)? (ii) Do the investigated seed orchards possess a broad genetic base for the future selection of superior parents? (iii) What are the characteristics of the core collection and do they conserve the entire germplasm gene pool?

2. Materials and Methods

2.1. Seed Orchard Characteristics and Location

A total of 246 European larch (*Larix decidua* L.) clones (vegetative copies) from five clonal seed orchards in the eastern part of Romania (Table 1) were sampled. The five seed orchards are part of the larch first-breeding generation initiated more than forty years ago by the grafting of plus trees selected from valuable stands, most of which were artificial and of unknown origin, although plus trees were also selected from the two main natural distribution centres of larch in Romania (Table 2). In the absence of provenance tests, the selection was made based on phenotypic criteria (i.e., growth traits and stem form). To confirm clonal identity and identify possible labelling errors, samples consisting of needles or cambium were collected in the summer of 2020 with four ramets per clone (except for the clones represented by three or fewer ramets) so that at total of 623 specimens were ultimately analysed.

Table 1. Study seed orchards and primary characteristics.

Seed Orchard Name	Seed Orchard ID *	Latitude (°N)	Longitude (°E)	Year of Installation	Area (ha)	No. of Clones	Sample Size
Siminicea	PS-LA-SV83	47°41′	26°22′	1983	5.0	56	119
Gârcina	PS-LA-NT82	47°03′	26°26′	1982	6.8	56	152
Hemeiuşi	PS-LA-BC67	46°37′	26°51′	1967	5.6	38	104
Beizadele	PS-LA-PH82	44°53′	25°53′	1982	4.0	42	99
Valea lui Ştefan	PS-LA-AG68	45°05′	25°04′	1968	5.0	54	149
Pooled						246	623

* Seed orchard ID is in accordance with the National Catalogue of Basic Material of the Forest Reproductive Materials of Romania [28].

Table 2. The larch clones studied.

Seed Orchard	Clone ID	Region of Provenances *	Forest District	Type of Stand
Hemeiuşi	1–36, NB, NS	G3	Fântânele	plantation
Gârcina	1–34	G3	Fântânele	plantation
	83, 86	G1	Pătrăuţi	plantation
	113, 119, 584, 586, 718–726, 1P-4P, NB, NP	A2	Gura Humorului	plantation
Siminicea	1–36	G3	Fântânele	plantation
	77, 78, 83–88	G1	Pătrăuţi	plantation
	102, 103, 112–115, 126	A2	Gura Humorului	plantation
Beizadele	2.1–2.18, 2.26	B1	Brasov	plantation
	3.6, 3.12	E3	Vidolm	natural
	5.1–5.11	B2	Sinaia	natural
Valea lui Ştefan	1L–30L	C2	Latorita	natural
	1S–24S	B2	Sinaia	natural, plantation

* Region of provenances in accordance with the National Catalogue of Basic Material of the Forest Reproductive Materials of Romania [28]. A2: Outer Eastern Carpathians; B1: Braşov Depression; B2: Curvature Carpathians; C2: Southern Carpathians, southern cline; E3: Apuseni Mountains, eastern cline; G1: Suceva/Siret/Iași Hills; G3: Bârlad Plateau.

2.2. SSR Analysis

Genomic DNA was extracted from needles dried with silica gel or cambium according to the ATMAB method [29]. DNA quantity and quality were determined with a Biophotometer Plus spectrophotometer (Eppendorf, Germany). A total of 15 nuclear microsatellites (nSSR) were developed according to the following references: [30] (markers bclK211, bclK228, bclK229, bclK189, bclK263, and bclK253), [31] (markers Ld31, Ld30, Ld50, Ld56, Ld42, Ld45, Ld58, Ld101), and [32] (marker UAKLLy6). They were organised in three multiplex reactions according to the expected length of the fragments and fluorescent labelling of the primer.

Polymerase chain reaction (PCR) was performed in a reaction volume of 15 µL containing 1× Qiagen Multiplex PCR MasterMix 2×, 1 to 3 µM of each primer and ultrapure water to final volume. Amplification conditions consisted of an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C (denaturation), 1.30 min at 56 °C, and 1 min at 72 °C, followed by a final extension step for 30 min at 60 °C. The amplification reaction was carried out using the MiniAmpPlus Thermal Cycler (ThermoFisher Scientific, Waltham, MA, USA). SRR fragments were separated by capillary electrophoresis using the automated sequencer GeXP analyser (Beckman-Coulter, Brea, CA, USA) and analysed with the use of an internal size standard (DNA Size Standard Kit: 400). The resulting raw data were processed, and the fragment lengths were determined using the fragment analysis tool GenomLab version 10.1 (Beckman-Coulter).

2.3. Data Analysis

To limit as much as possible any genotyping errors resulting from the presence of null alleles, in addition to stuttering and large allele dropout, each locus was investigated by means of bootstrapping with Micro-Checker v. 2.2.3 [33], based on 1000 bootstraps and 95% confidence intervals. The analysis of the SSR data included the following: the mean number of alleles per locus (Na), the effective number of alleles per locus (Ne), the observed (Ho) and expected (He) heterozygosity, the inbreeding coefficient (FIS), and the polymorphic information content (PIC). The determination of the frequency of null alleles and PIC was carried out in CERVUS [34], while the other measures were assessed with GENALEX v. 6.503 software [35]. Because some of the clones were common to several seed orchards, we verified the clonal identity in each seed orchard based on the obtained genotype in GENALEX v. 6.503 by selecting the option "matching or near matching genotypes", which indicates the presence of individual specimens with the same genetic profile. The number of foreign genotypes varied between 5 and 13 specimens, representing about 5% of the total sample size. We identified only 177 trees with unique genotypes, and these were used further to assess the genetic structure of the clones. After removing the common genotypes, the genetic structure was analysed to highlight the genetic relationships among the 177 European larch accessions (see below) due to the lack of information related to the putative origin of the source populations as well as to discover different parental genotypes that could be employed in future genetic and breeding efforts. The partitioning of the genetic variation between seed orchards was evaluated by analysis of molecular variances (AMOVA), a standard test with 999 permutations that was used to determine the significance of the differences. The pairwise FST values for each seed orchard were determined and plotted as a heat map graph.

In addition, we performed the analysis of the genetic structure of unique clones with three approaches. First, the Bayesian model-based clustering algorithm implemented in STRUCTURE ver. 2.3.1 was employed to evaluate the number of subpopulations to which the accessions were assigned. The subpopulations were identified according to different allelic frequencies, and the individuals were placed into specific subpopulations if the values of the estimated membership probability (Q) were higher than 0.7. In the present study, the value of K was set to be from 1 to 10, and 10 independent runs were performed, with a burn-in of 100,000 iterations, followed by 1,000,000 iterations for each value of K, assuming an admixture model with correlated allele frequencies. The most

probable K-value was determined with the highest ΔK [36] in STRUCTURE HARVESTER v. 0.6 [37]. The CLUMPAK web server was used to visualise the bar plot of the probability of membership from the results of the Q-matrix. Second, the discriminant analysis of principal component (DAPC), a multivariate method that uses a non-hierarchical approach for defining genetic clusters, was used to analyse the genetic structure of seed orchards. The DAPC was implemented in the adegenet package of R statistical software [38]. Finally, the genetic relationships between individual clones were assessed using an unweighted neighbour-joining method, with the use of MEGA 11 [39].

An optimised core collection, identified from the entire collection of larch clones (the "accessions"), was developed using Core Hunter 3 [40], a method that selects a representative fraction (the "entries") from the entire collection as a whole. Several allocation algorithms were used by the programme to choose core subsets by optimising a single genetic parameter or multiple parameters simultaneously. These were subsequently tested using the following methods [40–43]: (1) average accession-to-nearest-entry distance (A-NE), which considers the mean distance between each accession in the entire collection and the near selected accession, and the results in core accessions of maximum dissimilarity; (2) average entry-to-nearest-entry distance (E-NE), which optimises the average distance between each accession and the next closest other accession in the core and has, as a result, diverse cores of low redundancy; (3) allele coverage (Cov), which maximises the share of detected alleles in the complete dataset that are retained in the selected core and results in core accessions preserving the alleles; (4) expected heterozygosity (He), in which the anticipated percentage of heterozygous loci explicitly considers the variability within each locus, resulting in cores with a lower likelihood to be homozygous for a number of different loci (e.g., compared to using the Shannon index); (5) Shannon's diversity index: irrespective of their co-location within loci, the core subsets maintained as many unusual alleles as possible, with a maximum under the unique allele occurrence in the complete dataset; and (6) a combination of two methods, the average entry-to-nearest-entry distance (E-NE) and Cov using equal weights. Each method was applied to yield a sampling percent of up to 50% of the whole accessions, as previously reported [44]. Various parameters were calculated for assessing the capacity of the core to capture the diversity present in the entire germplasm collection: mean number of alleles per locus (Na), effective number of alleles (Ne), Shannon Information Index (I), Ho, and He. A one-sample *t*-test compared the genetic parameters of the core collections determined with the original collection, such that the smallest core subset presenting nonsignificant differences with the breeding population ($p \leq 0.05$) was selected as the final core germplasm collection.

3. Results

All of the 15 SSR markers analysed were polymorphic, with a total number of 185 alleles. The average number of alleles per locus was 12.33, ranging from 5 (Ld101) to 22 (bclK263), with an average effective allele per locus of 5.54 (Table 3). The mean value of Ho was 0.660, which is much lower than the He of 0.764. A single locus had an excess of heterozygotes (bclK263), while the rest of the loci showed heterozygote deficiency, leading to a positive fixation index (mean F_{IS} = 0.14). A high frequency of null alleles was identified in five loci, with null allele frequencies ranging from 0.102 to 0.267, with the null alleles frequencies being 0.077. The PIC values were 0.202 and 0.918 for Ld101 and bclK263, respectively. Other than two exceptions (marker Ld101 and bclK229), all the markers displayed good discriminating power (Table 3).

Genetic diversity averaged across loci by seed orchard is shown in Table 4. As expected, the three seed orchards sharing the same clones (HEM, GAR, and SMN; Table 1) presented similar levels of genetic diversity, compared to that of others (BZD, VST). Within the seed orchard, Na ranged from 8.9 (HEM) to 10.3 (GAR), with a mean of 9.8. Ne ranged from 5.1 (HEM) to 6.1 (VST), with a mean of 5.6, while I showed an average value of 1.83 (between 1.75 (HEM) and 1.90 (VST)) (Table 4). The observed Ho was generally lower than the He (0.690 vs. 0.790, respectively). Ho ranged from 0.633 (VST) to 0.729 (SMN), and He

ranged from 0.774 (HEM) to 0.809 (VST). Estimates of the overall inbreeding coefficient 0.132 (FIS) suggests a heterozygote deficit in the seed orchards evaluated, with extremes of 0.072 (HEM) and 0.223 (VST). Two seed orchards displayed a higher inbreeding level (BEI—FST = 0.206 and VST—FST = 0.223).

