forests

MDPI

Article

Genetic Diversity in Peripheral Pedunculate Oak (*Quercus robur* L.) Provenances—Potential Climate Change Mitigators in the Center of Distribution despite Challenges in Natural Populations

Maja Popović [1], Ida Katičić Bogdan [1,*], Filip Varga [2,3], Zlatko Šatović [2,3], Saša Bogdan [1] and Mladen Ivanković [4]

[1] Faculty of Forestry and Wood Technology, University of Zagreb, 10000 Zagreb, Croatia; mpopovic@sumfak.unizg.hr (M.P.); sbogdan@sumfak.hr (S.B.)
[2] Faculty of Agriculture, University of Zagreb, 10000 Zagreb, Croatia; fvarga@agr.hr (F.V.); zsatovic@agr.hr (Z.Š.)
[3] Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), 10000 Zagreb, Croatia
[4] Croatian Forest Research Institute, 10450 Jastrebarsko, Croatia; mladeni@sumins.hr
* Correspondence: ikaticic@sumfak.hr; Tel.: +385-98-9830-038

Abstract: Croatian pedunculate oak (*Quercus robur* L.) populations represent southern range peripheral populations, often viewed as sources of valuable diversity and drought-resistant ecotypes. At the same time, they endure stronger selection pressures as a result of climate change. The leaves of 20 individuals per population (17) were sampled in a field trial and analyzed using 10 nuclear and 9 chloroplast SSRs to determine the level of intrapopulation genetic variability and genetic differentiation. Analysis with nSSRs revealed deviation from HWE in seven populations. AMOVA showed a high intra-population diversity (98.53%) and a small but statistically significant inter-population differentiation. Isolation by distance explained 19.6% of differentiation. Average F_{ST} between populations was low (0.013) compared with usual values for peripheral populations. Populations were rich in cpSSR haplotypes, confirming the hotspot of diversity caused by the encounter of recolonization routes. Unbiased haplotype diversity (H_E) from 9 chloroplast SSRs and 325 individuals was (H_E = 0.440). Sixty-six different haplotypes were grouped in three maternal lineages by both a median-joining network and a neighbor-joining algorithm. AMOVA for cpSSRs showed statistically significant diversity among populations (70.23%), suggesting genetic differentiation, but also a probable anthropogenic effect. AMOVA of nSSRs within and between lineages showed that original recolonization patterns of nuclear diversity were subsequently erased by gene flow.

Keywords: peripheral populations; nSSRs; cpSSRs; inbreeding; differentiation; diversity hotspot; *Quercus robur* L.

Citation: Popović, M.; Katičić Bogdan, I.; Varga, F.; Šatović, Z.; Bogdan, S.; Ivanković, M. Genetic Diversity in Peripheral Pedunculate Oak (*Quercus robur* L.) Provenances—Potential Climate Change Mitigators in the Center of Distribution despite Challenges in Natural Populations. *Forests* **2023**, *14*, 2290. https://doi.org/10.3390/f14122290

Academic Editor: Claudia Mattioni

Received: 12 October 2023
Revised: 17 November 2023
Accepted: 20 November 2023
Published: 22 November 2023

1. Introduction

Genetic diversity is the foundation of biodiversity and a crucial tool for the adaptive potential, survival, and evolution of living organisms, including forest tree species, in changing environmental conditions [1,2]. Therefore, it is necessary to explore various aspects of genetic diversity to shed light on evolutionary processes and to evaluate the use and conservation of genetic resources of a species.

Forests cover 49.3% of Croatian territory (2,759,039 ha) out of which 76% of the forests is state owned and mostly managed by the public company Croatian forests Ltd. The pedunculate oak (*Quercus robur* L.) is one of the most significant tree species in Croatia, both ecologically and economically. It comprises about 11% of overall wood stock [3]. As a keystone species in the forest ecosystem, maintaining a high level of genetic diversity is essential to enhance its capacity for adaptation through natural selection and to ensure

its survival in a changing environment [4–6]. In Croatia, the majority of pedunculate oak stands reside in the northern part of the country, as larger complexes of forests along the Sava, Drava and Danube rivers (Figure 1). The exceptions are two separate populations; one in the Lika region (purposefully planted against erosion) and one in Istria, by the Mirna river.

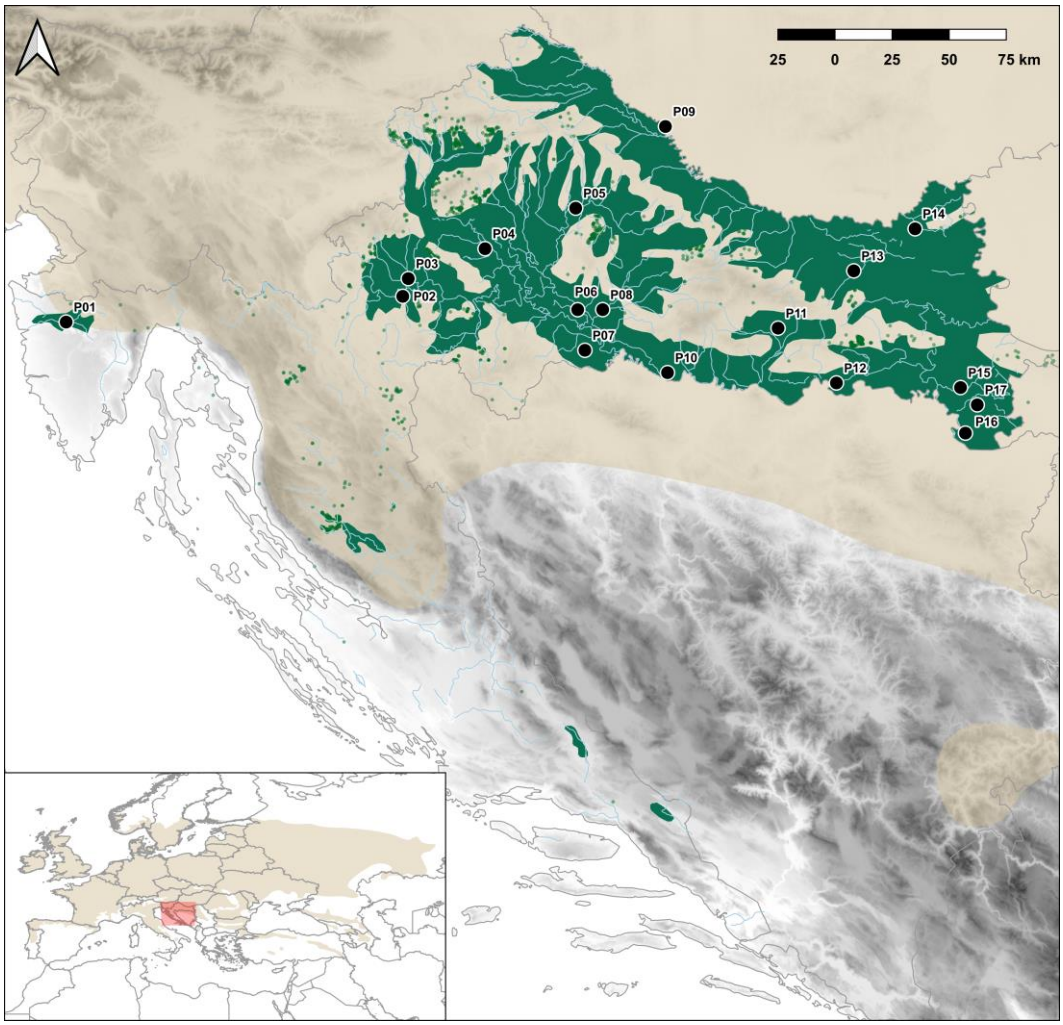

Figure 1. Sampling locations of the 17 pedunculate oak (*Quercus robur* L.) in Croatia. The distribution range of *Q. robur* in Europe, according to EUFORGEN (http://www.euforgen.org/wileyonlinelibrary.com (accessed on 20 August 2023)), is represented with a beige color, while distribution in Croatia according to publicly available data is shown in green.

Within the species *Quercus robur* L., taxonomic subspecies or ecological varieties have evolved over time in geographic sub-areas throughout its range. Regarded as such is the *Quercus robur* subsp. *slavonica* (Gáyer) Mátyás, distributed in lowland areas between the Drava and Sava rivers and from Zagreb to the far east of Croatia, forming the most valuable stands in the region. Slavonian oak is in increasing demand throughout Europe as an imported timber. This is because of its many good qualities, which include its high growth performance, straightness, long clear trunk and fine branches [7]. Additionally, its late flushing makes it significantly less affected by the European oak leaf roller (*Tortrix viridana* L.) and late spring frost [8].

In the past decades, pedunculate oak forests in Europe have faced numerous challenges, including low underground water levels enhanced by river regulations, climate extremes including drought stress and other effects of climate change, such as strong winds and pest and diseases, including the continuous pressure caused by lace bug (*Corythucha*

arcuata Say, 1832), the long-term effects of which are yet to be sufficiently explored. All of these negative impacts combined have resulted in the frequent deterioration of trees' health status, growth rate and lack of natural regeneration and sometimes even to their premature death. Furthermore, as climate change intensifies, there is potential for significant damage to pedunculate oak forests through interactions between causal agents, culminating in a complex phenomenon known as oak decline [9].

Pedunculate oak populations in Croatia, as well as generally in this part of Europe, represent peripheral populations at a southern range margin, which adds special importance to, and interest in, the state and form of their standing genetic diversity. Peripheral populations on the lower edge of species distribution often represent reservoirs of diversity, as well as a potential source of drought-resistant ecotypes. However, at the same time, studies have shown an increased negative effect of recent climate change on these populations, compared with the core populations, because of stronger selection pressures. Marginal populations more often experience various biotic and abiotic stress factors which negatively affect their reproductive success and survival [9]. As described by Temunović et al. [10], climate envelope models foresee significant extinctions among forest tree populations situated at the lower latitudinal margins of species' ranges in the coming decades, due to diminishing habitat suitability. However, because these populations have historically endured higher temperatures and reduced precipitation, genotypes that have evolved under such specific climatic conditions could offer valuable genetic reservoirs [11,12]. These reservoirs might play a crucial role in aiding the adaptation of more central populations to analogous climatic conditions expected in the near future due to ongoing climate change.

As a concrete example, there is already increasing interest in Slavonian oak in forestry due to the forest restructuring in Germany caused by climate change. Slavonian oaks have already been introduced into the western part of Germany, especially in the region around Münster, in the second half of the nineteenth century, as well as in other European countries. These Slavonian oak stands are the first generation stands in Germany and are regarded as a valuable potential tool for mitigating the possible effects of climate change in the future [8]. Considering economic value, as mentioned, it has a great number of valuable traits and has already been proven as broadly adaptable provenance which performs well even under different climatic conditions of plantation sites [13]. From a genetic point of view, recent research [10] on Croatian pedunculate oak populations has revealed more consistent associations with environmental variation than in previous pedunculate oak studies attempting to detect signatures of local adaptation in core populations. The results provided independent molecular evidence across multiple loci that important climate change-related factors, such as water availability and its seasonal variation, have already shaped the adaptive divergence among investigated populations on the southern range margin. These populations may contain advantageous genetic variants that might be important for the species' response to a rapidly changing climate. Additionally, these variants could be exchanged even between distant populations, contrary to common expectations for marginal populations. These genetic resources hold particular significance for long-lived stationary species like forest trees because they rely heavily on the existing standing genetic variation at evolutionarily significant loci to mount adaptive responses. Additionally, results obtained from the drought-prone field trial of pedunculate oak, located in eastern Croatia show that the studied progeny populations originating from the southeastern part of the pedunculate oak distribution range in Croatia exhibited high mean survival rates after being exposed to two successive years of substantial decrease in water availability at the field trial site. This finding indicates their possible adaptation to the relatively long-term arid conditions at the specific trial site [14].

Considering the existing and potential importance of Croatian populations, there was a need for detailed research into their genetic diversity. For the purpose of studying adaptive traits of different provenances of pedunculate oak, covering most of its distribution in Croatia, three provenance trials were established on sites with different environmental

conditions. The aim was to test the performance of all the provenances on sites covering the main range of pedunculate oak in Croatia.

