

Article

Genetic Analyses of a Mixed Oak Stand at the Xeric Limit of Forest Climate and Its General Consequences for *In Situ* Conservation Management

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Abstract: Forests in the Tolna region (Hungary) are distributed at the xeric limit of broadleaved forest zones and adapted to the arid ecological conditions of the wood-steppe climate. An 85-year-old *in situ* gene conservation stand of *Quercus virgiliana* mixed with other taxa of section *Quercus* was studied, which was regenerated naturally by both seedlings and coppicing. To analyze the phenotypes growing within the stand and the genetic structure of the population, a total of 138 trees were sampled. For taxonomic classification, a complex of morphological traits of oak taxa growing naturally in the region was used. Out of the 12 morphotype groups, only a few trees were classified as *Q. virgiliana* (eight individuals) or *Q. robur* (nine individuals), and the majority of the trees (121 individuals) were hybrid or introgressed phenotypes of *Q. virgiliana* adapted to xeric conditions by its xeromorphic traits. Despite the high number of coppiced trees (89 pcs vegetatively regenerated), the genetic variation was relatively high based on 16 nSSR markers used for analyses. Some of the trees were classified as non-autochthonous with Slavonian oak origin, both by morphological traits and SSR structure. Despite some alleles being lost, the allelic diversity of the seedling trees' group was similar to that of the group of parent generation (coppiced trees). The spatial structure of trees supported the results of morphologic classification, and *Q. virgiliana* and hybrid phenotypes were growing on xeric microhabitats of the stand, mostly on southeast-facing slopes or ridges of hills. Consequently, the stand might fulfill all the *in situ* gene conservation requirements based on the high genetic diversity measured and the high number of xeromorphic phenotypes in the context of climate change as well.

Keywords: SSR; gene conservation; climate change; taxonomy; introgression; *Quercus virgiliana*



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1. Introduction

Hungary is located in the central part of the Pannonian Basin, which is affected by continental, Atlantic, and (sub)Mediterranean climatic effects. Therefore, besides deciduous forest associations, the forest-steppe ecosystems are also naturally occurring, mainly at the xeric limit of the forest vegetation zone. As a consequence of climate change, populations at the xeric limit will be further reduced or probably shift into forested vegetation zones at higher altitudes [1–3]. Since the Middle Ages, the forest-steppe climatic zone has been

intensively used by agriculture [4], and habitats of forest-steppe ecosystems are therefore strongly reduced. Some remnants of forests or even wood pastures are left, composed dominantly of drought-tolerant oak taxa such as *Quercus dalechampii* Ten., *Quercus virgiliana* Ten. [5–7]. The relevance of evolutionary and genetic consequences of environmental changes on forest trees' distribution, especially at the xeric limit of forest zones, was already recognized [8] but has not been sufficiently studied yet. This study aimed to describe and analyze a remnant mixed oak forest stand at the edge of the Hungarian Great Plain.

In the temperate deciduous forest vegetation zone, taxa of the genus *Quercus* are the most crucial forest stand-forming species. The richness of oak species in Europe is relatively low compared to the American and Asian regions. The reasons can be rooted back to glacial periods, as the natural barrier effect of the east-west mountain ranges of the European high mountains (Pyrenees, Alps, Carpathians, etc.) caused a strong bottleneck effect on many plant species, including oak populations as well [9–12]. However, some microrefugial effects have also been observed in a few regions of Europe, such as in the Pannonian Basin [13], where various fossil data also support the hypothesis of micro- or secondary refugia [9,12,14,15]. Recently, three common oak species of Section *Quercus* [16] have naturally occurred in Hungary such as *Quercus robur* s.l., *Quercus pubescens* s.l., and *Quercus petraea* s.l. The sessile oak *Quercus petraea* s.l. in Hungary includes *Quercus dalechampii*, a taxon distributed in the Balkans and partly in the Apennine peninsula, and *Quercus polycarpa* Schur with an Eastern Mediterranean area distributed at the Balkan peninsula and further east (Turkey, Caucasus, and Iran) [17–19]. The taxonomic status of *Q. dalechampii* is controversial; Italian authors classified the taxon in the *Q. pubescens* species complex [20]. The occurrences in Southeast Europe and the Pannonian basin are considered as a sessile oak taxon classified into the *Q. petraea* complex, but its nomenclature is under discussion with synonyms: *Quercus banatus* [21,22] and *Quercus aurea* [22,23]. The taxonomic status of *Q. virgiliana* in the pubescent oak complex [17,24–26] and *Quercus pedunculiflora* K. Koch in the pedunculate oak complex (whose areas are similar to those of the previous taxa) is also disputed. These taxa from the Eastern Mediterranean region were found in semi-natural woodlands by local botanists 100–150 years ago [27,28] or are currently associated with the typical oak forest communities [29–31]. Hybrids and introgressed forms are also common in the *Quercus* section, which are not easily distinguishable by molecular genetic markers [20,32–34]. Nevertheless, the frequent hybridization is considered a driving force for the success of the genus *Quercus* [35,36].