Table 3. Summary of characteristics of the 15 nuclear microsatellite loci used in the analysis of five European larch seed orchards.

Locus	Observed Allele Size (bp) (Multiplex)	A	N_e	H_o	H_e	F_{IS}	PIC	f_{null}
Ld30	106–138 (A)	12	4.59	0.641	0.778	0.174	0.771	0.102 ***
Ld31	113–143 (A)	13	4.02	0.709	0.751	0.056	0.760	0.024
bclK189	146–172 (A)	12	6.88	0.823	0.854	0.037	0.845	0.038 ***
bclK211	188–232 (A)	21	4.54	0.703	0.777	0.098	0.789	0.011
bclK228	176–212 (A)	17	8.67	0.841	0.884	0.048	0.885	0.024
bclK253	204–226 (A)	11	7.06	0.691	0.853	0.196	0.845	0.115
Ld50	168–196 (B)	14	5.82	0.687	0.809	0.148	0.815	0.098 ***
Ld58	140–174 (B)	17	8.43	0.716	0.881	0.187	0.870	0.003
Ld45	203–219 (B)	9	4.48	0.673	0.767	0.117	0.755	0.070 **
Ld42	178–194 (B)	8	3.75	0.452	0.726	0.379	0.743	0.267 ***
Ld56	228–248 (B)	10	6.01	0.815	0.831	0.023	0.840	0.021
bclK263	185–243 (C)	22	10.65	0.908	0.906	−0.003	0.918	0.023
bclk229	93–125 (C)	8	3.09	0.603	0.675	0.109	0.611	0.070 **
Ld101	190–198 (C)	5	1.31	0.154	0.239	0.327	0.202	0.103
UAKLLy6	229–239 (C)	6	3.93	0.484	0.741	0.340	0.702	0.193 ***
Mean		12.33	5.54	0.660	0.764	0.149	0.756	0.077

Abbreviations: A, number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; FIS, inbreeding coefficient; PIC, polymorphic information content; f_{null}, frequency of null alleles. Significance: **—$p < 0.01$, ***—$p < 0.001$.

Table 4. Multilocus genetic diversity indices of European larch seed orchards.

Seed Orchard	N	N_a	N_e	I	H_o	H_e	F_{IS}
Hemeiuşi (HEM)	37	8.90	5.15	1.75	0.725	0.774	0.072
Gârcina (GAR)	55	10.27	5.51	1.81	0.725	0.783	0.082
Siminicea (SIM)	40	9.72	5.52	1.83	0.729	0.789	0.076
Beizadele (BEI)	37	10.00	5.94	1.88	0.637	0.797	0.206
Valea lui Ştefan (VST)	50	10.00	6.10	1.90	0.633	0.809	0.223
Mean	44	9.78	5.64	1.83	0.690	0.790	0.132

Abbreviations: N, number of clones; N_a, mean number of alleles per locus; N_e, effective number of alleles; H_o, observed heterozygosity; He, expected heterozygosity; I, Shannon Information Index; F_{IS}, inbreeding coefficient.

3.1. Genetic Structure of the Seed Orchards

AMOVA, indicating Wright's fixation indices (FST = 0.015, FIS = 0.135, and FIT = 0.148) (Table 5), revealed that only 2% of the variation observed was among the five seed orchards, whereas 85% of the variation was due to the variation among accessions used in the analysis, with the remainder (13%) due to the variation among the accessions within each seed orchard (Table 5).

Table 5. Results of analysis of molecular variance (AMOVA) of the genetic variation among and within five European larch seed orchards.

Source	df	SS	MS	Est. Var.	%	*p*
Among seed orchards	4	43.669	10.917	0.068	2%	0.001
Among accessions within seed orchards	214	1070.636	5.003	0.595	13%	0.001
Within accession	219	835.248	3.814	3.814	85%	0.001
Total	437	1949.553		4.476	100%	
F-statistics value F_{ST} 0.015 F_{IS} 0.135 F_{IT} 0.148						

Abbreviations: df, degrees of freedom; SS, sum of squares; MS, mean of the squares; Est. Var., estimated variance of components; %, percentage of total variance contributed by each component.

However, significant moderate differences were found not only between the Valea lui Ștefan seed orchard and the others, but also between the Beizadele, Hemeiuși, and Gârcina seed orchards (Table 6). The highest level of differentiation was found between the Valea lui Ștefan and Hemeiuși seed orchards (FST = 0.046, $p < 0.001$). As expected, the lowest differentiation was between Siminicea and Gârcina seed orchards, which share both clones originating from Fântânele and clones originating from Gura Humorului.

Table 6. Pairwise seed orchard F_{ST} values.

Hemeiuşi	Gârcina	Siminicea	Beizadele	Valea lui Ştefan	
0.000					Hemeiuşi
0.004	0.000				Gârcina
0.005	0.003	0.000			Siminicea
0.011 **	0.010 **	0.007	0.000		Beizadele
0.046 ***	0.043 ***	0.030 ***	0.022 ***	0.000	Valea lui Ştefan

Abbreviations: significance **—$p < 0.01$, ***—$p < 0.001$.

The estimation of ΔK using the Bayesian clustering method implemented by the software STRUCTURE from the 177 individual genotypes showed the highest value for K = 3 (ΔK = 31.62) (Figure 1), although high values were also obtained for K = 6 (ΔK = 15.22) and K = 2 (ΔK = 6.51). At K = 2, the European larch clones (accessions) are clearly divided into two subpopulations: native (A group) and planted (NA group), according to the putative origin of the plus trees. The A group includes the highest number of clones from the Valea lui Ștefan seed orchard, with the clones originating from Latorița centre displaying the highest purity (Q > 0.80) (Figure 2), followed by Sinaia centre (native) and a few from the Siminicea seed orchard. The NA group includes the clones originating from plus trees from artificial seed stands of unknown origin located in northeastern and southeastern Romania. At K = 3, the NA group is split into two subgroups, one corresponding to clones with origins in the Fântânele seed stand and in artificial stands from the southeastern Romanian Carpathians (Sinaia, Brașov), while the other corresponds to northeastern Romania (Gura Humorului, Pătrauți) and western Romania (Alba). According to the Q mean value (i.e., the estimated membership coefficient to a certain cluster), more than 75% of clones that were assigned to cluster 1, which corresponds to an autochthonous origin of plus trees, had a Q > 0.7, while the proportion admixed in the other two groups was approximately 50% (Figure 1C).

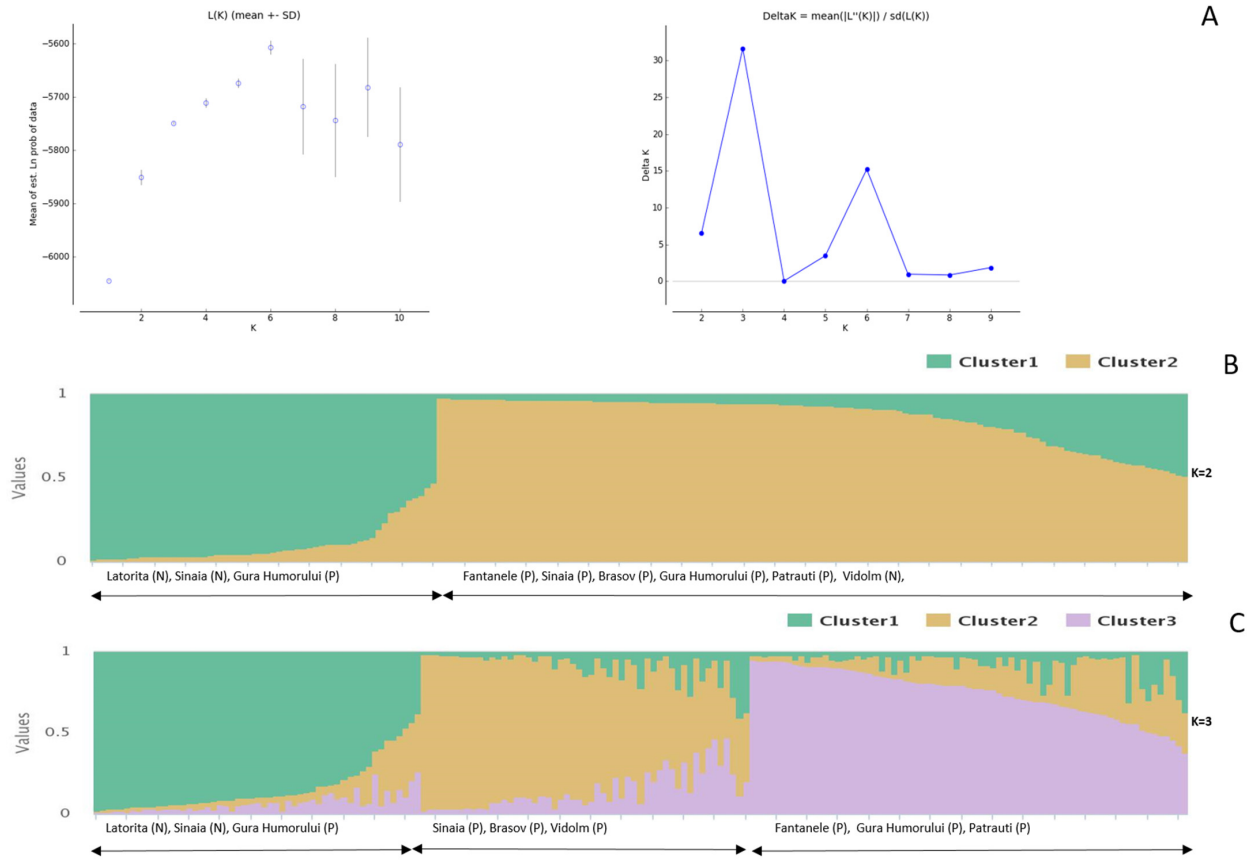

Figure 1. Results of population genetic structure analysis of European larch accessions: estimated population structure (K = 2 and K = 3) (**A**), estimation of the best subpopulation numbers based on DK and mean L(K) ± SD values (**B**), and the stacked bar plots of genetic structure for K = 2 and K = 3 (**C**). The provenance of plus tree is in horizontal line.