The objective of this study is to determine the level of intrapopulation genetic variability and the extent of genetic differentiation of pedunculate oak natural populations in Croatia on the samples collected in one of the genetic trials, using DNA nuclear and chloroplast microsatellites (SSRs), and to propose possible recommendations for the conservation of genetic diversity of these populations near the species' range margin, connecting the results with existing research on adaptive traits [15,16] and candidate gene SNP variation [10] conducted on the same trial i.e., DNA samples.

2. Materials and Methods

2.1. Plant Material

Young leaves of pedunculate oak for this study were collected during May 2012, in a genetic field trial established with two-year-old seedlings from 16 seed stands (FRM category selected) and one normal managed stand (identified source; P15) across the Croatian distribution range of the species (Figure 1 and Supplement S1). Genetic field trial Jastrebarsko was established by the Croatian Forest Research Institute (CFI) in 2008 in the area of Forest Management Karlovac, forest unit Jastrebarski lugovi (N 45.644; E 15.699, elevation 111 m a.s.l.). The acorns for establishment were collected in the year 2006, recognized as year of full and high-quality crop of pedunculate oak in Croatia [17]. To avoid kinship, acorns were collected from mother trees, at least 50 m apart. The trial consisted of three repetitions, with each population represented per repetition by 20 half-sib families of five plants each [15,16]. The samples for each population were collected in one repetition, with one individual sampled per family i.e., 20 individuals per population (340 samples in total). Therefore, the samples represent the collection of the progeny of 2006 from the sampled populations, not the adult individuals from those populations. Such sampling was chosen for better connectivity of the obtained data with measurements of adaptive traits conducted in the trial, under the presumption that the diversity of the progeny from a full crop year is a good representative of populations' standing genetic diversity, as noted in certain studies [7].

2.2. DNA Isolation and PCR

DNA isolation was undertaken with DNeasy Plant Mini Kit (QIAGEN®, Hilden, Germany).

For cpSSR analysis, we used nine primer pairs: *µdt1*, *µdt3*, *µdt4*, *µcd4*, *µcd5*, *µkk3* and *µkk4* from Deguilloux et al. [18], and *ccmp10* and *ccmp6* from Weising and Gardner [19]. The PCRs were carried out following a slightly changed protocol from Deguilloux et al. [18].

The nSSR primer pairs used in this study were: seven primers (*ssrQrZAG96*, *ssrQrZAG7*, *ssrQrZAG11*, *ssrQrZAG87*, *ssrQrZAG112*, *ssrQrZAG101* and *ssrQrZAG30*) from Kampfer et al. [20], two primers (*ssrQpZAG9* and *ssrQpZAG16*) from Steinkellner et al. [21], and one primer pair (*MSQ13*) from Dow and Ashley [22]. We followed a slightly changed protocol from Kampfer et al. [20] for all primer pairs except *ssrQrZAG112*, for which PCR was undertaken according to Guichoux et al. [23].

PCR was performed with Mastercycler® ep, Eppendorf, Hamburg, Germany. Capillary electrophoresis was performed by Macrogen Europe Inc., Amsterdam, The Netherlands. Alleles were scored using GeneMapper 5.0 (Applied Biosystems®, Waltham, MA, USA).

2.3. Statistical Analysis

2.3.1. Chloroplast Microsatellites (cpSSR)

Haplotype Diversity

Haplotype diversity was assessed in each population and in the metapopulation by calculating the number of haplotypes (n_h), the number of haplotypes per number of individuals (n_h/n), the number of private haplotypes (n_{ph}), the effective number of haplotypes (n_E), the haplotypic richness (n_{hr}) and the unbiased haplotype diversity (H_E)

using the program HAPLOTYPE ANALYSIS v1.05, University of Goetingen, Goetingen, Germany [24].

Genetic Differentiation

Genetic differentiation was assessed by calculating three coefficients, G_{ST}, N_{ST} and R_{ST}, using the program PERMUT and cpSSR v2.0, Paris, France [25]. G_{ST} [26] is based solely on haplotype frequencies (unordered haplotypes), N_{ST} [25] considers the genetic similarities between haplotypes based on the proportion of common alleles (ordered haplotypes), while R_{ST} [27] involves the calculation of genetic distances based on the individual cpSSR loci assuming the stepwise mutation model (SMM) of microsatellite evolution. The differences between G_{ST} and N_{ST} values and between G_{ST} and R_{ST} values were tested using 10,000 permutations. Significantly higher N_{ST} or R_{ST} values compared with G_{ST} indicate a phylogeographic structure in populations [25].

Total genetic variance based on cpSSR haplotypes (i.e., including a single individual per haplotype and excluding individuals sharing the same cpSSR haplotype) was partitioned (1) within and among populations and (2) among and within the three maternal lineages (L1–L3) using AMOVA in Arlequin v. 3.5.2.2, University of Bern, Bern, Switzerland [28] and the significance of variance components was tested based on 10,000 permutations.

Network/Maternal Lineages

A median-joining (MJ) network [29] was constructed based on cpSSR haplotypes found in more than one individual using the program PopArt v1.7, University of Otago, Dunedin, New Zealand [30]. In order to group similar cpSSR haplotypes into maternal lineages, genetic distances between all haplotype pairs were calculated based on nine cpSSRs using the proportion of shared allele distances (D_{PSA}) [31] in the MICROSAT, University of Washington, Washington, USA [32]. Cluster analysis was performed using the neighbor-joining method implemented in the program NEIGHBOR of the package PHYLIP v3.698, University of Washington, Washington, USA [33]. The reliability of the tree topology was assessed by bootstrapping [34] based on 1000 replicates generated by MICROSAT and subsequently used in the NEIGHBOR and CONSENSE programs of PHYLIP.

2.3.2. Nuclear Microsatellites (nSSR)

Microsatellite Diversity

The total number of alleles (N_a), polymorphic information content (PIC) and probability of identity (PI) of each nuclear microsatellite locus (nSSR) were calculated using Cervus v. 3.0 [35]. The microsatellite data were checked for the presence of null alleles using Micro-Checker v. 2.2.3, University of Hull, Hull, UK [36]. The frequency of null alleles was estimated using the expectation-maximization algorithm in FreeNA, INRAE, Paris, France [37].

Within-Population Diversity

Genetic diversity within each population was determined by calculating the average number of alleles (N_{av}) and the number of private alleles (N_{pr}). Genepop v. 4.7, University of Montpellier, Montpellier, France [38] was used to calculate the genetic parameters of the populations, including observed heterozygosity (H_O), expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS}). Possible deviations from the Hardy–Weinberg equilibrium were tested in Genepop. The significance level was adjusted after sequential Bonferroni corrections for multiple testing using SAS v. 9.4 [39]. Allele frequencies adjusted for the presence of null alleles were used to recalculate expected heterozygosity values ($H_{E(null)}$) and compare them to the original values using the Wilcoxon signed-rank test in SAS. Evidence of recent genetic bottleneck in each population was tested using the Wilcoxon signed-rank test [40] and the two-phase model (TPM) assuming 22% multistep changes and a variance of 11.92 as recommended by Peery et al. [41] using BOTTLENECK v. 1.2.02, INRAE, Paris, France [42,43].

Population Differentiation and Structure

FSTAT v. 2.9.3.2, University of Lausanne, Lausanne, Switzerland [44] was used to calculate the pairwise F_{ST} values. Significance levels were calculated after 10,000 random permutations. Pairwise F_{ST} values were also estimated after correction for the presence of null alleles using the ENA correction method (excluding null alleles; $F_{ST(null)}$) implemented in FreeNA and compared with the original values using the Wilcoxon signed-rank test in SAS. Analysis of molecular variance (AMOVA) [45] was used to partition the total genetic variance (A) among and within populations, and (B) among and within three maternal lineages (L1–L3) inferred from cpSSRs. The variance components were tested with 10,000 permutations in Arlequin. The genetic structure of the populations was examined using a Bayesian clustering approach implemented in STRUCTURE v. 2.3.4, Stanford University, Stanford, CA, USA [46]. Runs were performed assuming admixture and using the correlated allele frequencies both with and without the LOCPRIOR option [47]. STRUCTURE was run for K = 1–11 with 30 replicates for each K with a burn-in period of 200,000 and 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. The optimal number of clusters was determined by calculating the average estimates of the likelihood of the data ($ln\ P(X \mid K)$) and ΔK [48] using STRUCTURE HARVESTER v. 0.6.94, University of Los Angeles (UCLA), Los Angeles, CA, USA [49]. The results were further analyzed using CLUMPAK, University of Tel Aviv, Tel Aviv, Israel [50].

Spatial Genetics

The method of Rousset [51] was used to test isolation by distance (IBD) among populations. The matrix of pairwise $F_{ST}/(1 - F_{ST})$ ratios and that of the natural logarithm of geographical distances (ln km) between pairs of populations were subjected to a Mantel test using 1,000,000 permutations in NTSYS-pc v. 2.21L, Exeter Software, New York, USA [52].

Correlation with SNP and Quantitative Traits Data

To complement the results of previous analyses of quantitative traits in the trial [15,16], as well as the results of the study by Temunović et al. [10] (with SNPs selected from candidate genes, conducted on the same DNA samples) with our data on presumably neutral genetic diversity, we tested the Pearson correlation of our populations' genetic parameters (N_{av}, H_O, He, F_{IS} for nSSRs) with parameters of interest from the quantitative data and the SNP data (F_{IS}). Correlations were calculated using SAS [39].

Considering the SNP data, we used the genetic variance values for all analyzed SNP loci (both outliers and non-outliers), demonstrated by node sizes, obtained from conditional graph distance (cGD) approach (Figure 2 in [10]). Node sizes are determined by the size of intrapopulation variance of a population, relative to other populations included in the network. We correlated the sizes with our F_{IS} to compare whether the populations showing possible signs of inbreeding at neutral loci also show signs of decreased intrapopulation variability at all analyzed SNPs. We also performed the same correlation only for non-outlier loci.

To establish connections between SSR and quantitative data and explore whether the narrowed neutral diversity is somehow reflected in a diversity of quantitative traits, we correlated N_a, H_O, H_E and F_{IS} values with populations' arithmetic means, as well as standard deviations for the following traits; flushing phenology (plant ages 5, 6 and 7 years) and height and survival (plant ages 4, 5 and 6 years). As explained in Section 2.1, the DNA samples were taken from one repetition in the trial and each of the 20 families per population included in the repetition was represented by one member (altogether 340 individuals). The quantitative data were measured and calculated from the entire trial, with 17 populations being represented by the same 20 families of 5 individuals each, in 3 repetitions (altogether 5100 individuals). We used previously published quantitative traits data [15,16].

3. Results

3.1. Chloroplast Microsatellites (cpSSR)

The analysis of 325 individuals obtained 66 different haplotypes. Fifteen individuals were excluded due to missing data. Nine (9) haplotypes were the most common and were found in 196 individuals (60%). Thirty-four individuals had unique haplotypes found in no other individual.

3.1.1. Haplotype Diversity

As seen in Table 1, the largest number of haplotypes (n_h) (12) was found in the population P07 (Sunja, FA Sisak), while the highest haplotype diversity (n_h/n) was recorded in the P08 population (Lipovljani, FA Zagreb) (0.733 haplotypes per individual). The most uniform population, with only 0.150 haplotypes per individual, P04 (Velika Gorica, FA Zagreb), also had the lowest diversity of haplotypes (0.195). The population P12 (Trnjani, FA Nova Gradiška) had the most private haplotypes (n_{ph} = 7). For other diversity parameters, population P08 had the highest values and population P04 the lowest.

Table 1. Genetic diversity of 17 pedunculate oak (*Quercus robur* L.) populations in Croatia based on nine chloroplast microsatellite loci.

Population	Forestry	n	n_h	n_h/n	n_{ph}	n_E	n_{hr}	H_E
P01	Buzet	18	7	0.389	2	2.893	5.294	0.693
P02	Karlovac 1	19	4	0.211	2	2.560	2.754	0.643
P03	Karlovac 2	20	9	0.450	5	6.667	6.921	0.895
P04	Velika Gorica	20	3	0.150	0	1.227	1.500	0.195
P05	Vrbovec	19	5	0.263	1	2.843	3.575	0.684
P06	Kutina	20	11	0.550	2	5.714	7.939	0.868
P07	Sunja	18	12	0.667	4	9.000	9.496	0.941
P08	Lipovljani	15	11	0.733	2	9.783	10.000	0.962
P09	Repaš	19	9	0.474	1	5.085	6.873	0.848
P10	Stara Gradiška	20	5	0.250	1	2.469	3.636	0.626
P11	Požega	20	9	0.450	2	6.061	6.877	0.879
P12	Trnjani	18	11	0.611	7	6.231	8.627	0.889
P13	Koška	20	7	0.350	3	3.774	4.947	0.774
P14	Darda	20	9	0.450	3	4.651	6.636	0.826
P15	Otok 1	19	11	0.579	3	5.554	8.246	0.865
P16	Gunja	20	4	0.200	2	2.469	2.697	0.626
P17	Otok 2	20	8	0.400	1	2.985	5.645	0.700

n—sample size, n_h—number of haplotypes, n_h/n—number of haplotypes per individual, n_{ph}—number of private haplotypes, n_e—effective number of haplotypes, n_{hr}—haplotypic richness, H_E—unbiased haplotype diversity.

3.1.2. Genetic Differentiation

Genetic differentiation was assessed by calculating three coefficients, G_{ST}, N_{ST} and R_{ST} (G_{ST} = 0.203, N_{ST} = 0.193, R_{ST} = 0.073). There was no statistical significance between N_{ST} and R_{ST} compared with G_{ST}, the G_{ST} was also larger than N_{ST} and R_{ST}. This leads to the conclusion that there is no phylogeographic structure of the populations.

Total genetic variance based on cpSSR haplotypes (i.e., including a single individual per haplotype and excluding individuals sharing the same cpSSR haplotype) was statistically significant for both partitioned analyses ((1) within and among populations and (2) among and within the three maternal lineages (L1–L3)) shown in Table 1. Highly statistically significant diversity among populations suggested genetic differentiation between 17 pedunculate oak (*Quercus robur* L.) populations in Croatia.

3.1.3. Network/Maternal Lineages

Figure 2A shows the network of H01–H32 haplotypes i.e., haplotypes found in more than one individual (n > 1). Haplotypes were grouped into three lineages. The size of the circles is proportional to the number of individuals representing that haplotype. The first

four haplotypes represent the main network. Haplotypes found in only one individual are not shown in the picture, due to the unnecessary 'noise' they create when visualized.

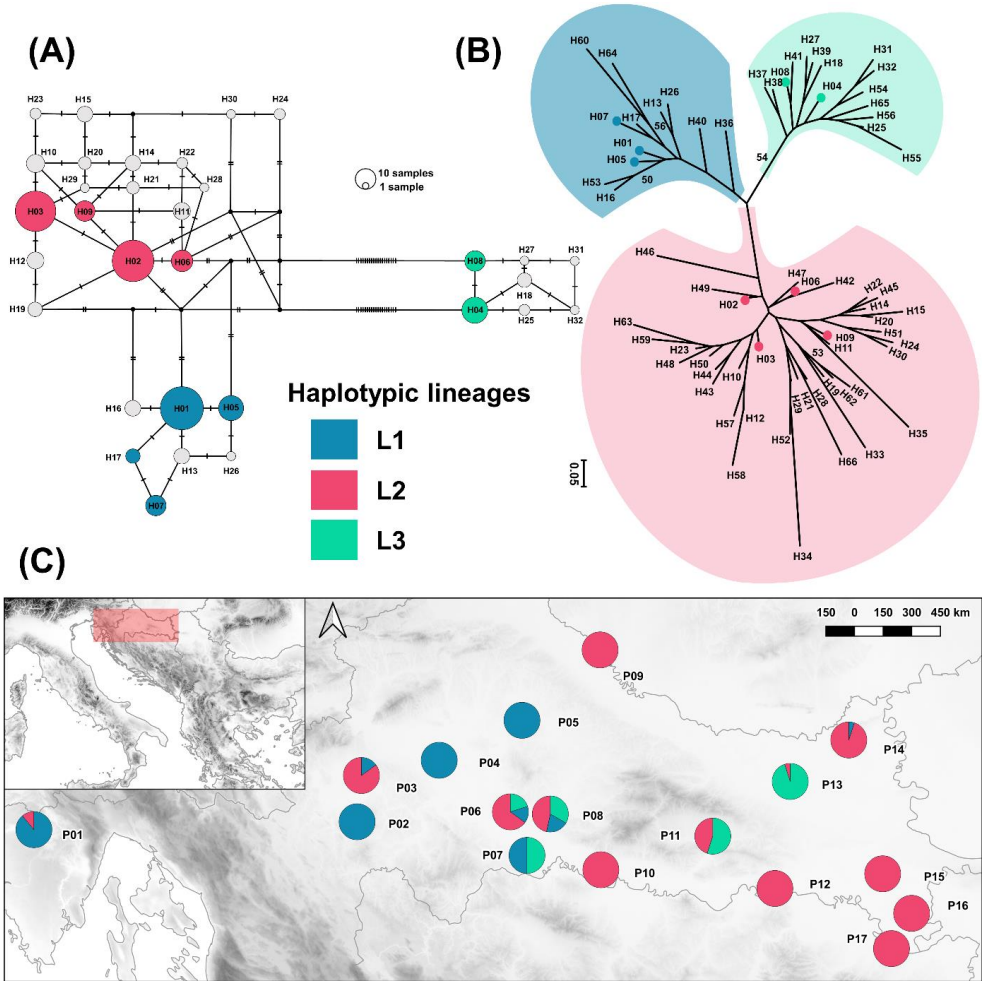

Figure 2. (**A**) Median-joining (MJ) network based on 32 cpSSR haplotypes found in more than one sample in 17 pedunculate oak (*Quercus robur* L.) populations in Croatia. The size of the circles is proportional to the number of individuals representing that haplotype. (**B**) Neighbor-joining tree of 66 cpSSR haplotypes found showing three maternal lineages (L1–L3). (**C**) Proportion of individuals in populations with cpSSR haplotypes belonging to three maternal lineage (L1–L3) haplotypes found in more than one sample.

The distribution of maternal lineages by population is shown in Figure 2C. Populations P02 (Karlovac 1), P04 (Velika Gorica) and P05 (Vrbovec) belong only to lineage L1 (blue). Populations P09 (Repaš), P10 (Stara Gradiška), P12 (Trnjani), P15 (Otok 1), P16 (Gunja) and P17 (Otok 2) only to line L2 (red). The L3 lineage (green) appears in populations exclusively in combination with the other two lineages, with the largest share in the population P13 (Koška).

3.2. Nuclear Microsatellites (nSSR)

3.2.1. Microsatellite Diversity

An analysis of ten microsatellite loci revealed a total of 251 detected alleles (N_a). The PCR product with the highest number of alleles was *ssrQrZAG30* (42), and with the lowest *ssrQpZAG9* (13). The average number of alleles was 25.10. Genetic diversity ranged from 0.418 for *ssrQrZAG96* to 0.954 for *ssrQrZAG30* (Supplement S1). The PCR

products of *ssrQrZAG87*, *ssrQrZAG96*, and *MSQ13* had a significant frequency of null alleles (Supplement S2).

3.2.2. Within-Population Diversity

Population parameters describing the intra-population diversity of 17 pedunculate oak populations are shown in Table 2. The average number of alleles per locus (N_{av}) in populations ranged from 10.30 (P02) to 13.70 (P15). A total of 44 private alleles (N_{pr}) were identified, the population P01 had the largest number (10), and populations P04, P07 and P14 had no private alleles. The observed heterozygosity (H_O) ranged from 0.760 (P16) to 0.814 (P12). In all populations, the expected heterozygosity (H_E) values were higher and ranged from 0.797 (P17) to 0.846 (P10). Differences between expected heterozygosity (H_E) and expected heterozygosity after estimation of null alleles ($H_{E(null)}$) were tested with the Wilcoxon test and were not significant, so we conclude that the null alleles did not significantly influence the values of expected heterozygosity. The multilocus test found a significant deviation from the Hardy–Weinberg equilibrium in seven populations: P01, P03, P08, P10, P11, P13 and P16.

Table 2. Genetic diversity of 17 pedunculate oak (*Quercus robur* L.) populations in Croatia based on 10 nuclear microsatellite loci.

Population	Forestry	n	N_{av}	N_{pa}	H_O	H_E	$H_{E(null)}$	F_{IS}	$P(F_{IS})$	$P_{Bottleneck}$
P01	Buzet	20	11.90	10	0.782	0.839	0.840	0.067	***	0.988
P02	Karlovac 1	20	10.30	2	0.790	0.825	0.831	0.042	ns	0.813
P03	Karlovac 2	20	12.10	6	0.768	0.846	0.854	0.092	***	0.999
P04	Velika Gorica	20	10.70	0	0.785	0.809	0.817	0.029	ns	0.813
P05	Vrbovec	20	11.50	3	0.785	0.812	0.816	0.033	ns	0.652
P06	Kutina	20	12.20	1	0.785	0.817	0.822	0.040	ns	0.348
P07	Sunja	20	11.80	0	0.790	0.827	0.833	0.045	ns	0.615
P08	Lipovljani	20	11.80	1	0.770	0.840	0.847	0.084	**	0.313
P09	Repaš	20	11.60	3	0.794	0.816	0.819	0.027	ns	0.500
P10	Stara Gradiška	20	13.00	2	0.765	0.846	0.849	0.096	**	0.903
P11	Požega	20	13.00	2	0.760	0.831	0.840	0.086	**	0.993
P12	Trnjani	20	11.80	4	0.814	0.844	0.826	0.036	ns	0.615
P13	Koška	20	10.80	3	0.770	0.820	0.830	0.061	**	0.652
P14	Darda	20	11.90	0	0.785	0.839	0.855	0.064	ns	0.839
P15	Otok 1	20	13.70	4	0.807	0.836	0.846	0.035	ns	0.652
P16	Gunja	20	13.10	2	0.760	0.838	0.849	0.093	***	0.920
P17	Otok 2	20	12.10	1	0.785	0.797	0.801	0.015	ns	0.884

n—sample size; N_{av}—average number of alleles; N_{pr}—number of private alleles; H_O—observed heterozygosity; H_E—expected heterozygosity; $H_{E(null)}$—expected heterozygosity based on allele frequencies corrected for null alleles; F_{IS}—inbreeding coefficient (significance levels: ns—non-significant value; **—significant at $p < 0.01$; ***—significant at $p < 0.001$); $P_{Bottleneck}$—probability of a Wilcoxon signed-rank test for population bottleneck.

3.2.3. Population Differentiation and Structure

Out of 136 pairs of populations 50 were significantly differentiated. The most differentiated population with statistically significant F_{ST} values from almost all other populations was P01 (Buzet, Istria), followed by P02 (Karlovac 1), P03 (Karlovac 2) and P04 (Velika Gorica) (Supplement S3). These populations are located in the more western part of the distribution and are significantly differentiated from most of the more eastern ones, as well as amongst themselves.

The results of the AMOVA (Table 3) were calculated for two assumed levels of structure: (A) among and within populations and (B) the diversity of nuclear microsatellites within and among maternal lineages L1–L3, obtained in an analysis of chloroplast haplotypes (cpSSR). Although the percentage of variance caused by differences among populations/maternal lineages was highly significant ($p < 0.0001$), both for A and B analysis, the percentage of variance within populations (98.53% (A) and 99.62% (B)) showed that the

differences between populations were very small and that there was almost no preserved connection between haplotype diversity and diversity of nuclear loci.

Table 3. Analysis of variance (AMOVA) of 17 pedunculate oak (*Quercus robur* L.) populations in Croatia using nuclear (nSSR) and chloroplast (cpSSR) microsatellites. The partitioning of nSSR diversity (A) among and within populations and (B) among and within three maternal lineages (L1–L3) and cpSSR diversity (C) among and within populations and (D) among and within three maternal lineages (L1–L3).

Analysis	Source of Variation	df	Variance Components	% Total Variance	φ_{ST}	$P(\varphi_{ST})$
(A) nSSR	Among populations	16	0.060	1.47	0.015	<0.0001
	Within populations	663	4.057	98.53		
(B)	Among maternal lineages	2	0.016	0.38	0.004	<0.0001
	Within maternal lineages	647	4.101	99.62		
(C) cpSSR	Among populations	16	0.661	29.77	0.298	<0.0001
	Within populations	118	1.559	70.23		
(D)	Among maternal lineages	2	1.718	54.23	0.542	<0.0001
	Within maternal lineages	63	1.450	45.77		

$P(\varphi_{ST})$—significance after 10,000 random permutations.