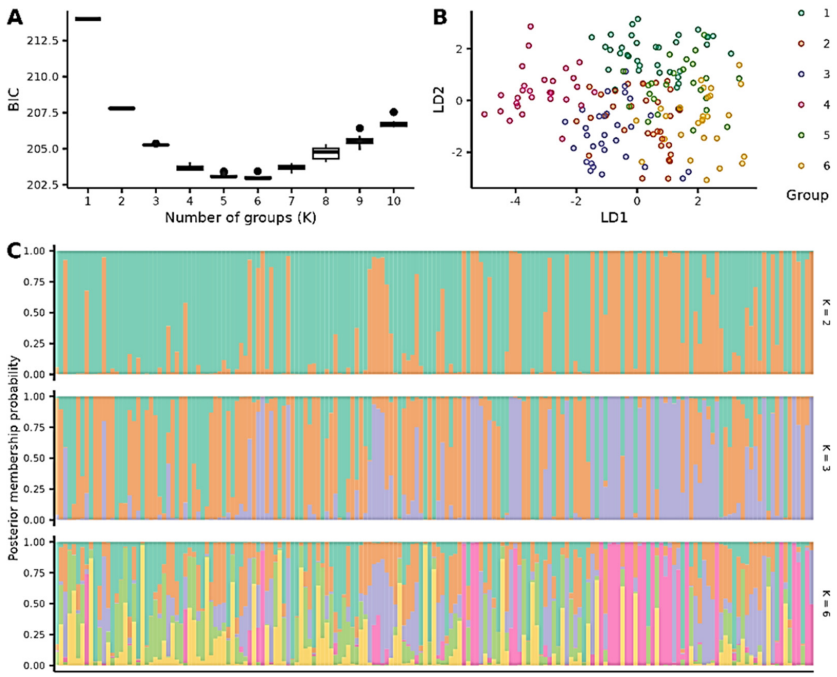

Figure 2. Discriminant analysis of principal components (DAPC) of the 177 European larch genotypes (**A**) and estimation of the number of clusters by Bayesian information criterion (BIC) (**B**,**C**).

The BIC (Bayesian information criterion) of the DAPC indicated six (K = 6) as the most probable number of clusters of the 177 genotypes (Figure 2A); for each genotype, the posterior membership probability relative to 2, 3, and 6 clusters is presented in Figure 2C. Clusters 1 and 4 were clearly differentiated using the two main discriminant functions (Figure 2B), compared to clusters 2 and 5, and are located in the middle of the graph. The clone membership to the clusters of STRUCTURE and DAPC is presented in Supplementary Material Table S1.

The neighbour-joining dendrogram supports the clustering of Latorița, the autochthonous Sinaia clones from the Valea lui Ștefan and Beizadele seed orchards, and three clones from the Gârcina and Siminicea seed orchard (Figure 3). Moreover, admixed genotypes were distributed in all clusters.

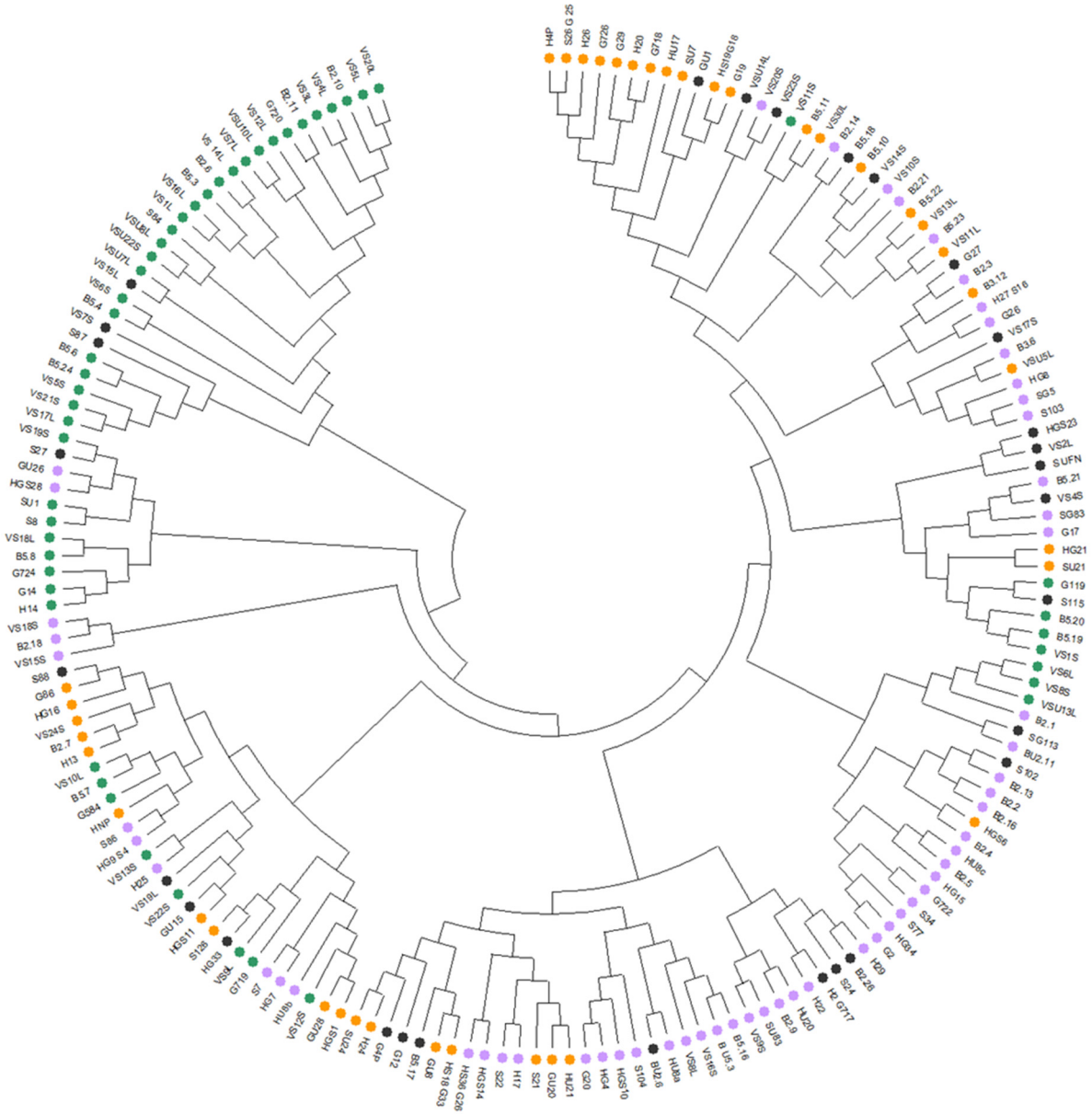

Figure 3. Neighbour-joining dendrogram based on genetic distances between clones. Each clone is identified by the first letter of the seed orchard and the corresponding code. For each genotype, the colours are in accordance with their assignment to the different clusters, as inferred by STRUCTURE software at K = 3. The black symbols are the admixed genotypes.

3.2. Construction of the European Larch Core Collection

The sampling strategies, intensities (from 10% to 50% in steps of 10, and 90%), and diversity parameters (Na, Ne, I, Ho, and He) applied to 177 accessions resulted in 36 core subsets (Table 7). When comparing the core collections to the original collection (e.g., including all accessions), Na was susceptible to the sampling strategy when using the genetic distances-based method, and was not significantly different under the method maximising allelic richness. Except for the A-NE strategy, in Ne, I and He were found at significantly larger ($p < 0.05$) values compared to the original collection, whereas in Ho, no significant differences were observed. Across the sampling intensities, the minimum core collection captured at least 90% of the number of alleles of the entire collection and included 40 entries (with the simple, Cov method) or 50 entries (with the combined E-NE + Cov method). The values of Ne, I, and He parameters peaked at 30 accessions. The final core germplasm collection established by the CoV method consisted of 40/50 clones (22% and 28% sampling intensity), ensuring a retention of 90.1% and 99.1% of Na and Ho values, respectively, while higher values were captured in Ne, I, and He (117.2%, 107.4%, and 104.3%, respectively). These results support the general finding that the core collection is more diverse than the original collection; thus, the constructed core collection includes clones representing each seed orchard and each of the identified genetic clusters.

Table 7. Variability of genetic diversity parameters for various core subsets.

Method	Subset Size	Sampling Intensity	Na	Ne	I	Ho	He
Whole	177	100	13.2	6.57	2.01	0.699	0.820
A-NE	10	5	6.8	4.69	1.57	**0.660**	0.720
	20	11	9.4	6.07	1.88	**0.687**	0.794
	30	16	10.2	6.02	1.90	**0.711**	0.799
	40	22	11.1	**6.33**	1.96	**0.706**	0.810
	50	28	10.9	6.22	1.96	**0.699**	0.810
E-NE	10	5	8.9	7.08	**2.02**	**0.730**	0.849
	20	11	9.9	7.01	**2.04**	**0.700**	0.842
	30	16	11.1	7.19	2.08	**0.687**	0.844
	40	22	10.7	6.92	2.05	**0.693**	0.841
	50	28	11.8	7.02	2.08	**0.703**	0.843
Cov	10	5	9.5	7.23	**2.06**	**0.740**	0.85
	20	11	11.4	7.23	2.09	0.670	0.842
	30	16	12.4	7.41	2.11	**0.702**	0.84
	40	22	**13**	7.16	2.10	**0.706**	0.835
	50	28	**13.1**	6.99	2.09	**0.704**	0.836
He	10	5	9.5	7.63	2.10	**0.670**	0.86
	20	11	10.9	7.74	2.12	0.645	0.858
	30	16	11.4	7.78	2.14	0.667	0.857
	40	22	12.0	7.99	2.16	**0.685**	0.858
	50	28	12.1	7.98	2.15	**0.691**	0.857
Shannon	10	5	9.3	7.44	2.07	**0.680**	0.856
	20	11	11.2	8.02	2.15	**0.675**	0.859
	30	16	11.8	8.08	2.16	0.669	0.857
	40	22	12.2	8.09	2.17	**0.688**	0.856
	50	28	12.4	7.97	2.16	**0.688**	0.853
E-NE+Co	10	5	8.8	7.05	**2.05**	0.650	0.855
	20	11	11.1	7.60	2.13	**0.695**	0.849
	30	16	11.9	7.70	2.16	**0.693**	0.856
	40	22	12.4	7.48	2.14	**0.710**	0.849
	50	28	**12.9**	7.36	2.13	**0.705**	0.84

Abbreviations: Na, mean number of alleles per locus; Ne, effective number of alleles; I, Shannon Information Index; Ho, observed heterozygosity; He, expected heterozygosity. The values shown in bold were not differentiated from the whole in a simple *t*-test. The rest of values are significant at $p < 0.05$.

4. Discussion

The selection of optimal samples with an increased potential for adaptation and the preservation of broad genetic diversity are prerequisites for future forest ecosystems that are resilient and resistant under predicted changes to the climate [1,2]. In the European larch breeding programme in Romania, numerous plus trees from different seed stands, both autochthonous and of unknown origin (although presumably alpine), were selected, grafted, and grown in seed orchards that contained different numbers of clones and genetic composition. To characterise the European larch germplasm, 15 nuclear SSR markers were employed. The PIC value obtained indicated that microsatellites are highly polymorphic and informative for discriminating individual ramets and to accurately establish the clonal genotype, a finding that is consistent with results reported in other studies [27]. Overall, the frequency of null alleles was relatively low; however, some loci with a high frequency of null alleles were removed from processing to avoid affecting the estimation of genetic structure and differentiation [44]. For the remaining 12 microsatellite loci, the proportion of null alleles was reduced by only 4.5%, and the PIC value was even higher, due to the very low informative value of the Ld101 locus.