Bayesian analysis using STRUCTURE without LOCPRIOR option showed no genetic structure between analyzed populations (Figure 3). With the LOCPRIOR option populations showing slight differences. The highest ΔK value was observed for $K = 2$ (21.85), followed by that for $K = 3$ (5.42) (Supplement S4). At LOCPRIOR $K = 2$, almost all individuals in population P01 (Buzet) and part of the individuals in populations P03 (Karlovac 2) and P12 (Trnjani) belong to the cluster A (red). At LOCPRIOR $K = 3$, majority of individuals in populations P04 belongs to cluster C (green), as well a part of populations P10 and P14, while the distribution of individuals' percentage in cluster A (red) remains almost unchanged.

3.2.4. Spatial Genetics

The correlation between the matrix of $F_{ST}/(1 - F_{ST})$ values and the matrix of natural logarithms of geographic distances (in km) between the analyzed populations was $r = 0.443$ and was highly significant ($P_{Mantel} = 0.0006$). The coefficient of determination was $R^2 = 0.196$, revealing that 19.6% of the genetic differentiation between the analyzed populations can be explained by their spatial distance (Supplement S5).

3.2.5. Correlation with SNP and Quantitative Traits Data

We found no significant correlation between the node sizes for all analyzed SNPs and F_{IS} for nSSR data, but performing the same correlation only for non-outlier loci (Figure 3 in [10]), which are also expected to represent neutral diversity, we obtained statistically significant positive correlation ($R = -0.563$, $p < 0.05$) (Supplement S7). The negative correlation with F_{IS} means that the populations showing possible signs of inbreeding also had narrower intrapopulation genetic variance for non-outlier SNPs.

F_{ST} matrices for SNP and SSR data (for all SNPs, both outlier and non-outlier, as available from Temunović et al. [10]) were significantly correlated ($R = 0.682$, $p < 0.0001$).

Considering the quantitative traits data, the height was not correlated with any of the SSR parameters. H_E was also not correlated with any of the quantitative traits, nor was the N_a.

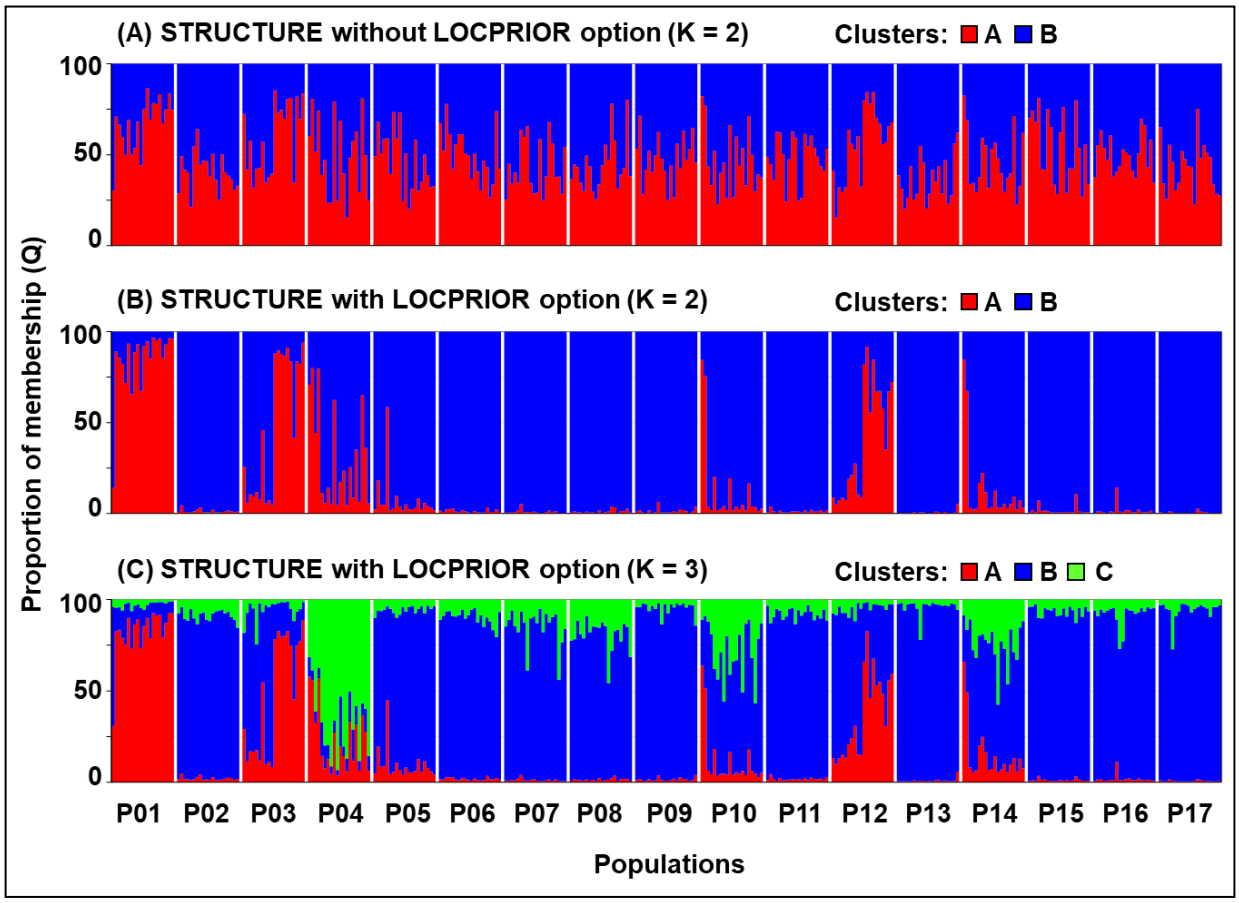

Figure 3. Genetic structure of 17 pedunculate oak (*Quercus robur* L.) populations in Croatia derived from Bayesian analysis using STRUCTURE (**A**) without LOCPRIOR option at $K = 2$, (**B**) with LOCPRIOR option at $K = 2$, and (**C**) with LOCPRIOR option at $K = 3$. Each individual is represented by a column, and the color corresponds to the percentage of membership (Q-values) of the individual belonging to a particular genetic cluster.

However, for all three years there was a significant positive correlation of H_O and for years 6 and 7 negative correlations of F_{IS} with standard deviation of flushing phenology (Supplement S7). This means that populations with higher F_{IS} i.e., lower H_O were significantly more uniform in flushing phenology (timing of bud burst), which is generally well correlated with flowering.

In the first measurement, after the establishment of the trial, H_O was not significantly correlated with populations' survival means and standard deviations, but in two consequent years their negative (means) i.e., positive (Std) correlation with H_O (year 6 ($p < 0.05$) and 7 ($p < 0.01$)) became significant and increased with age.

4. Discussion

4.1. Chloroplast Microsatellites

The chloroplast chromosome is non-recombinant, haploid and in angiosperms mostly inherited from the maternal line. Thus, chloroplast markers are transferred to new generations by seed. Such inheritance greatly contributes to the study of postglacial migration pathways, i.e., the recolonization pathways of species after the last glaciation [53]. Comprehensive research of chloroplast diversity of the main European oak species from 2613 populations was conducted with PCR-RFLP analysis. Since cpRFLP analysis is complicated, laborious and sometimes has replicability issues, the cpSSR markers were developed and comparatively tested with cpRFLP markers in the same populations. It was found that the results match, if not more precisely with cpSSRs, with resolution increasing with added

cpSSR loci [29]. In this study we used cpSSRs to analyze the haplotype diversity of Croatian populations and tried to infer the origin of populations from the nearest glacial refugia.

The differences between G_{ST} and N_{ST} values and between G_{ST} and R_{ST} values were not statistically significant. This leads to the conclusion that there is diminished phylogeographic structure of the populations. The chloroplasts and chloroplast markers are inherited by the maternal line, i.e., the seeds, we assume that these results indicate an anthropogenic impact by transferring genetic material within the pedunculate oak area of distribution in Croatia, or that some of the analyzed seed stands are not, in the true sense of the idea, of natural origin.

Diversity among populations based on cpSSR haplotypes has proven to be highly statistically significant, suggesting genetic differentiation between populations.

The total genetic diversity (h_T) estimated from 9 cpSSRs and 325 individuals for all populations was quite high (h_T = 0.953) [15]. This is similar to results in the study of Katičić Bogdan et al. [54] where the total diversity of clonal seed orchards, h_T, is 0.945. Other parameters, average within-population diversity, total diversity, and genetic differentiation, are comparable between these two studies. A smaller discrepancy in value is evident when using the ordered allele method, considering the genetic similarities between haplotypes, i.e., the proportion of common alleles [25]. In this study there was no statistically significant differences for three different methods of genetic differentiation, while Katičić Bogdan et al. [54] report statistically significant differences at the level 0.05–0.01 for the N_{ST} value, thus confirming genetic differentiation between plantations. Both studies have higher values of diversity over genetic differentiation.

Slade et al. [55] used a restriction fragment length polymorphism for the analysis of four chloroplast DNA sections (cpRFLP). Due to the method used, the data are not compatible for comparison, but the average intra-population diversity for oak in that study was 0.200, while in the present study h_S is 0.760 [15]. Genetic differentiation also differed and was much higher in the Slade et al. study [55] G_{ST} = 0.717, and here G_{ST} = 0.203 [15].

A study of pedunculate oak in Poland [56] identified 67 haplotypes on 3938 individuals with 14 cpSSRs. The effective number of haplotypes was n_E = 5.543, and the diversity of haplotypes was H_E = 0.820. These results can be compared with the results obtained in this study for the total average of all individuals for all loci (n_E = 4.702 and H_E = 0.760—Table 1). Expected values are slightly lower due to the smaller sample size than in the study by Chmielewski et al. [56].

Deguilloux et al. [18] showed that a comparison of different chloroplast markers (chloroplast restriction fragment length polymorphisms (cpRFLP) and chloroplast microsatellites (cpSSR)) is possible. Likewise, in a recent study, Chmielewski et al. [56] state that, despite the greater number of haplotypes and the greater polymorphism of cpSSRs, the cpRFLPs can be clearly compared and that cpSSRs can be classified into three major lineages originating from glacial refugia [57–59].

In the study of Slade et al. [55], seven haplotypes and five cpRFLP subtypes for the Central Balkan region were found. As the cpRFLPs are less polymorphic than the cpSSRs, it was expected that the number of haplotypes will be significantly smaller, but that the origin is the same (three main glacial refugia). According to the authors, different recolonization lines (Balkan and Apennine) are encountered in the territory of Croatia. Large number of haplotypes detected in this study confirm the large cpSSR diversity expected in areas close to the refugia, where different lineages encounter each other (n = 66, Supplement S6). Katičić Bogdan et al. [54] found 28 haplotypes in 124 individuals in the narrower part of the core distribution in Croatia, which also confirms the expected larger diversity compared with the core of overall pedunculate oak distribution.

The median-joining network of haplotypes (Figure 3A), and neighbor-joining tree (Figure 3B), separate 66 detected haplotypes into three lineages. The distribution of maternal lineages across the studied populations, shown in Figure 3C, indicates that line L2, which also contains the largest number of haplotypes (Figure 3A,B), extends from east to west, line L1 is most represented in the western part of the pedunculate oak distribution in Croatia,

while L3 occupies only the central part of the distribution range. Compared with the study of Katičić Bogdan et al. [54] the lineages L1 and L2 from that study are pooled in L2 in this study, L3 is L1 and L4 is L3.

AMOVA of haplotypic diversity in populations showed highly statistically significant diversity among populations, with interpopulation variance of 30% (Table 3). AMOVA of haplotypic diversity for maternal lineages showed the high value of intra-lineage diversity of 54%, which further supports the allocation of haplotypes to the three lineages.

According to Petit et al. [57] and Slade et al. [55], 6 cpRFLP haplotypes of pedunculate oak are found in Croatia; 17, 2 and 5 belong to the Italian peninsula refugium; and 4, 6 and 7 to Balkan refugium. Our lineage L1 could be connected to haplotype 2, originating from the Italian peninsula and lineage L2 with haplotype 5 also from Italy. L3 could be connected to haplotype 6, originating from the Balkan peninsula, but because of the great admixture of different cpRFLP haplotypes it is hard to exactly delineate their distribution in cpSSR lineages.