The values of genetic diversity of the European larch seed orchards, in terms of Ho and He, are slightly higher than the averages obtained in natural populations: 0.650 and 0.720, respectively, in the Swiss Alps [45], 0.739 and 0.761, respectively, in the French Alps [46], and 0.715 and 0.761, respectively, in the Tyrol region of Austria [25]. For an old core Polish larch, the values obtained were Ho = 0.720 and He = 0.752 [47]. For Romanian larch natural populations, the available studies are limited; however, the values obtained are lower than that of the average values for all the seed orchards together or for every individual seed orchard considered separately: Ho = 0.620 and He = 0.693 [27]. When values corresponding to the artificial populations of unknown origin are considered, He = 0.738 [27]. All studies cited were based on a set of markers with a high degree of polymorphism, developed by Wagner et al. [31], and have in common at least eight SSR loci.

Overall, the seed orchards displayed heterozygote deficiency, similarly to results reported in other studies in both *Larix decidua* or other *Larix* spp. [27,48,49], which can be associated with a strong selective pressure on populations in a strongly fragmented area. The allelic diversity within seed orchards was found to be closely associated with the source of plus trees and, to a lesser degree, with the number of clones that established the seed orchards. Although the native populations of the Romanian Carpathians reveal a low level of genetic diversity, which can be explained by their small size, isolation, and evolution [50], but may also be associated with interesting genotypes [2], the participation of clones of autochthonous origin increased the allelic richness in the seed orchards where they were found. It should be mentioned that only three (Latoriţa, Bucegi, and Apuseni) out of the five naturally occurring areas of larch in Romania are found in breeding populations. On the other hand, we still lack information about the level of diversity of the source populations of plus trees, with the exception of the Baciu seed orchard (northwestern part of Romania) [51].

AMOVA showed that only 2% of the total genetic variation occurring among seed orchards, even if they consisted of diverse germplasm, located in different regions and with different histories and evolution. However, the degree of differentiation between individual specimens within the seed orchards was similar to that obtained by Mihai et al. [26] when analysing populations originating from the Tyrolean Alps and Southern Carpathians, with a percentage of variation between groups of 11.03%, compared to 13% in the present study. Our results were corroborated by DAPC, STRUCTURE, and neighbour-joining analyses, which indicated that the degree of inclusion in the germplasm of accessions of unknown or suspected distant origin is quite pronounced in the national larch breeding programme included in the analysis, and that the consequences of translocation on adaptability are not sufficiently understood to date.

STRUCTURE analysis identified three distinct clusters among the non-duplicate accessions, and the membership coefficient revealed that only 17.5% of the individual specimens

possessed an uncertain affiliation to genetic clusters. Moreover, cluster assignment did not match entirely with the passport data from all accessions; for example, the provenance mentioned in the records from the installation of the seed orchards. In this regard, the present study is important as it clarifies or completes this information. For example, for the Hemeiuși seed orchard (the first seed orchard installed in Romania almost 60 years ago), the information regarding the origin of the plus trees is general in nature and refers to the region and the name of the forest district but does not specify exactly which clone comes from which area. Due to our study's results, the correct assignment has been successfully made. In addition, a clone with the presumed local origin was identified. The divergences in this designation could be due to several factors, including the fact that the only reference population with known origin is Latorița, and that not much is known about the origin of low-altitude larch populations. What can be concluded from our study is that the clones originating from stands to the north of the Romanian Carpathians (Gura Humorului, Pătrăuți) are distinct from those to the south of the Carpathians, and probably come from a region other than the Tyrolean Alps. This hypothesis needs to be investigated in more detail, however, using comparative analysis with reference to populations from different European gene pools.

Numerous studies investigating the genetic variability of larch provenances in terms of growth traits, wood quality, and adaptability suggest that the European larch presents a higher degree pf genetic variation both among and within populations, which is beneficial for additional assisted migration and breeding programmes [16,52]. Although the tests for identifying the offspring of seed orchards are still at the beginning stage [20], genotypic evaluation is useful for correcting inherent errors related to mature seed orchards (e.g., the origin of clones) and for providing alternative support for the interpretation of the performances in phenotypic traits. With the exception of the Baciu seed orchard (from the northwestern region of Romania) [51], we know little about the diversity of the source populations of plus trees. In evaluating the germplasm collections constructed to obtain desirable traits, the final extracted core collection increased the rate of allele preservation, incorporated every allele from the entire collection, and provided candidate resources for the enhanced breeding of larch. The practice of transferring reproductive material as the result of different constraints (e.g., reduced availability/productivity of local seeds sources or budget) has been recognised since at least the 19th century [1]. This area requires the expansion of existing knowledge through comparative analysis with reference populations from different European gene pools, for example, which would improve the ability to manage local populations; for example, by selecting better trees for advanced breeding, maximising genetic diversity, and showing more concern for ecological adaptability or the anticipated reactions to climate change. New approaches are available, providing rigorous genetic evaluation and enabling the improvement and conservation of commercial and non-commercial species under a range of environmental constraints [53]. Assisted gene flow is not risk-free, however, and current requirements (e.g., European Council Directive 1999/105/EC) stipulate the traceability of forest reproductive material, with the aim of better use (e.g., originating from stands "preadapted" to a future climate) or to anticipate potential issues of maladaptation or other related ecological consequences [1].

5. Conclusions

The overall degree of genetic diversity of the European larch seed orchards is slightly higher than that found in natural populations, while the heterozygote deficiency is comparable to that reported in other studies of *Larix decidua* or other *Larix* spp., a finding that can be explained by the highly fragmented area that exerts a strong selective pressure on these populations. The allelic richness of seed orchards increased whenever clones of autochthonous origin were found. The degree of differentiation between individual specimens within the seed orchards is similar to that of populations originating from the Tyrolean Alps and Southern Carpathians (11.03% vs. 13% in the present study). An important output of the present study is the use of assignment based on clustering, which in some

cases suggest differences compared to that of the passport data (provenance), as well as the distinctiveness between clones originating from stands located in the northern Romanian Carpathians and those in the southern region, with a possible origin from an area other than the Tyrolean Alps. The final constructed core collection includes 50 entries, corresponding to 28% of the sampling effort, in which each seed orchard and determined cluster are represented, a strategy that increases the rate of allele preservation and incorporates every allele from the entire collection. The recognised practice of transferring European larch reproductive material has altered the genetics of European larch populations in the Eastern Carpathians. Nevertheless, more knowledge is still needed, however, to better manage local populations, maximise genetic diversity, and face the challenge of future climate change.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14081575/s1. Table S1: List of larch unique genotypes, the seed orchards where they are found and the group of clustering according to STRUCTURE and DAPC analysis.

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Article

Large Differences in Bud Burst and Senescence between Low- and High-Altitude European Beech Populations along an Altitudinal Transect in the South-Eastern Carpathians

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Abstract: Phenology is considered an indicator of environmental changes, with direct implications in the length of the growing season; therefore, it offers essential information for a better understanding of the tree–environment relationships that could lead to the right decisions for forests' sustainable use and conservation. A better understanding of how European beech (*Fagus sylvatica*) phenology responds to predicted climate change effects is important for forest management. This study aimed to assess bud burst and senescence among and within beech populations located along a steep elevational gradient. Phenological observations were carried out on 150 beech individuals along an altitudinal transect in the south-eastern Carpathian Mountains, from 550 to 1450 m, in five study sites in two consecutive years. The start of the bud burst, of senescence, and the duration of the growing season varied inversely proportionally to the elevational gradient in both monitored years. Individuals located at the highest altitude need 28 more days to start the growing season than those at the lowest altitude. There is an average difference of 14 days at the start of the growing season in the same beech populations between the two consecutive years. The first stage of senescence (yellowing of leaves) lasted longer in 2021 (21–32 days) than in 2022 (18–25 days), with a difference of 16%–28%, proportional to the increase in altitude. The association of field phenological data with meteorological data indicates that the start of the growing season occurs when the thermal threshold of 10 °C is exceeded, with an accumulation of a least 60 GDD (growing degree days) with a threshold of 0 °C in the last 7 days as a complementary condition. The appearance of the first stage of senescence, the yellowing of the leaves, was also influenced by the temperature and the accumulation of at least 72 SDD (senescence degree days) with a threshold of 0 °C in the last 7 days. Our results confirm that the temperature is the triggering meteorological factor for the onset of bud burst and leaf senescence in European beech.

Keywords: leaf phenology; *Fagus sylvatica*; altitudinal transect; local adaptation

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1. Introduction

Seasonal changes in terrestrial ecosystems are becoming more and more influenced by the effects of climate change, especially in the middle and higher latitudes [1]. In this context, the trend of temperature increase has become more noticeable. It can be directly linked with processes such as desertification, melting glaciers, reduced snow cover, intensifying heavy rainfall, and rising sea levels, and indirectly with soil erosion [2] and changing habitat areas for plants and animals. As a result of these effects of climate

change, species in forest ecosystems are forced to adapt and react through their regulatory mechanisms (physiological adaptation) or are even pushed to their survival limit [3].

Climate change alters the timing and length of the spring and autumn periods, which can significantly affect vegetation [4]. These seasonal changes in vegetation are determined by plant phenology [5], which is defined as the "synchronization of seasonal activities of plants and animals" [6,7]. The phenology of forest tree species is one of the most responsive and easily observable traits in response to climate effects. The survival rate, reproductive performance, persistence, and, therefore, the distribution range of a forest tree species are affected by phenological timing [8].

Temperature is a primary driver of forest tree species' growth and development. It influences the rates of chemical reactions in physiological processes [1], although its specific effects vary among organs [7]. Increases in air temperature due to the anthropogenic greenhouse effect can be detected easily in the phenological data of Europe within the last four decades [9]. In many cases, a higher temperature has been shown to accelerate a tree's development, with each degree increase in the spring temperature causing an advanced start of the leaf-out process by 2–7 days [5], which leads to an earlier transition to the next phenophase [1]. A longer growing season starts with advanced forest phenology driven by global warming [10]. Early spring and higher summer temperatures advance leaf yellowing [11]. On the contrary, warmer autumn temperatures delay leaf yellowing; an increase with each degree in autumn temperature causes a delayed senescence date by up to 8 days for some forest tree species [5]. The Earth's climate has warmed by approximately 0.6 °C over the past 100 years, with two main warming periods, between 1910 and 1945 and from 1976 onwards [6].

Taking into account only the crown condition (defoliation), European beech (*Fagus sylvatica* L.) is the healthiest major forest tree species in Europe [12], but it is one of the most sensitive hardwood species to uprooting produced by snow, ice, and wind [13,14]. European beech is Europe's most widespread forest tree species [15], including Romania (NFI Cycle II—[16]). It has high economic and ecological value, becoming a thoroughly investigated European tree species [17,18].

Aridity and warming have produced a decline in the growth rate of beech across Europe, except in the extreme north and high-altitude regions [19]. European beech decline was followed by severe droughts and heatwaves [20], flooding, and water excess [21,22]. Additional biotic factors (*Neonectria* sp. or *Phytophthora* sp.) have been aggravating factors of the decline [23,24]. Some ecological interactions between forest tree species and various communities of fungi and insects are influenced by the timing of leaf-out phenology [25].

Based on field observation and mathematical models, phenology can provide an algorithm/model to explain the reaction of forest tree species and the capacity to adapt to new site conditions. Even though field observations of tree phenology are labor-intensive, they offer valuable information regarding tree-level monitoring [26]. Time-series observations of spring phenology and senescence may lead to a better understanding of climate variability or climate change effects on plant responses as a direct correlation with meteorological data, especially temperature [27]. Understanding the impact of climate change on vegetation depends on how accurate the monitoring of phenology is and the precise delimitation of the start of the growing season and the end of it [28].

The onset of the bud burst of European beech is predominantly influenced by the seasonal course of temperature in late winter and early spring [29]. Prolonged droughts cause a premature onset of senescence in European beech, and this tendency is no longer only visible in low-altitude regions; it has also begun to appear in medium-altitude areas [30,31], directly involving a shorter growing season.

In the actual context of climate change, forest management must focus on species that have high adaptability and phenotypically plastic responses to new conditions, resistance to diseases and pests, and a wide range of uses for wood. Under climate change, the local adaptation and survival of European beech forests depend on their genetic variation and high adaptability to new environmental conditions [32]. These arguments justify the

choice of this species as a viable solution and the need to gain new information about its adaptation in as much detail as possible.

In this study, we aimed to assess the phenological variation in European beech along a complex altitudinal transect spanning 900 m of elevation during two years of monitoring through field observations and to associate these data with meteorological ones, especially temperature. The main objectives were to assess the phenological differences between the two consecutive years, among populations, between populations located at the extremities of the transect, and the intrapopulation variation, and to associate these data with the most appropriate meteorological indicator. The appearance of bud burst has already been linked with exceeding the threshold of 10 °C [33]; we evaluated the temperature variation (average daily temperature, and maximum and minimum daily temperature) from the last seven days until leaf flush. The association of temperature with the onset of senescence (yellowing of the leaves) was also evaluated.

2. Materials and Methods

2.1. Study Area

The study was conducted in the south-eastern Carpathian Mountains along an altitude transect at five study sites at an elevation between 550 and 1450 m (Figure 1; Table 1). One hundred fifty individuals were selected, thirty at each study site, located at a minimum distance of 25 m from each other and on north-facing slopes. This altitudinal transect with 900 m elevation overlapped with the natural range of *Fagus sylvatica* in the Brasov area, where it forms mixtures with other deciduous trees (*Acer pseudoplatanus* or *Carpinus betulus*) and conifers (*Picea abies* or *Abies alba*) with an age range of 80–120 years.

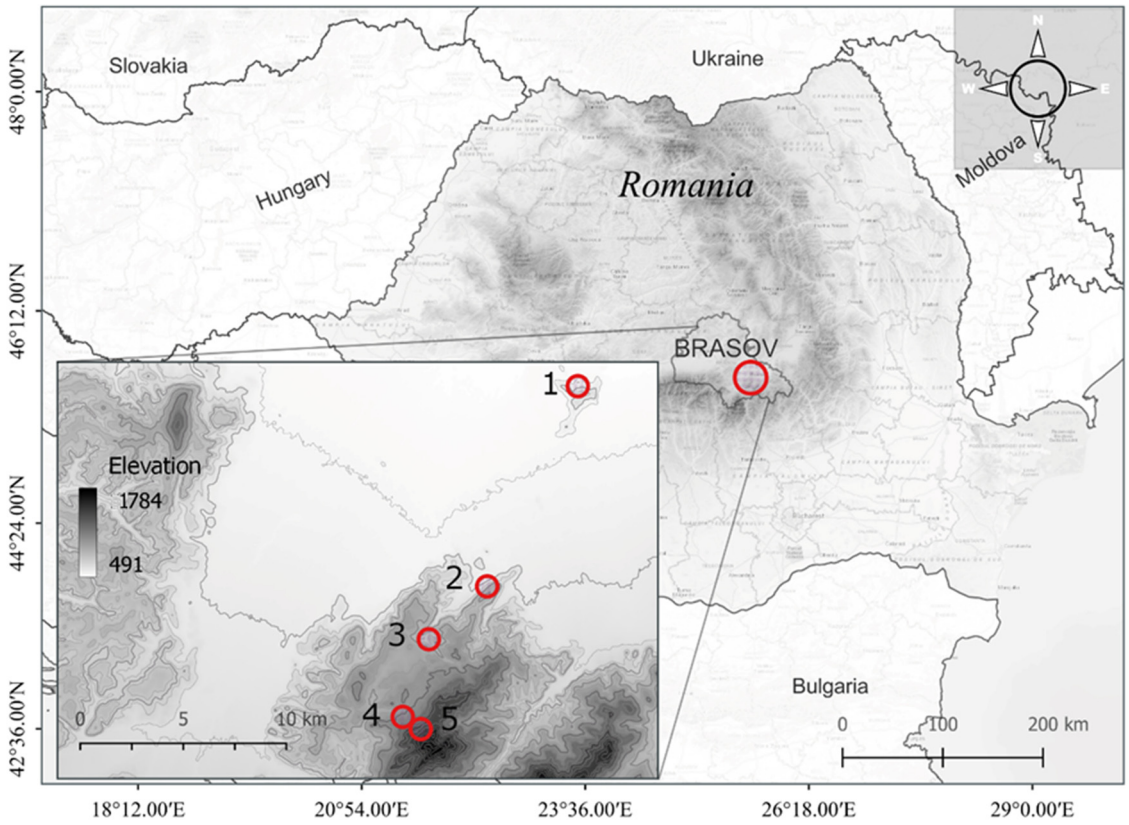

Figure 1. The geographic location of the studied European beech (*Fagus sylvatica* L.) populations across the altitudinal gradient from the south-eastern Carpathian Mountains (1—Lempes, 2—Tampa, 3—Solomon, 4—P. Lupului, and 5—Ruia).

Table 1. The geographic location of the studied European beech (*Fagus sylvatica* L.) populations from the south-eastern Carpathian Mountains and their altitude range.

Population ID	Geographic Coordinates	Altitude Range (m)
Lempes	45°43′34.88″ N 25°39′30.66″ E	550–650
Tampa	45°38′18.86″ N 25°35′38.56″ E	650–750
Solomon	45°36′59.75″ N 25°33′39.87″ E	800–1000
P. Lupului	45°34′54.64″ N 25°32′36.43″ E	1000–1150
Ruia	45°34′25.41″ N 25°33′11.67″ E	1300–1450

2.2. Phenological Data

Phenological observations from the field were performed based on the methodology proposed by Vitasse et al. [34]. Every population from each study site was visited twice a week, from April to June (spring phenology) and September to November (senescence). These observations were always carried out by the same observer, with the naked eye or using binoculars, approximately 15 m away from the tree. The leaf unfolding (LU) process was divided into four stages of development (Table 2). Each tree received a stage depending on the majority proportion (>50%) of the buds from the upper third of the crown at that moment. Each study site population received an LU stage based on the average of the estimated stages for the 30 sampled individuals. Further, the qualitative scale of the bud-opening and leaf-unfolding process was converted into a quantitative one (according to the range of the percentage of green cover; Table 2) to have higher precision in delimiting the stages (a finer scale) and to adapt it to the same unit of measure (%) as that of senescence.

Table 2. Leaf-unfolding stages linked to the observed leaf development and green cover range.

Code	0	1	2	3
Phenological stage				
Phenological stage	Dormant winter bud	Bud swollen	Bud burst	At least one leaf unfolding
BBCH correspondent	BBCH 00	BBCH 01	BBCH 07	BBCH 09
Range of the percentage of green cover (%)	<25	26–50	51–75	>75

The senescence (%CFL) was calculated based on two variables, the percentage of colored leaves and the percentage of missing (fallen) leaves, according to the following formula (1):

$$\%CFL = (\%CL \times (100 - \%FL))/100 + \%FL \quad (1)$$

where %CFL is the % of colored or fallen leaves (senescence), %CL is the % of colored leaves, and %FL is the % of fallen leaves.

The start of an individual's growing season was considered when it reached stage 2 (bud burst) according to the bud-opening and leaf-unfolding process [35,36]. The reporting in the growing season at the bud burst stage was carried out because this was the first visible one from the observer's level identified at the time of field observations [37]. This

stage also has the equivalent with the same name on the BBCH scale [38]. The start of the senescence was considered when the leaves started to yellow.

2.3. Meteorological Data

The process of acquiring raw meteorological data differed for each of the two monitoring years. In the first year, 2021, raw data were extracted from a database from meteorological stations near the plots where the studies were performed. In the second year, 2022, three temperature and relative humidity sensors were installed inside the stand, in representative points, for each study site (two HOBO loggers and one iButton logger). Each sensor was calibrated to record temperature and relative humidity values at a frequency of 30 min (48 values/day), later being compared with those from the nearby meteorological stations. Subsequently, these raw data were processed, and several meteorological indices were calculated based on them. The average daily temperature (°C) and relative humidity (%) values were calculated as the average of the 48 daily measurements recorded at a frequency of 30 min. From these 48 daily measurements (both for temperature and relative humidity), the maximum and minimum values were selected for calculating the maximum and minimum daily temperature (T_max and T_min) and maximum and minimum daily relative humidity (RH_max and RH_min). GDD (growing degree days) was used as a meteorological indicator for spring phenology, and it was calculated by subtracting the thresholds of 0 °C (GDD_0), 5 °C (GDD_5), and 10 °C (GDD_10) from the daily average temperature values. SDD (senescence degree days) was used as a meteorological indicator for autumn phenology, and it was calculated by subtracting the thresholds of 0 °C from the daily average temperature values.

2.4. Data Analysis

We performed a normality test and used variance analysis (ANOVA) to test for differences between populations in spring and autumn phenology. For the variance study, changes along the altitudinal gradient were considered according to each phenophase stage (bud burst, yellowing of the leaves) for the two years of monitoring. We also tested for correlations in phenological sensitivity to meteorological indicators (temperature, GDD, RH). To test for significant differences between the populations, we used a *t*-test.

All statistical analyses were computed in R software v. 4.3.1 [39]. The results were graphically displayed using the "ggplot2" and "corrplot" packages.

3. Results

3.1. Phenological Data

3.1.1. Spring Phenology

Using the methodology of Vitasse et al. [34], we monitored the phenophases during the spring and autumn of 2021 and 2022. The duration amplitude in the bud burst stage was similar between the populations in 2021 and 2022 (29 days). In relative terms, individuals located at the highest altitude (Ruia) need 28 (23%) more days to start the growing season than those from the lowest altitude (Lempes). Significant differences exist in the dynamic of spring foliage phenology between these two years (Figure 2). In 2021, during the transition between the first phenophases, the stages' duration was faster than in 2022, at 2–3 days (7 days for the individuals at the maximum altitude). In the second year, the phenophases lasted longer, at 5–7 days (3 days for the individuals at the minimum altitude), representing a slower dynamic of the entire process starting in the growing season (10–13 days). There was also the same delay of 29 days between the two populations located at the extremities of the study area. Between the two monitored years, there was a difference of 14 days at the start of the growing season of the same populations.