Although AMOVA for distribution of nSSR diversity between cpSSR lineages showed statistically significant inter-lineage variance, this component was only 0.38% (Table 3—among maternal lineages) and therefore shows no signs of real connection between the two genomes.

In oaks there is a pronounced asymmetry in gene flow by pollen compared with the seed. Pollination is carried out by the wind, while relatively large and heavy seeds can be transmitted over long distances mainly by animals (primarily birds) or humans. Studies have shown that pollen migration is approximately 200 times more effective than seed migration [57,60–62]. This difference is visible in the limited population differentiation for nuclear biparentally inherited markers. Meanwhile, differentiation for cytoplasmic, maternally inherited markers (such as chloroplast markers in angiosperms) is more noticeable. The restricted seed migration has likely contributed to the formation of spatial patterns, but any potential patterns at neutral nuclear loci are presumably largely erased due to the consistent gene flow through pollen and/or by selection. In the case of these particular populations, the lack of congruence between the two genomes' diversity was further confirmed by the study of Temunović et al. [10]. Comparing SNP and cpSSR variation and finding no effects of shared ancestry owing to the species' postglacial history within the sensitive population network obtained by cGD approach, the authors have suggested that gene flow since the initial establishment of the investigated populations has probably played a much bigger role than shared ancestry in shaping populations' current genetic variation.

4.2. Nuclear Microsatellites

The investigation of the molecular genetic diversity of the pedunculate oak has been substantial, particularly in central Europe, as demonstrated by numerous studies [60,63–73]. Collectively, these studies reveal significant intrapopulation diversity, with much lower interpopulation differentiation. Molecular studies on pedunculate oak within Croatia have been limited with several contributions to the field [10,54,55,74–78].

Simple sequence repeats (SSRs), also known as microsatellites, are short repetitive sequences found throughout eukaryotic genomes. Nuclear SSRs (nSSRs) are used to detect genetic variability within individuals and populations [21], are codominant, easy to genotype, highly polymorphic, and suitable for studying population structures [79,80]. The range lengths of the amplified fragments for microsatellite loci (Supplement S1) are consistent with the references in the literature they were sourced from [20–22]. The genetic diversity (expected heterozygosity) for the *ssrQrZAG7* in this study (H_E = 0.849) is in the middle of the expected heterozygosity range reported by Neophytou et al. [65], while it is very high compared with Steinkellner et al. (H_E = 0.65) [21]. *SsrQpZAG9* has higher H_E values (0.872) than those in Neophytou et al. [65] but lower than those in Steinkellner et al. [21]. The expected heterozygosity values in this study for the *ssrQrZAG11*, *ssrQpZAG16*, and *ssrQrZAG101* are in the middle of the values reported for these loci in Neophytou et al. [65], while they were higher for the *ssrQrZAG30*, *ssrQrZAG87*, *ssrQrZAG96*, and *ssrQrZAG112*. Kesić et al. [9] have reported similar but slightly lower values for diversity when con-

sidering shared loci (*ssrQpZAG9*, *ssrQrZAG11*, *ssrQrZAG30*, *ssrQrZAG87*, *ssrQrZAG96*, *ssrQrZAG101*, *ssrQrZAG112*, and *MSQ13*).

Overall genetic diversity parameters, averaged for all 17 populations (H_E = 0.828, N_{av} = 11.96) were similar to other studies of pedunculate oak in Europe (Netherlands, Germany, Bulgaria, France, Belgium, Serbia etc.) [9,65,81,82], at the higher margin of usually observed values. It has been previously observed that these values are quite similar throughout the pedunculate oak range and that, for pedunculate oak in general, convincing evidence for intraspecific genetic structure is rarely found at neutral loci. In accordance with these findings, and in the case of our study, there was virtually no geographic structure shown in these Croatian populations, i.e., some weak structure was found only at K = 3 with the LOCPRIOR option (Figure 3). The low genetic differentiation within this species, which is widespread in Europe, is not surprising. Even if the forests of pedunculate oak are not continuous, large distance gene flow, current or historical, might account for a high degree of genetic exchange among populations resulting in a rather homogenous gene pool, compared with some other species. Additionally, at the neutral nSSR loci used in our study, as well as those in used in the abovementioned studies, their effect on genetic variation as a result of adaptation is almost never found, regardless of whether there were large study areas with various site conditions [67].

Although generally peripheral populations tend to show higher differentiation than those from core distribution [9,10], this was not the case in our study. The average F_{ST} value between our populations (0.013) was even smaller than reported for central European populations (0.024) [53]. The difference is even more pronounced compared with other peripheral populations. Kesić et al. [9] have reported an average F_{ST} of 0.032 between seven Serbian populations, Ballian et al. [83] 0.051 for twelve populations in Bosnia and Herzegovina and Craciunesc et al. [84] 0.45 for four populations in Romania. In this study, significant differentiation was observed for 50 out of 136 population pairs, but mostly between the most isolated P01 population, Buzet, and all but one other population and between more western and more eastern populations. Two more eastern populations had almost no significant differentiation amongst themselves, while the more western were also significantly differentiated amongst each other (Supplement S2). The crucial equalizing factor in more eastern populations was gene flow by pollen, as the predominantly low topography and sufficient continuity of the pedunculate oak's complexes in this area provided a corridor for gene transfer between undifferentiated populations. Namely, the area between the Drava, Sava, and Danube rivers, where the more eastern analyzed populations are located, belonged to the Military Frontier until the second half of the 19th century, which spared it from the intensive and unplanned exploitation that led to the fragmentation of many other oak forests in Europe [10,54]. Therefore, historically, these populations were certainly part of the same connected complex. However, the powerful among-population network analysis based on the cGD approach [85] in SNP analysis of the same populations indicated that the populations in the lower Drava and Sava basins experience reduced levels of genetic connectivity relative to neutral IBD expectations [10], which could be related to more contemporary events.

The proportion of variance between populations (AMOVA, Table 3) was statistically significant but amounted to only 1.47%. This was mostly driven by the differentiation of other populations with P01 Buzet and the other two most western populations, as, in Katičić Bogdan et al. 2018 [54], where seed regions from the core of the pedunculate oak distribution in Croatia were analyzed, between-region proportion was only 0.04% and not significant. The neutral nSSR variance partition of pedunculate oak populations often shows similar values, even at much greater between population distances [69]. Of course, it is also influenced by the number of SSR loci used [83].

Spatial analysis by the method of Rousset [51] revealed that 19.6% of genetic differentiation can be explained by geographic distance while spatial correlation analysis demonstrated that individuals were significantly genetically closer at distances within 40 km, and significantly more genetically distant than random pairs of individuals after

245 km of distance [15]. In Temunović et al. [10], the isolation by distance (IBD) signals for non-outlier SNP loci, which usually follow similar patterns of genetic diversity as those of neutral nSSR loci, explained 8% of the genetic variation.

As mentioned, as the samples were collected in the genetic trial, one of our goals was to complement the results of the previous analysis of quantitative traits in the trial [15,16], as well as the results of the study by Temunović et al. [10] (with SNPs selected from candidate genes from the same DNA samples), with data on presumably neutral genetic diversity.

Considering the correlation of SNP data with our F_{IS}, we found no significant correlation between intrapopulation variance of all SNP loci and F_{IS}; however, when performing the same correlation only for non-outlier loci, which are also expected to represent neutral diversity, we obtained statistically significant positive correlation ($R = -0.563$, $p < 0.05$) (Supplement S7). The negative correlation with F_{IS} means that the populations showing possible signs of inbreeding also had narrower intrapopulation genetic variance for non-outlier SNPs.

F_{ST} matrices for SNP and SSR data (for all SNPs, both outlier and non-outlier) were significantly correlated ($R = 0.682$, $p < 0.0001$). Certainly, the correlation would be even higher considering only non-outlier data. A significant correlation between SSR and non-outlier SNP data is expected, as they both represent neutral diversity [86]. Because the correlation of all SNPs genetic variance with F_{IS} was not significant, this further confirms the effects of selection on outlier SNP loci, as discussed by Temunović et al. [10].

In our study, seven populations had significant F_{IS} values. P01 (Buzet) is a completely isolated complex of pedunculate oak in Istria and we presume that this is the reason for increased F_{IS} values. P03 (Karlovac 2) is an isolated stand surrounded by agricultural land, although not very far from the main complex to which P02 (Karlovac 1) belongs. However, looking at the cpSSR data from this research, it is clear that this stand was established with reproductive material from the eastern complex, as almost all the sampled individuals belong to maternal lineage 2, with a much smaller percentage of lineage 1 individuals, mainly found in all of the more western populations in this study, including P02. Therefore, we presume that, in this case, deviation from HWE is due to the different origin than the surrounding stands, possibly causing reproductive isolation from the main complex. This is similar to Germany, where indigenous stands of pedunculate oak grow side by side with introduced Slavonian oak stands but sustain separate gene pools, mainly due to phenological differences [7]. In the case of P08 (Lipovljani) we think that the lower H_O might be the result of sampling. Those pedunculate oak stands belong to a very large complex and it seems unlikely that they would exhibit deviation from HWE, although this entire area has suffered great oak dieback in recent decades [87]. In this particular case, the 20 mother tree samples were taken from three different parts of the complex, approximately 10 km distance by air apart from each other, each part being on the edge of the complex. We propose that it is possible that such sampling resulted in artificial homozygotes excess caused by subpopulation sampling; the so-called Wahlund effect. Only for this population is the $P_{Bottleneck}$ value low (0.313) when compared with other populations with significant F_{IS} values, whose $P_{Bottleneck}$ values are mostly very high (Table 2). P11 (Požega) is situated in the Forest management unit in Požega basin, where pedunculate oak stands come in small patches surrounded by agricultural land. The entire basin is surrounded by hills with sessile oak stands, presenting a probable gene flow barrier to other pedunculate oak complexes in Slavonia. We presume this is the reason for the increased F_{IS} in this population. Population P13 (Koška) has been reported to be in very bad condition with intensive dieback, probably due to a decrease in underground water. Additionally, it is situated on the lowest edge of the complex, surrounded by agricultural land, which might be the reason for the decreased heterozygosity. Populations P10 (Stara Gradiška) and P16 (Gunja) are both situated in Sava catchment, where pedunculate oak is mixed with narrow leaved ash and are both late flushing populations [16]. P16 is situated at the lower tail of the Spačva complex and F_{ST} values for both SSRs and SNPs show no differentiation with the closest populations P15 and P17, nor the other populations in the eastern part. However, Temunović et al. [10]

used a conditional graph distance (cGD) approach derived from population networks to establish patterns of differentiation, retaining only connections between populations that are due to significant conditional genetic covariance. This approach aims to correct the false impression of ongoing gene flow between populations whose low F_{ST} values are in fact the result of ancestry from the same mainland [85]. In the graph topology such connections are lost, which in this case was demonstrated for the relationship between P16 and P17 (P15 was not included in the Temunović study), as it was for the next closest, P12 (Trnjani), for the non-outlier SNP loci that are compatible with SSRs. P16 is a very late flushing population, which could be the reason for its reproductive isolation and increased H_O. The part of the forest complex where P16 is situated is frequently flooded in one part of the year, while at the same time it experiences high temperatures and extreme drought at the other. It is an exact example of the floodplain habitat that subjects oak populations to stronger selective pressures than those in many other environments within its range. This is due to the extreme and frequent spatiotemporal variation in water availability that may mimic some of the extreme conditions predicted in climate change scenarios for Central Europe, such as increases in heavy precipitation intensity and drought risk. Although it demonstrates narrowed intrapopulation variance both at SSR and non-outlier SNP loci, it harbors wider than average intrapopulation variance at outlier loci, which are affected by selection [10]. The reasons for increased F_{IS} in P10 (Stara Gradiška) could be similar to those of P16 but could also have been partially caused by a great dieback of oak in this area, recorded between 1910 and 1925 [88], around the time of the P10 establishment.

Attempting to establish connections between SSR and quantitative data and explore whether the narrowed neutral diversity is somehow reflected in the diversity of quantitative traits, we correlated N_a, H_O, H_E and F_{IS} values with populations' arithmetic means, as well as standard deviations for several quantitative traits. We found no significant correlation with growth (height), which is not unusual as height is a trait heavily influenced by environment, especially at a young age.