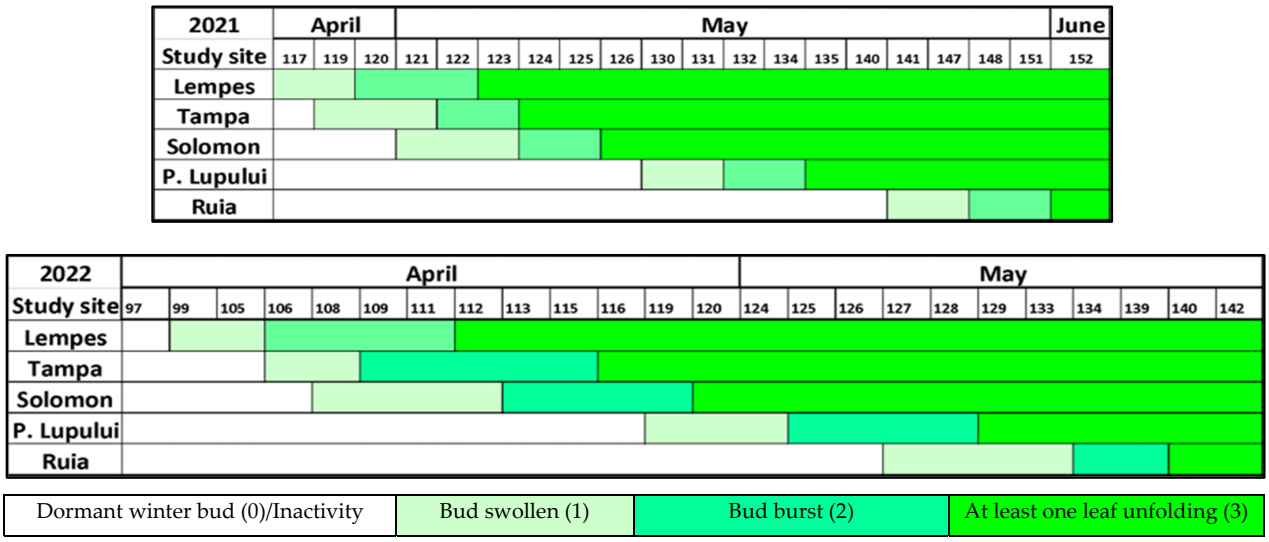

Figure 2. The dynamic of spring foliage phenology in 2021 and 2022 at each study site, based on mean values, along D.O.Y (day of the year).

Interpopulation variation (Figure 3) was significant in all cases ($p < 0.0001$ ANOVA test). The start of each phenophase at the population level varied according to the altitudinal gradient. There were significant differences between the two monitored years (ANOVA, $p < 0.0001$) in reaching the specific stage of each phenophase.

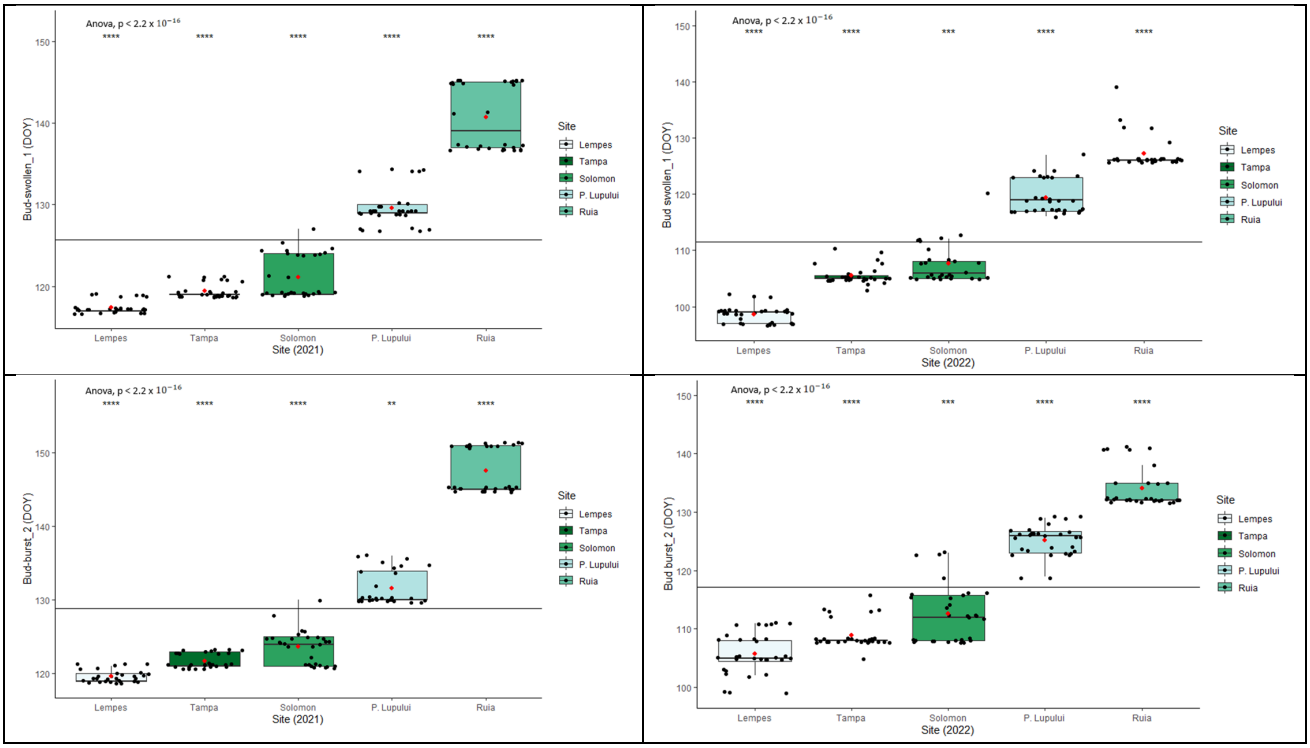

Figure 3. *Cont.*

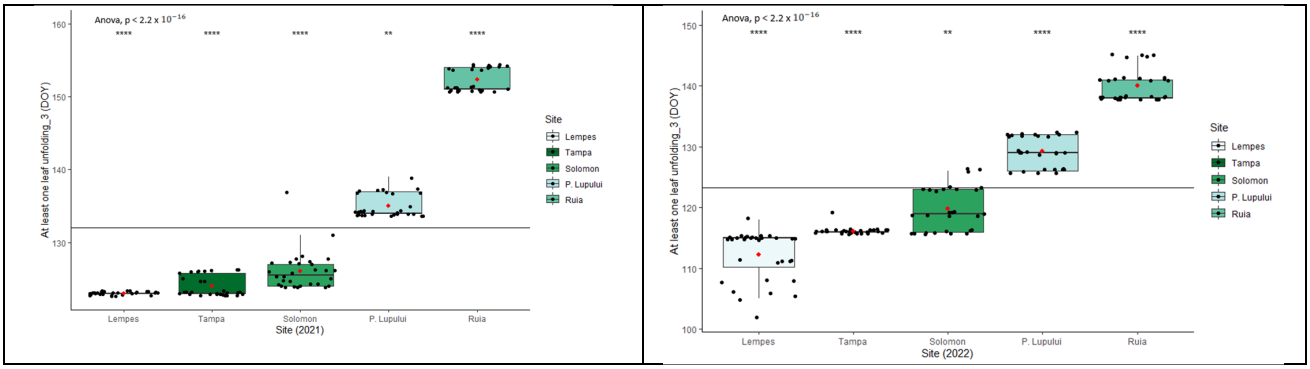

Figure 3. Intra- and interpopulation variation in European beech for each spring's phenophase in the two monitored years (****—$p \leq 0.0001$, ***—$p \leq 0.001$ and **—$p \leq 0.01$).

3.1.2. Autumn Phenology/Senescence

The dynamic of autumn foliage phenology varied during the two monitored years (Figure 4). In this case, the senescence was estimated by quantifying the two leading indicators of phenophases, yellowing and falling of the leaves. The phenophase of leaf yellowing took a more extended period in 2021 (21–32 days) than in 2022 (18–25 days), with a difference between them of 16%–28%, proportional to the increase in altitude. As in the case of spring phenology, the variation was directly proportional to the elevational gradient, with some exceptions (2021) found in the sites with special stationary conditions (wind exposure, stand density).

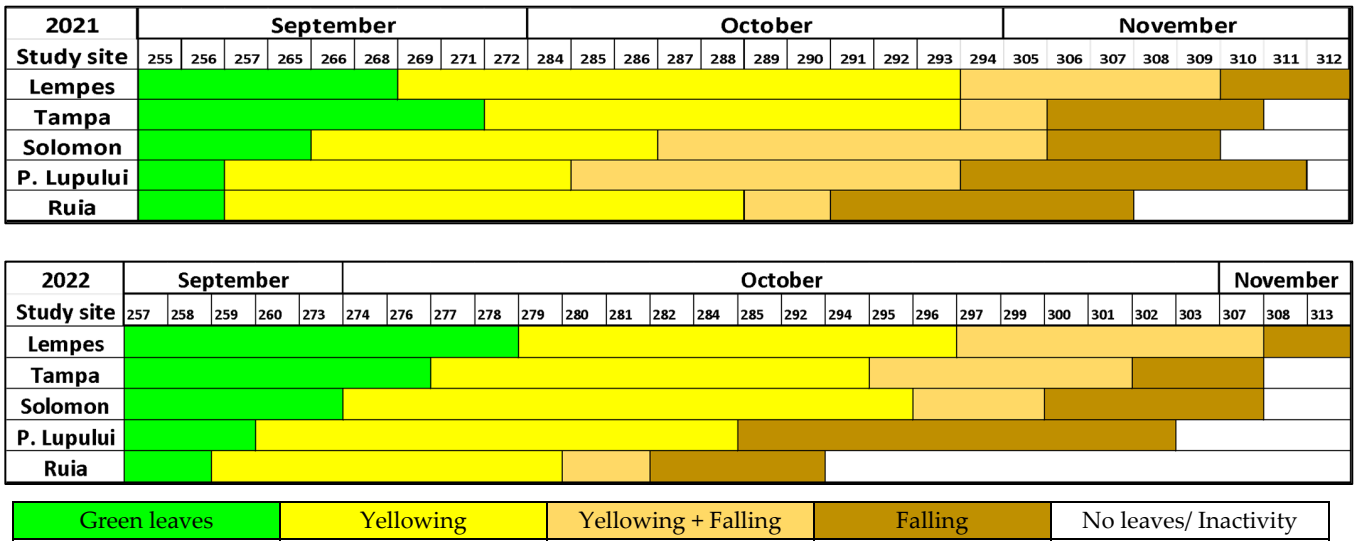

Figure 4. The dynamic of autumn foliage phenology during 2021 and 2022 at each study site, based on mean values, along D.O.Y (day of the year).

Interpopulation variation (Figure 5) was significant in all cases ($p < 0.0001$). In the same stationary conditions (same site), individuals with an early onset in the growing season showed the same early behavior in senescence. There were significant differences between the two monitored years (ANOVA, $p < 0.0001$) in reaching the specific stage of each phenophase.

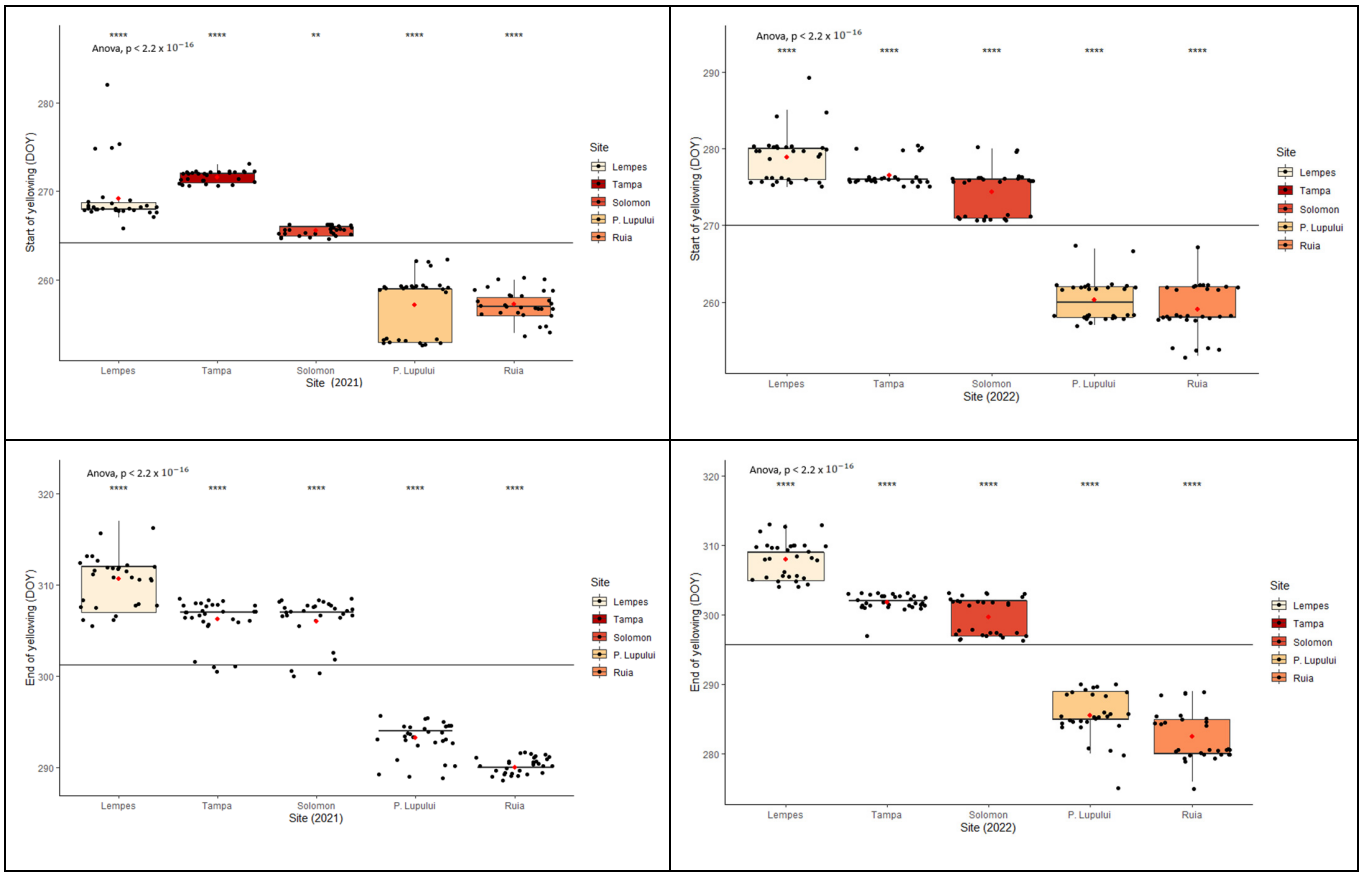

Figure 5. Intra- and interpopulation variation in European beech for the yellowing of leaves phenophase in the two monitored years (****—$p \leq 0.0001$ and **—$p \leq 0.01$).

The analysis of leaf fall of the individuals from these five study sites revealed significant interpopulation variation (Figure 6), with significant differences between all populations ($p < 0.0001$ ANOVA test). There were significant differences between the two monitored years (ANOVA, $p < 0.0001$) in reaching the specific stage of each phenophase.

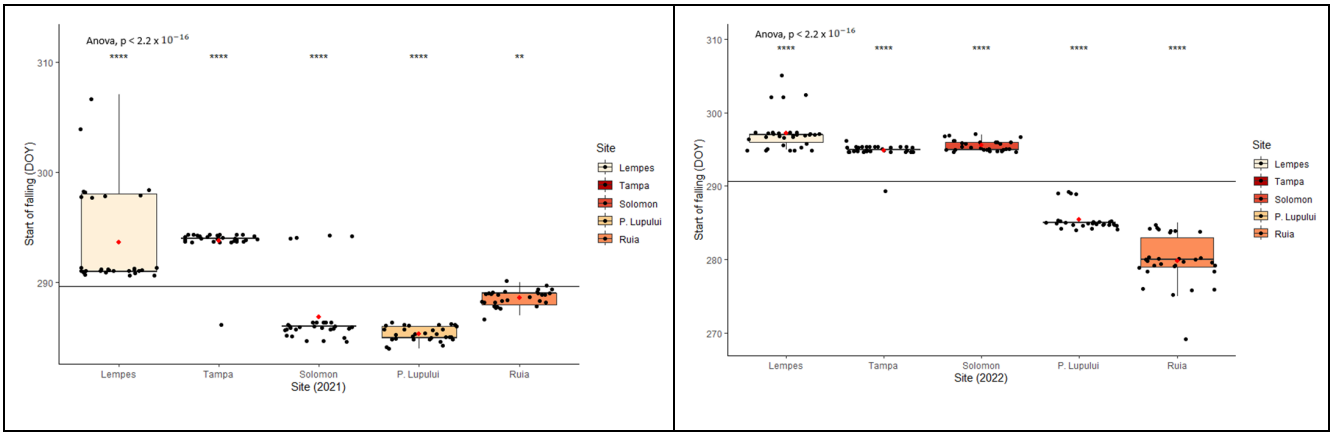

Figure 6. *Cont.*

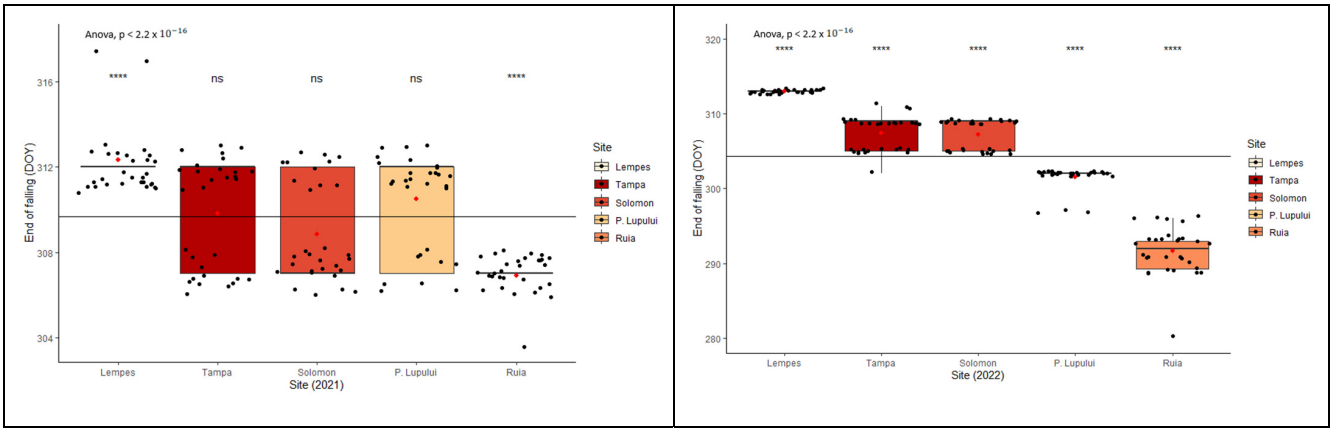

Figure 6. Intra- and interpopulation variation in European beech for the falling of leaves phenophase in the two monitored years (****—$p \leq 0.0001$, **—$p \leq 0.01$ and ns—$p > 0.05$).

3.1.3. Length of Growing Season

The length of the growing season of individuals (defined as a period between the average onset of bud burst and leaf-yellowing phenophases) varied during the two years of monitoring (Figure 7). There were significant differences between the two monitored years (ANOVA, $p < 0.0001$). An increase in altitude causes a later bud burst, a more premature onset of senescence, and a shorter growing season. A delay in the start of the spring phenophases, such as in the case of 2021, implies a shorter growing season. The average growing season duration ranged from 109 to 150 days in 2021 and 125 to 176 days in 2022. On average, about a 37% shorter growing season was observed at the highest altitudes compared to the lowest in both monitored years, and about a 14% longer growing season in 2022 compared with 2021.

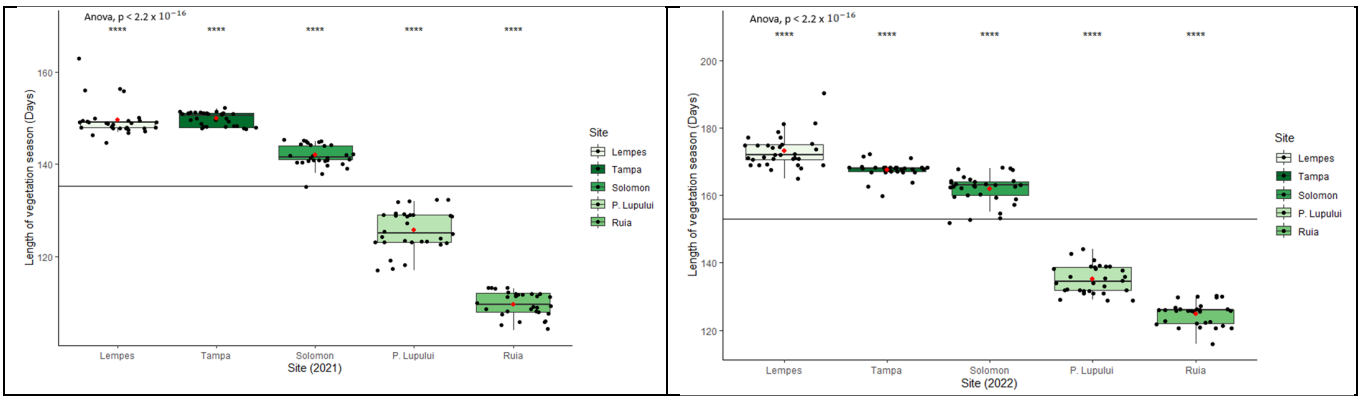

Figure 7. Intra- and interpopulation variation in European beech for the length of growing season in the two monitored years (****—$p \leq 0.0001$).