However, for all three years there was a significant positive correlation of H_O and for years 6 and 7 negative correlations of F_{IS} with standard deviation of flushing phenology i.e., populations with higher F_{IS} and lower H_O were significantly more uniform in flushing phenology (timing of bud burst), which is generally well correlated with flowering. This trend of greater phenological uniformity of the stands with lower H_O could point to the partial reproductive isolation of some of the affected populations due to phenological incompatibility with nearby stands, therefore forming a more unified phenological structure within those populations.

We also found significant correlation between H_O and survival in years 6 and 7 and the correlation showed a rising trend with trial age. It has been noted that inbreeding depression causes weak survival of inbred individuals in oak, contributing to weaker geographic structure [61,89]. Our result brings additional evidence that decreased heterozygosity detectable at neutral loci implies lower survival abilities and should be taken in strong consideration in choosing forest reproductive material for forest stand regeneration. This is especially important in the context of climate change. According to some experimental studies, climate change-induced drought stress may exacerbate the detrimental genetic consequences of heterozygosity loss, as the response to low levels of individual heterozygosity is stronger under environmental stress than under optimal conditions. In the study of Vranckx [90] heterozygosity–fitness correlations were examined by correlating the recorded traits of individual seedlings to their multi-locus heterozygosity (MLH), assessed by nSSRs and by studying their response to drought stress. Weak but significant effects of MLH on several fitness traits were found, which were stronger for transpiration variables than for the recorded growth traits. This was also the case in our study, where we found no correlation of heterozygosity and height. The authors therefore stress the necessity to maximize individual multi-locus heterozygosity in forest tree breeding programs.

4.3. Current State and Recommendations

Genetic diversity of pedunculate oak stands in Croatia is shaped by a number of various factors. Analysis by different approaches in this genetic trial has given us an insight into how these factors contributed to different aspects of diversity. In this study we used nSSRs to analyze the neutral diversity and population dynamics and cpSSRs for postglacial recolonization dynamics and possible influences of anthropogenic transfer of forest reproductive material. The same samples were analyzed before with SNPs from candidate genes connected to drought stress, water availability and similar factors connected to climate change. Additionally, in this trial a number of quantitative traits were measured over years, which altogether gave us the chance to compare the results of different approaches on the same samples.

Although the distribution of pedunculate oak in Croatia represents peripheral populations at the southern-range margin, contrary to a number of studies indicating stronger differentiation between such populations, Croatian populations are weakly differentiated, partly due to the inclusion of most of the valuable complexes in the Military Frontier until the second half of the 19th century, which spared them from intensive and unplanned exploitation that led to the fragmentation of many other oak forests in Europe. Additionally, pedunculate oak in this area formed large complexes in general, as opposed to the smaller and fragmented populations often associated with range margins.

As these populations are situated near the refugia and in the area where different recolonization routes encountered each other, there is a hotspot of diversity that is still recognizable today by its pronounced haplotypic richness. However, as proven by comparison of both nSSR and SNP with cpSSR analysis, original recolonization patterns of nuclear diversity were erased by gene flow and the possible effects of selection. Signals of selection were visible on outlier SNP loci with very small congruence between the population networks based on positive outliers vs. non-outliers, indicating that they had been driven by different evolutionary processes. Positive outliers may have been at least partly shaped by local selection and non-outliers, with nSSRs mostly shaped by neutral demographic processes that contributed less to the shaping of outlier diversity. Analyses of quantitative adaptive traits have also shown signals of local adaptation, providing additional important information for gene conservation and management, while their connection to neutral diversity has demonstrated the negative effects of decreased heterozygosity on the ultimate indicator of fitness, which is survival.

The specific selection pressures present in these floodplain populations possibly predispose their adaptive diversity for climate change effects in the more western and northern parts of Europe, which is why some countries there are considering Slavonian oak as a means with which to mitigate climate change. Because of its widely recognized quality it has already been historically and extensively planted in other countries and used for high quality products and this potential adaptational advantage makes it even more desirable.

But what about home? Slavonian and generally Croatian floodplain populations of pedunculate oak are faced with many challenges in their natural habitat, first and foremost climate change, negatively effecting many species at the southern margin of distribution. Climate extremes, changes in groundwater level, regulation of rivers and pests are putting pressure on the populations, affecting their fitness and regeneration, therefore directly calling the future structure of these populations into question. Pronounced oak dieback has been present in this area throughout the last century and has particularly quickened its pace since the 1980s [88]. The latest extreme event, the supercell storm in July 2023, caused major damage to the most valuable stands in Spačva basin, destroying a number of trees and causing unforeseeable problems with the natural regeneration of these stands. Both nSSRs and non-outlier SNPs indicated the possibility that intensive geneflow by long-distance pollen dispersal, which minimizes phenological mismatches between populations, might be decreased compared with the past. This could possibly be affected by fragmentation after the Military Frontier or by flowering problems due to environmental challenges. This is not detectable by F_{ST} but was partially caught by population graphs and hinted at by the

significant F_{IS} values in certain populations where we did not expect signs of inbreeding, considering the mainly flat topology of the terrain. There was significant correlation between the level of observed heterozygosity and survival in the trial, at the early age of 5 and 6, pointing to the importance of introducing genetically diverse reproductive material into stands in lieu of the increasingly frequent absence of natural regeneration. The lack of crops represents one of the greatest challenges in managing oak stands in Croatia [91]. Results from genetic trials established with the same populations and families, but in more challenging environmental conditions [92] (later partially confirmed by SNP analysis) suggest an ecotypic pattern of the adaptive genetic differentiation among studied populations and indicate a possible problem even in the transfer of western Croatian populations' reproductive material to the eastern part, a result of their adaptability to wetter habitats. Taking into consideration the fact that, even within the country, problems with maladaptation may occur with west-to-east transfer, extreme caution should be taken when discussing the import of oak reproductive material from outside sources. Even if the outsourced reproductive material is taken from compatible habitats and is able to adapt to local conditions in Croatia, a toll would certainly be paid in the decreased quality of timber compared with the well-recognized quality of Slavonian oak. Thus, emphasis should be put on ensuring the best regeneration by local sources by careful handling of local reproductive material in order to maximize the successful utilization of collected crops and survival of progeny in the stands. Special care should be taken to ensure the broad base of genetic diversity for forest regeneration, which also means using the crops from different years and optimizing storage techniques to preserve the quality of seed. Greater effort should be placed in the nursery production of saplings from the collected crops, especially those of higher genetic quality, collected in three clonal seed orchards of pedunculate oak. Increased regeneration by saplings should complement the prevalent regeneration by scattering acorns. As it is no longer realistic to expect the full crop every few years, a comprehensive change in the treatment of the available resources is necessary to preserve the viability of reproductive material. Additionally, in order to minimize the detrimental effect of extreme events such as destructive storms, there may be a need to change the established management practices and switch to cuttings and regeneration on smaller surfaces. These are all great challenges, of both technical and logistical natures, but certainly it is becoming impossible to avoid them, considering the changed conditions that the forests are facing. The main goal should be the conservation of their sustainability and adaptability. The existing clonal seed orchards should be extensively used as polygons for monitoring the influence of various factors on acorn yield and should be properly managed in order to maximize production of high-quality reproductive material and act as valuable ex-situ conservation units. The in-situ gene conservation units (registered seed stands) that demonstrated signs of inbreeding should be reconsidered and replaced by more appropriate ones. Our research, as well as some previous research on the importance of multi-locus heterozygosity points to the importance of choosing appropriate stands as seed sources to ensure long term survival and adaptability of future stands.

This study and other studies by the same and other authors [10,15,16,92] underline the importance of genetic trials and different approaches to studying their genetic diversity in order to ensure a scientific background for gene conservation and management in challenging conditions.

5. Conclusions

This study investigated the genetic diversity of Croatian pedunculate oak populations in a genetic trial, employing nSSR and cpSSR loci to analyze neutral diversity, population structure and post-glacial recolonization. Genetic diversity of both nSSR and cpSSR loci was high and most pronounced at intra-population level. The weak differentiation among Croatian populations is attributed to topography and historical protection in the Military Frontier until the 19th century, preventing intensive exploitation. Large oak complexes and recolonization near refugia contributed to a diversity hotspot, but original recolonization

patterns at nSSR loci were obscured by gene flow and selection. Diversity parameters at nSSR loci were partly correlated to diversity of SNPs from candidate genes as well as results from previous studies of quantitative traits in the same genetic trial. Observed heterozygosity was significantly and positively correlated to survival in the trial. Signals of local adaptation are evident in adaptive traits, suggesting potential adaptability to climate change in other parts of Europe. However, in their native habitat, Croatian oak populations face challenges such as climate change, extreme events and pests, impacting fitness and regeneration. Oak dieback, exacerbated by a 2023 storm, poses significant threats, including potential decreases in gene flow and regeneration. The study emphasizes the importance of introducing genetically diverse reproductive material to counteract inbreeding and challenges in managing oak stands. It underscores the need for caution and evidence-based consideration in transferring reproductive material across regions and emphasizes local sourcing for optimal regeneration and survival.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14122290/s1, Supplement S1: S1_Pop_Data; Supplement S2: S2_NullAlleles; Supplement S3: S3_FST; Supplement S4: S4_Evanno_with and without; Supplement S5: S5_Spatial; Supplement S6: S6_Pop_cpSSR_Data; Supplement S7: S7_correlations.

Author Contributions: Conceptualization, M.I. and S.B.; methodology, S.B.; software, Z.Š.; validation, S.B. and Z.Š.; formal analysis, M.P. and I.K.B.; investigation, M.P. and I.K.B.; resources, M.I.; data curation, Z.Š.; writing—original draft preparation, M.P. and I.K.B.; writing—review and editing, F.V. and Z.Š.; visualization, F.V.; supervision, S.B.; funding acquisition, M.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was part of scientific project MZOS 024-0242108-2099—"Breeding and forestry seed production" (Ministry of Science, Education and Sports of the Republic of Croatia), scientific research project "Mass and individual selection, establishment and evaluation of seed facilities" (Croatian Forests Ltd. Zagreb) and the scientific research project "Testing of seed stands of pedunculate (*Quercus robur* L.) by comparative genetic tests" (TEST HR) (Croatian Forests Ltd. Zagreb, Forest Management Karlovac). As part of these projects, genetic test was established and data were collected. The work was also co-financed by the Croatian Science Foundation within project Conservation of genetic resources of forest tree in light of climate changes (8131) during which collected data were analyzed. The authors are grateful for the financial support.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to large data set on nucSSR. The data presented in this study are available in Supplementary Materials (Supplement S6 S6_Pop_cpSSR_Data).

Acknowledgments: We would like to thank all employees of the Croatian Forests Ltd. Zagreb, who participated in the organization and collection of seed material in seed stands. Special thanks to Dubravko Perez and Tomislav Beg from Croatian Forest Research Institute for help in management and measurements in field trial. Thanks to Martina Temunović for useful explanations.

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

References

1. Atkins, K.E.; Travis, J.M.J. Local Adaptation and the Evolution of Species' Ranges under Climate Change. *J. Theor. Biol.* **2010**, *266*, 449–457. [CrossRef] [PubMed]
2. Valladares, F.; Matesanz, S.; Guilhaumon, F.; Araújo, M.B.; Balaguer, L.; Benito-Garzón, M.; Cornwell, W.; Gianoli, E.; van Kleunen, M.; Naya, D.E.; et al. The Effects of Phenotypic Plasticity and Local Adaptation on Forecasts of Species Range Shifts under Climate Change. *Ecol. Lett.* **2014**, *17*, 1351–1364. [CrossRef] [PubMed]
3. Croatian Forests Ltd. Forests in Croatia. Available online: https://www.hrsume.hr/sume/sume-u-hrvatskoj/ (accessed on 11 September 2023).
4. Rauš, Đ. Forest Associatio of Pedunculate Oak. In *Pedunculate Oak (Quercus robur L.) in Croatia*; Klepac, D., Ed.; Croatian Academy of Sciences and Arts, Croatian Forests: Zagreb, Croatia, 1996; pp. 27–54.
5. White, T.L.; Adams, W.T.; Neale, D.B. *Forest Genetics*; CABI: Wallingford, UK, 2007.
6. Vukelić, J.; Mikac, S.; Baričević, D.; Bakšić, D.; Rosavec, R. *Šumska Staništa i Šumske Zajednice u Hrvatskoj Nacionalna Ekološka Mreža*; State Department for Nature Protection: Zagreb, Croatia, 2008; ISBN 978-953-7169-42-8.

7. Burger, K.; Gailing, O. Genetic Variability of Indigenous (*Quercus robur* L.) and Late Flushing Oak (*Quercus robur* L. Subsp. Slavonica (Gáyer) Mátyás) in Adult Stands Compared with Their Natural Regeneration. *Eur. J. For. Res.* **2022**, *141*, 1073–1088. [CrossRef]
8. Burger, K.; Müller, M.; Rogge, M.; Gailing, O. Genetic Differentiation of Indigenous (*Quercus robur* L.) and Late Flushing Oak Stands (Q. Robur L. Subsp. Slavonica (Gáyer) Mátyás) in Western Germany (North Rhine-Westphalia). *Eur. J. For. Res.* **2021**, *140*, 1179–1194. [CrossRef]
9. Kesić, L.; Cseke, K.; Orlović, S.; Stojanović, D.B.; Kostić, S.; Benke, A.; Borovics, A.; Stojnić, S.; Avramidou, E.V. Genetic Diversity and Differentiation of Pedunculate Oak (*Quercus robur* L.) Populations at the Southern Margin of Its Distribution Range—Implications for Conservation. *Divers* **2021**, *13*, 371. [CrossRef]
10. Temunović, M.; Garnier-Géré, P.; Morić, M.; Franjić, J.; Ivanković, M.; Bogdan, S.; Hampe, A. Candidate Gene SNP Variation in Floodplain Populations of Pedunculate Oak (*Quercus robur* L.) near the Species' Southern Range Margin: Weak Differentiation yet Distinct Associations with Water Availability. *Mol. Ecol.* **2020**, *29*, 2359–2378. [CrossRef] [PubMed]
11. Lindner, M.; Maroschek, M.; Netherer, S.; Kremer, A.; Barbati, A.; Garcia-Gonzalo, J.; Seidl, R.; Delzon, S.; Corona, P.; Kolström, M.; et al. Climate Change Impacts, Adaptive Capacity, and Vulnerability of European Forest Ecosystems. *For. Ecol. Manag.* **2010**, *259*, 698–709. [CrossRef]
12. Lindner, M.; Fitzgerald, J.B.; Zimmermann, N.E.; Reyer, C.; Delzon, S.; van der Maaten, E.; Schelhaas, M.J.; Lasch, P.; Eggers, J.; van der Maaten-Theunissen, M.; et al. Climate Change and European Forests: What Do We Know, What Are the Uncertainties, and What Are the Implications for Forest Management? *J. Environ. Manag.* **2014**, *146*, 69–83. [CrossRef]
13. Kleinschmit, J. Intraspecific Variation of Growth and Adaptive Traits in European Oak Species. *Ann. Sci. For.* **1993**, *50*, 166s–185s. [CrossRef]
14. Popović, M.; Ivanković, M.; Bogdan, S. Variability of Height Growth and Survival of Progenies from Pedunculate Oak (*Quercus robur* L.) Seed Stands at the Field Trial 'Jastrebarski Lugovi'–First Results. *Šumarski List. Znan. Staleško Glas. Hrvat. Šumarskog Društva* **2014**, *138*, 155–165.
15. Morić, M. Genetska Raznolikost Hrasta Lužnjaka (*Quercus robur* L.) u Pokusnim Nasadima s Potomstvom Iz Odabranih Sjemenskih Sastojina. Ph.D. Thesis, University of Zagreb, Faculty of Forestry and Wood Technology, Zagreb, Croatia, 2016.
16. Morić, M.; Bogdan, S.; Ivanković, M. Kvantitativna Genetska Diferencijacija Populacija Hrasta Lužnjaka (*Quercus robur* L.) u Pokusnom Nasadu »Jastrebarski Lugovi. *Nov. Meh. Šumarstva Časopis Teor. Praksu Šumarskoga Inženjerstva* **2018**, *39*, 35–45.
17. Gradečki-Poštenjak, M.; Novak Agbaba, S.; Licht, R.; Posarić, D. Dynamics OfAcorn Production and Quality of English OakAcorn (*Quercus robur* L.) in Disrupted Ecological Conditions. *Šumarski List* **2011**, *135*, 169–180.
18. Deguilloux, M.F.; Pemonge, M.H.; Petit, R.J. Use of Chloroplast Microsatellites to Differentiate Oak Populations. *Ann. For. Sci.* **2004**, *61*, 825–830. [CrossRef]
19. Weising, K.; Gardner, R.C. A Set of Conserved PCR Primers for the Analysis of Simple Sequence Repeat Polymorphisms in Chloroplast Genomes of Dicotyledonous Angiosperms. *Genome* **1999**, *42*, 9–19. [CrossRef] [PubMed]
20. Kampfer, S.; Lexer, C.; Glössl, J.; Steinkellner, H. Characterization of (GA)n Microsatellite Loci from *Quercus robur*. *Hereditas* **1998**, *129*, 183–186. [CrossRef]
21. Steinkellner, H.; Fluch, S.; Turetschek, E.; Lexer, C.; Streiff, R.; Kremer, A.; Burg, K.; Glössl, J. Identification and Characterization of (GA/CT)n-Microsatellite Loci from Quercus Petraea. *Plant Mol. Biol.* **1997**, *33*, 1093–1096. [CrossRef]
22. Dow, B.D.; Ashley, M.V. Microsatellite Analysis of Seed Dispersal and Parentage of Saplings in Bur Oak, Quercus Macrocarpa. *Mol. Ecol.* **1996**, *5*, 615–627. [CrossRef]
23. Guichoux, E.; Lagache, L.; Wagner, S.; Chaumeil, P.; Léger, P.; Lepais, O.; Lepoittevin, C.; Malausa, T.; Revardel, E.; Salin, F.; et al. Current Trends in Microsatellite Genotyping. *Mol. Ecol. Resour.* **2011**, *11*, 591–611. [CrossRef]
24. Eliades, N.-G.H.; Eliades, D.G. *HAPLOTYPE ANALYSIS: Software for Analysis of Haplotype Data*; Georg-August University Goettingen: Goettingen, Germany, 2009.
25. Pons, O.; Petit, R.J. Measuring and Testing Genetic Differentiation with Ordered versus Unordered Alleles. *Genetics* **1996**, *144*, 1237. [CrossRef]
26. Nei, M. *Molecular Evolutionary Genetics*; Columbia University Press: New York, NY, USA, 1987; ISBN 0231063210.
27. Slatkin, M. A Measure of Population Subdivision Based on Microsatellite Allele Frequencies. *Genetics* **1995**, *139*, 457–462. [CrossRef]
28. Excoffier, L.; Lischer, H.E.L. Arlequin Suite Ver 3.5: A New Series of Programs to Perform Population Genetics Analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef] [PubMed]
29. Bandelt, H.J.; Forster, P.; Röhl, A. Median-Joining Networks for Inferring Intraspecific Phylogenies. *Mol. Biol. Evol.* **1999**, *16*, 37–48. [CrossRef] [PubMed]
30. Leigh, J.W.; Bryant, D. Popart: Full-Feature Software for Haplotype Network Construction. *Methods Ecol. Evol.* **2015**, *6*, 1110–1116. [CrossRef]
31. Bowcock, A.M.; Ruiz-Linares, A.; Tomfohrde, J.; Minch, E.; Kidd, J.R.; Cavalli-Sforza, L.L. High Resolution of Human Evolutionary Trees with Polymorphic Microsatellites. *Nature* **1994**, *368*, 455–457. [CrossRef] [PubMed]
32. Minch, E.; Ruiz-Linares, A.; Goldstein, D.; Feldman, M.; Cavalli-Sforza, L.L. *MICROSAT. A Computer Program for Calculating Various Statistics on Microsatellite Allele Data*; Stanford University: Palo Alto, CA, USA, 1997.
33. Felsenstein, J. *PHYLIP-Phylogeny Inference Package, Version 3.69*; University of Washington: Seattle, WA, USA, 2009.

34. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef] [PubMed]
35. Kalinowski, S.T.; Taper, M.L.; Marshall, T.C. Revising How the Computer Program CERVUS Accommodates Genotyping Error Increases Success in Paternity Assignment. *Mol. Ecol.* **2007**, *16*, 1099–1106. [CrossRef] [PubMed]
36. van Oosterhout, C.; Hutchinson, W.F.; Wills, D.P.M.; Shipely, P. Micro-Checker: Software for Identifying and Correcting Genotyping Errors in Microsatellite Data. *Mol. Ecol. Notes* **2004**, *4*, 535–538. [CrossRef]
37. Chapuis, M.P.; Estoup, A. Microsatellite Null Alleles and Estimation of Population Differentiation. *Mol. Biol. Evol.* **2007**, *24*, 621–631. [CrossRef]
38. Rousset, F. Genepop'007: A Complete Re-Implementation of the Genepop Software for Windows and Linux. *Mol. Ecol. Resour.* **2008**, *8*, 103–106. [CrossRef]
39. SAS Institute Inc. *The SAS System for Windows, Release 9.4*; Statistical Analysis Systems Institute: Cary, NC, USA, 2013; 556p.
40. Luikart, G.; Allendorf, F.W.; Cornuet, J.M.; Sherwin, W.B. Distortion of Allele Frequency Distributions Provides a Test for Recent Population Bottlenecks. *J. Hered.* **1998**, *89*, 238–247. [CrossRef]
41. Peery, Z.M.; Kirby, R.; Reid, B.N.; Stoelting, R.; Doucet-Bëer, E.; Robinson, S.; Vásquez-Carrillo, C.; Pauli, J.N.; Palsboll, P.J. Reliability of Genetic Bottleneck Tests for Detecting Recent Population Declines. *Mol. Ecol.* **2012**, *21*, 3403–3418. [CrossRef] [PubMed]
42. Cornuet, J.M.; Luikart, G. Description and Power Analysis of Two Tests for Detecting Recent Population Bottlenecks from Allele Frequency Data. *Genetics* **1996**, *144*, 2001–2014. [CrossRef] [PubMed]
43. Piry, S.; Luikart, G.; Cornuet, J.-M. BOTTLENECK: A Computer Program for Detecting Recent Effective Population Size Reductions from Allele Data Frequencies. *J. Hered.* **1999**, *89*, 502–503. [CrossRef]
44. Goudet, J. FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *J. Hered.* **1995**, *86*, 485–486. [CrossRef]
45. Excoffier, L.; Smouse, P.E.; Quattro, J.M. Analysis of Molecular Variance Inferred from Metric Distances among DNA Haplotypes-Application to Human Mitochondrial-DNA Restriction Data. *Genetics* **1992**, *131*, 479–491. [CrossRef] [PubMed]
46. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of Population Structure Using Multilocus Genotype Data. *Genetics* **2000**, *155*, 945–959. [CrossRef] [PubMed]
47. Hubisz, M.J.; Falush, D.; Stephens, M.; Pritchard, J.K. Inferring Weak Population Structure with the Assistance of Sample Group Information. *Mol. Ecol. Resour.* **2009**, *9*, 1322–1332. [CrossRef] [PubMed]
48. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the Number of Clusters of Individuals Using the Software STRUCTURE: A Simulation Study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef]
49. Earl, D.A.; Vonholdt, B.M. STRUCTURE HARVESTER: A Website and Program for Visualizing STRUCTURE Output and Implementing the Evanno Method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
50. Kopelman, N.M.; Mayzel, J.; Jakobsson, M.; Rosenberg, N.A.; Mayrose, I. Clumpak: A Program for Identifying Clustering Modes and Packaging Population Structure Inferences across K. *Mol. Ecol. Resour.* **2015**, *15*, 1179–1191. [CrossRef]
51. Rousset, F. Genetic Differentiation and Estimation of Gene Flow from F-Statistics under Isolation by Distance. *Genetics* **1997**, *145*, 1219–1228. [CrossRef] [PubMed]
52. Rohlf, F.J. *NTSYS-Pc: Numerical Taxonomy and Multivariate Analysis System*; Applied Biostatistics, Inc.: Setauket, NY, USA, 2009.
53. Petit, R.J.; Pineau, E.; Demesure, B.; Bacilieri, R.; Ducousso, A.; Kremer, A. Chloroplast DNA Footprints of Postglacial Recolonization by Oaks. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 9996–10001. [CrossRef] [PubMed]
54. Katičić Bogdan, I.; Kajba, D.; Šatović, Z.; Schüler, S.; Bogdan, S. Genetic Diversity of Pedunculate Oak (*Quercus robur* L.) in Clonal Seed Orchards in Croatia, Assessed by Nuclear and Chloroplast Microsatellites. *South-East Eur. For.* **2018**, *9*, 29–46. [CrossRef]
55. Slade, D.; Škvorc, Ž.; Ballian, D.; Gračan, J.; Papeš, D. The Chloroplast DNA Polymorphisms of White Oaks of Section Quercus in the Central Balkans. *Silvae Genet.* **2008**, *57*, 227–234. [CrossRef]
56. Chmielewski, M.; Meyza, K.; Chybicki, I.J.; Dzialuk, A.; Litkowiec, M.; Burczyk, J. Chloroplast Microsatellites as a Tool for Phylogeographic Studies: The Case of White Oaks in Poland. *iForest-Biogeosci. For.* **2015**, *8*, 765. [CrossRef]
57. Petit, R.J.; Brewer, S.; Bordács, S.; Burg, K.; Cheddadi, R.; Coart, E.; Cottrell, J.; Csaikl, U.M.; Van Dam, B.; Deans, J.D.; et al. Identification of Refugia and Post-Glacial Colonisation Routes of European White Oaks Based on Chloroplast DNA and Fossil Pollen Evidence. *For. Ecol. Manag.* **2002**, *156*, 49–74. [CrossRef]
58. Dumolin-Lapègue, S.; Démesure, B.; Fineschi, S.; Le Corre, V.; Petit, R.J. Phylogeographic Structure of White Oaks throughout the European Continent. *Genetics* **1997**, *146*, 1475–1487. [CrossRef]
59. Bordács, S.; Popescu, F.; Slade, D.; Csaikl, U.M.; Lesur, I.; Borovics, A.; Kézdy, P.; König, A.O.; Gömöry, D.; Brewer, S.; et al. Chloroplast DNA Variation of White Oaks in Northern Balkans and in the Carpathian Basin. *For. Ecol. Manag.* **2002**, *156*, 197–209. [CrossRef]
60. Petit, R.J.; Csaikl, U.M.; Bordács, S.; Burg, K.; Coart, E.; Cottrell, J.; Van Dam, B.; Deans, J.D.; Dumolin-Lapègue, S.; Fineschi, S.; et al. Chloroplast DNA Variation in European White Oaks: Phylogeography and Patterns of Diversity Based on Data from over 2600 Populations. *For. Ecol. Manag.* **2002**, *156*, 5–26. [CrossRef]
61. Streiff, R.; Labbe, T.; Bacilieri, R.; Steinkellner, H.; Glossl, J.; Kremer, A. Within-Population Genetic Structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. Assessed with Isozymes and Microsatellites. *Mol. Ecol.* **1998**, *7*, 317–328. [CrossRef]
62. Zanetto, A.; Roussel, G.; Kremer, A. Geographic Variation of Inter-Specific Differentiation between *Quercus robur* L. and Quercus Petraea (Matt.) Liebl. *For. Genet.* **1994**, *1*, 111–123.