3.2. Meteorological Data

Historical temperature records were obtained from 1970 to 2000 from the WorldClim database [40] (Figure 8) for each study site. Compared with the values of 2021 and 2022, there is a trend of increasing annual temperatures. Along this elevational gradient, the temperature decreases by 0.5 °C with 100 m increasing altitude A linear regression model was fitted to the temperature variation along this altitudinal gradient ($y = -0.99x + 11.03$; $R^2 = 0.9486$).

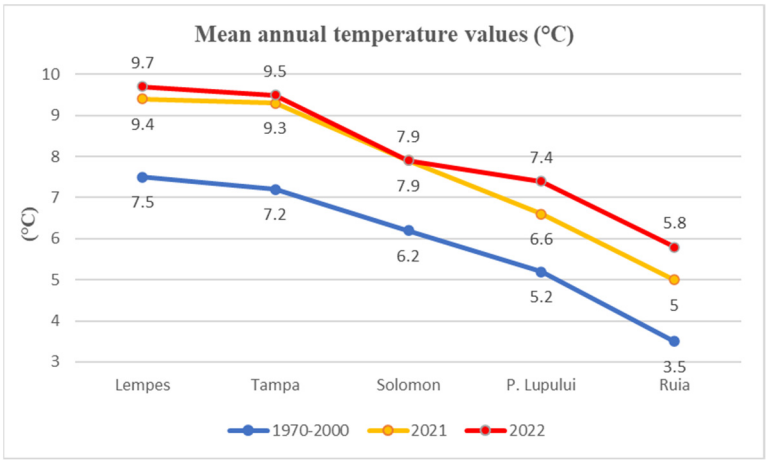

Figure 8. Mean temperature values for each study site for 1970–2000 compared with monitored years of 2021 and 2022.

3.3. Relationships between Phenological and Meteorological Data

The association between phenological and meteorological data shows that the daily average temperature triggers the start of the growing season the most (Figure 9). The other indicators (daily maximum and minimum values, growing degree days with the threshold of 0 °C, 5 °C, and 10 °C) obtained equally good values (>0.8), the explanation being their calculation method, which is also based on the daily average temperature. The correlation of phenological data with those of humidity (daily average value and daily maximum value) obtained a very low Pearson correlation value of −0.22 and −0.10, respectively.

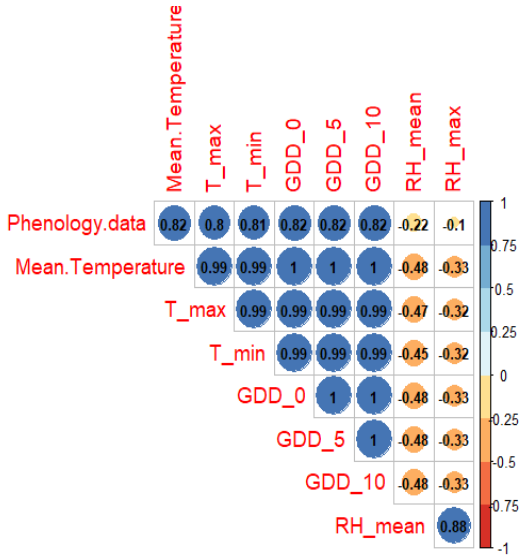

Figure 9. Correlogram of the Pearson correlation between phenological and meteorological data. The significance of the probability hypothesis (correlation) is indicated by the Pearson correlation value, the color (according to the color gradient from the right), and the size of the circles.

Temperature is the triggering factor for bud burst. When the thermal threshold of 10 °C is exceeded, bud burst occurs, but this condition is complementary to exceeding a specific accumulation of GDD (with a threshold of 0 °C). During the two monitored years, flush occurred after accumulating at least 60 GDD in the last 7 days (Figure 10).

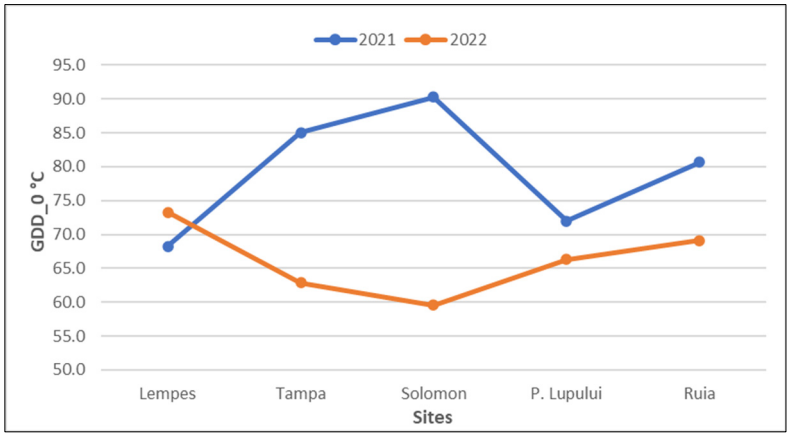

Figure 10. Cumulative GDD (growing degree days) with a threshold of 0 °C in the two monitored years for bud burst (associated with the start of the growing season) in the last seven days for each population (ascending altitudinal order).

Senescence is also influenced by temperature. The first phenophase of autumn phenology, the yellowing of the leaves, is triggered by an accumulation of at least 72 SDD with a threshold of 0 °C in the last 7 days (Table 3).

Table 3. Cumulative SDD (senescence degree days) with a threshold of 0 °C in the two monitored years on European beech reaching the yellowing of the leaves phenophase (associated with the start of the senescence) in the last seven days for each population (ascending altitudinal order).

Study Site	2021	2022
Lempes	72.14	81.49
Tampa	80.25	89.79
Solomon	72.87	85.47
P. Lupului	86.55	75.86
Ruia	78.97	78.35

The second phenophase of senescence, leaf fall, is not influenced to the same extent as the yellowing of the leaves by the accumulation of the average temperatures of the last seven days nor by the appearance of frost (the decrease in the thermal threshold of 0 °C). It is still inversely proportional to the altitudinal gradient (Figure 11).

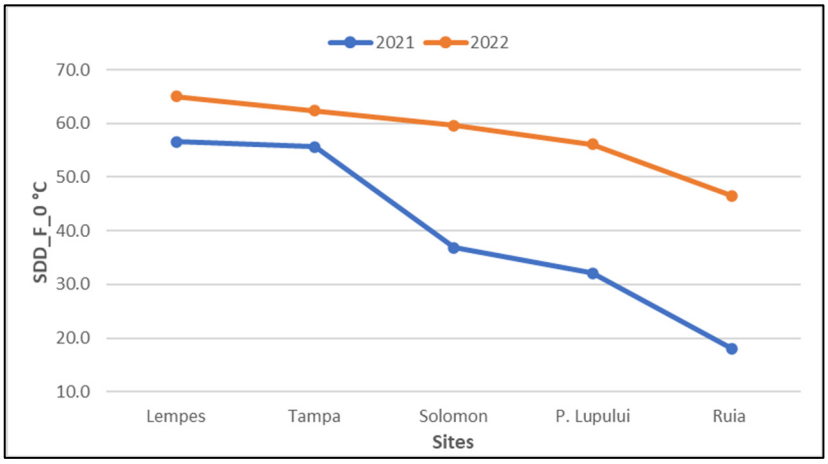

Figure 11. Cumulative SDD (senescence degree days) with a threshold of 0 °C in the two monitored years on European beech reaching falling of the leaves phenophase in the last seven days for each population (ascending altitudinal order).

4. Discussion

This study aimed to bring forth new information about European beech phenological behavior along an elevational gradient and predict the effects of climate change on this species' future distributions in the south-eastern Carpathians [30]. We performed phenological observations in the field and correlated the phenological data with the meteorological data to see which climatic indicator (temperature or humidity) predominantly influences the phenology of this species.

The two monitored years were phenologically different: in 2021, the start of the growing season occurred with a delay, associated with a faster transition between phenophases; this also implies a shorter growing season, compared to 2022, where the bud burst occurred earlier, the transition from one phenophase to another was slower, and the growing season was more extended [41].

The onset of spring phenology occurs when the daily temperature exceeds the thermal threshold of 10 °C [33], confirming the fact that reaching the leaf-unfolding phenophase in European beech is strongly influenced by temperature [9,29,42] and less sensitive to micro-topographical factors [43]. Our study shows that a day or two in which the daily average temperature exceeds the threshold of 10 °C is not enough to trigger this process. A specific accumulation of at least 60 GDD (growing degree days) in the last 7 days is necessary. Altitude is the main macroecological factor influencing leaf unfolding [44] because it also involves temperature variation.

Our results suggest that temperature is also the main driver of senescence, more precisely in its first phenophase, the yellowing of the leaves, confirming other similar studies [45,46]. However, there is still no information about autumn phenophases and their correlation with environmental factors [47,48]. It may be more difficult to detect the correlation between senescence and other meteorological factors, mainly due to the less precise quantification of the yellowing of the leaves from the upper third of the crown [49] and the less concrete delimitation of the influence on the yellowing of the leaves caused by senescence or drought. Our results showed that marcescence is not influenced to the same extent as the yellowing of the leaves by the accumulation of a certain SDD in the last seven days, nor by the appearance of frost (a decrease in the thermal threshold of 0 °C), and that it can be strongly related to micro-topographical factors (the location of the tree/stand and its exposure to air currents/wind).

The variation between individuals of the same populations is wider in the case of senescence, compared with spring phenology (see also Vilhar et al. [50]), due to the existence of several other factors that influence this process and the lack of precision in delimiting the impact of each one.

The spatial variability of temperature is related to the elevational gradient, with increasing altitude, the temperature decreases [36].

5. Conclusions

Monitoring tree phenology and analyzing these data are essential for predicting the effects of climate change on forest ecosystems. The association of phenology with meteorological data confirmed that temperature is the triggering factor for both spring phenology and senescence. Our study showed a variation in phenological stages on European beech along an altitudinal gradient, with individuals at low altitudes exhibiting an earlier onset of bud burst and a faster transition through the phenophases of spring phenology. The variation in the same individuals through senescence is also due to altitudinal and thermal differences.

The results of this study have important implications for understanding the phenological responses of European beech to climate change. As temperatures continue to increase, the start of the growing season is expected to occur earlier, while senescence is expected to be delayed. This could lead to a longer growing season overall, but it could also increase the risk of drought stress during the early stages of the growing season.

However, further investigation into phenological patterns is needed to develop models to predict how these factors will have implications. This may contribute to creating a strategy for beech forest management practices and conservation.

Author Contributions: M.I.C.C. and A.L.C. designed the experiment. M.I.C.C. performed the fieldwork. M.I.C.C., E.C., G.R.R., V.D.P. and E.B. analyzed the data. M.I.C.C. wrote the manuscript. E.C., D.C., G.R.R., V.D.P., E.B., O.G.Z., O.G. and A.L.C. reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data used in this work are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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