63. Kremer, A.; Petit, R.J. Gene Diversity in Natural Populations of Oak Species. *Ann. Sci. For.* **1993**, *50*, 186–202. [CrossRef]
64. Gregorius, H.R.; Degen, B.; König, A. Problems in the Analysis of Genetic Differentiation among Populations—A Case Study in *Quercus robur*. *Silvae Genet.* **2007**, *56*, 190–199. [CrossRef]
65. Neophytou, C.; Aravanopoulos, F.A.; Fink, S.; Dounavi, A. Detecting Interspecific and Geographic Differentiation Patterns in Two Interfertile Oak Species (*Quercus petraea* (Matt.) Liebl. and *Q. robur* L.) Using Small Sets of Microsatellite Markers. *For. Ecol. Manag.* **2010**, *259*, 2026–2035. [CrossRef]
66. Mattila, A.; Pakkanen, A.; Raisio, J.; Vakkari, P. Genetic Variation in English Oak (*Quercus robur*) in Finland. *Silva Fenn.* **1994**, *28*, 251–256. [CrossRef]
67. Neophytou, C.; Gärtner, S.M.; Vargas-Gaete, R.; Michiels, H.G. Genetic Variation of Central European Oaks: Shaped by Evolutionary Factors and Human Intervention? *Tree Genet. Genomes* **2015**, *11*, 79. [CrossRef]
68. Buche, G.; Colas, C.; Fougère, L.; Destandau, E. Oak Species *Quercus robur* L. and Quercus Petraea Liebl. Identification Based on UHPLC-HRMS/MS Molecular Networks. *Metabolites* **2021**, *11*, 684. [CrossRef]
69. Degen, B.; Yanbaev, Y.; Ianbaev, R.; Bakhtina, S.; Tagirova, A. Genetic Diversity and Differentiation among Populations of the Pedunculate Oak (*Quercus robur*) at the Eastern Margin of Its Range Based on a New Set of 95 SNP Loci. *J. For. Res.* **2021**, *32*, 2237–2243. [CrossRef]
70. Degen, B.; Streiff, R.; Ziegenhagen, B. Comparative Study of Genetic Variation and Differentiation of Two Pedunculate Oak (*Quercus robur*) Stands Using Microsatellite and Allozyme Loci. *Hered* **1999**, *83*, 597–603. [CrossRef]
71. Lexer, C.; Heinze, B.; Gerber, S.; Macalka-Kampfer, S.; Steinkellner, H.; Kremer, A.; Glössl, J. Microsatellite Analysis of Maternal Half-Sib Families of *Quercus robur*, Pedunculate Oak: II. Inferring the Number of Pollen Donors from the Offspring. *Theor. Appl. Genet.* **2000**, *100*, 858–865. [CrossRef]
72. Gömöry, D.; Yakovlev, I.; Zhelev, P.; Jedináková, J.; Paule, L. Genetic Differentiation of Oak Populations within the *Quercus robur* /Quercus Petraea Complex in Central and Eastern Europe. *Hered* **2001**, *86*, 557–563. [CrossRef] [PubMed]
73. Kremer, A.; Kleinschmit, J.; Cottrell, J.; Cundall, E.P.; Deans, J.D.; Ducousso, A.; König, A.O.; Lowe, A.J.; Munro, R.C.; Petit, R.J.; et al. Is There a Correlation between Chloroplastic and Nuclear Divergence, or What Are the Roles of History and Selection on Genetic Diversity in European Oaks? *For. Ecol. Manag.* **2002**, *156*, 75–87. [CrossRef]
74. Zoldoš, V.; Littvay, T.; Besendorfer, V.; Jelenić, S.; Lorković, Z.; Papeš, D. *Primjena Citogenetskih i Biokemijskih Analiza u Utvrđivanju Stupnja Oštećenja Šuma Hrasta Lužnjaka*; Rad. Šumarskog Instituta Jastrebarsko: Jastrebarsko, Croatia, 1994; Volume 29, pp. 151–160.
75. Zoldoš, V.; Besendorfer, V.; Littvay, T.; Papeš, D. The Common Oak (*Quercus robur* L.) as a Potential Test Plant for Cytotoxicity Monitoring. *Period. Biol.* **1995**, *96*, 490–492.
76. Besendorfer, V.; Zoldos, V.; Peskan, T.; Krsnik-Rasol, M.; Littvay, T.; Papes, D. Identification of Potential Cytogenetical and Biochemical Markers in Bioindication of Common Oak Forests. *Phyton* **1996**, *36*, 139–146.
77. Slade, D. *Phylogenetic Origin and Distribution of Pedunculate Oak (Quercus robur L.), Sessile Oak (Q. petraea Liebl.), Pubescent Oak (Q. pubescens Thuill.) and Hungarian Oak (Q. frainetto L.) in Croatia*; Rad. Hrvatski Šumarski Institut: Jastrebarsko, Croatia, 1999; Volume 34, pp. 121–131.
78. Slade, D. *Distribucija Haplotipova Hrasta Lužnjaka (Quercus robur L.) u Hrvatskoj*; University of Zagreb: Zagreb, Croatia, 2001.
79. Abdul-Muneer, P.M. Application of Microsatellite Markers in Conservation Genetics and Fisheries Management: Recent Advances in Population Structure Analysis and Conservation Strategies. *Genet. Res. Int.* **2014**, *2014*, 1–11. [CrossRef] [PubMed]
80. Marwal, A.; Gaur, R.K. Molecular Markers: Tool for Genetic Analysis. *Anim. Biotechnol. Model. Discov. Transl.* **2020**, 353–372. [CrossRef]
81. Vranckx, G.; Jacquemyn, H.; Mergeay, J.; Cox, K.; Kint, V.; Muys, B.; Honnay, O. Transmission of Genetic Variation from the Adult Generation to Naturally Established Seedling Cohorts in Small Forest Stands of Pedunculate Oak (*Quercus robur* L.). *For. Ecol. Manag.* **2014**, *312*, 19–27. [CrossRef]
82. Bakker, E.G.; Van Dam, B.C.; Van Eeuwijk, F.A.; Jacobsen, E. Population Genetics of Indigenous *Quercus robur* L. Populations and of Derived Half-Sib Families Has Implications for the Reproductive Management of the Species. *Plant Biol.* **2003**, *5*, 393–399. [CrossRef]
83. Ballian, D.; Belletti, P.; Ferrazzini, D.; Bogunić, F.; Kajba, D. Genetic Variability of Pedunculate Oak (*Quercus robur* L.) in Bosnia and Herzegovina. *Period. Biol.* **2010**, *112*, 353–362.
84. Craciunesc, I.; Ciocirlan, E.; Sofletea, N.; Curtu, A.L. Genetic Diversity of Pedunculate Oak (*Quercus robur* L.) in Prejmer Natural Reserve. *Bull. Transilv. Univ. Brasov. Ser. II For. Wood Ind. Agric. Food Eng.* **2011**, *4*, 15–20.
85. Dyer, R.J. Population Graphs and Landscape Genetics. *Annu. Rev. Ecol. Evol. Syst.* **2015**, *46*, 327–342. [CrossRef]
86. Zhang, J.; Yang, J.; Lv, Y.; Zhang, X.; Xia, C.; Zhao, H.; Wen, C. Genetic Diversity Analysis and Variety Identification Using SSR and SNP Markers in Melon. *BMC Plant Biol.* **2023**, *23*, 39. [CrossRef] [PubMed]
87. Teslak, K.; Čavlović, J.; Božić, M.; Beljan, K. Pedunculate Oak (*Quercus robur* L.) Trees Qualitative Structure as a Criterion of the Stand Regeneration Planning. *Šumarski List* **2013**, *137*, 367–377.
88. Mikac, S.; Žmegač, A.; Trlin, D.; Paulić, V.; Oršanić, M.; Anić, I. Drought-Induced Shift in Tree Response to Climate in Floodplain Forests of Southeastern Europe. *Sci. Rep.* **2018**, *8*, 16495. [CrossRef] [PubMed]

89. Cottrell, J.E.; Munro, R.C.; Tabbener, H.E.; Milner, A.D.; Forrest, G.I.; Lowe, A.J. Comparison of Fine-Scale Genetic Structure Using Nuclear Microsatellites within Two British Oakwoods Differing in Population History. *For. Ecol. Manag.* **2003**, *176*, 287–303. [CrossRef]
90. Vranckx, G. Genetic Diversity, Gene Flow and Inbreeding in Pedunculate Oak (*Quercus robur* L.) in Fragmented Forest Stands. Ph.D. Thesis, K.U.Leuven, Faculty of Science, Leuven, Belgium, 2014.
91. Markić, A.G.; Bogdan, S.; Poštenjak, M.G.; Lanšćak, M.; Vujnović, Z.; Bogunović, S.; Ivanković, M. Acorn Yields and Seed Viability of Pedunculate Oak in a 10-Year Period in Forest Seed Objects across Croatia. *South-East Eur. For.* **2022**, *13*, 27–36. [CrossRef]
92. Bogdan, S.; Ivanković, M.; Temunović, M.; Morić, M.; Franjić, J.; Katičić Bogdan, I. Adaptive Genetic Variability and Differentiation of Croatian and Austrian *Quercus robur* L. Populations at a Drought Prone Field Trial. *Ann. For. Res.* **2017**, *60*, 33–46. [CrossRef]