Oaks are typically stand-forming, wind-pollinated species whose pollen is produced in large quantities and can travel long distances, even at high atmospheric levels. Several studies have been conducted to determine the effective population size and the extent of the external-internal pollen flow [37–39]. As in the case of other wind-pollinated species, the progeny populations are typically derived from a few dominant pollinators and mother trees, and the external pollen ratio can be as high as 60% [37–42]. External pollen effects both increase and maintain genetic diversity [37,40] and increase the chances of hybridization [33,35,39,41,43]. In the case of oaks, both factors have a strong influence, which is also reflected in the oak genome [43] and also plays a relevant role in the conservation strategies of peripheral populations [44].

Besides their well-balanced generative mating system, vegetative reproduction is the other key factor forming population structure in the case of the genus *Quercus*. This capability was often used when oak forests were clear-cut for timber production, and they were mostly coppiced (vegetatively regenerated) [45]. In the coppiced stands, where seed-originated trees can only occasionally be found, vegetative individuals can occur over several generations. It can be highly important for gene conservation activities, as

representatives of the former natural population and its gene pool [46], whether *in situ* or *ex situ* conservation methods are used.

The conservation and use of forest genetic resources have recently reflected a growing interest. The drought-tolerant oak taxa are also in focus for the pan-European gene conservation strategies and efforts [EUFGIS database, <http://portal.eufgis.org/>, accessed on 28 May 2025), EUFORGEN <https://www.euforgen.org/species>, accessed on 28 May 2025)]. In the Tolna Region (Hungary), a complex gene conservation program for *Q. virgiliana* was started in 2017. According to EUFORGEN criteria, remnants of old oak forests were inventoried as possible *in situ* gene conservation stands. In many cases, plus trees were also selected for further *ex situ* gene conservation activities [7,47].

As a part of this gene conservation program, a mixed oak stand, composed partly of coppiced *Q. virgiliana* trees, was selected and registered as an *in situ* conservation stand. To verify the minimum criteria of the EUFGIS conservation units (autochthony, target species, etc.) and to study the genetic constitution of the conservation stand, individuals of all taxa of section *Quercus* were selected and analyzed to get answers to the following questions:

- What is the proportion of the drought-tolerant *Q. virgiliana* and/or putative hybrids of other taxa of the section *Quercus* based on morphological traits vs. genetic markers?
- What is the proportion of trees within the population that are of vegetative (previous parent generation) and seed (progeny generation) origin, and what is the proportion of ‘identical genotypes’ (clones)?
- Are there any differences in the genetic structure (gene pool?) between the previous generation (coppiced trees) and the offspring generation, e.g., allele number, heterozygosity values, possible loss of alleles, or the emergence of new alleles? Are there any genetic differences due to foreign origin (planted trees)?
- Is the spatial distribution of trees, taxa, or putatively specific genotypes influenced by any microhabitat factors (topography, exposure, soil hydrology)?
- Finally, is the gene pool of this population suitable for effective and long-term *in situ* gene conservation, and what aspects and factors should be considered? What silvicultural methods should or can be applied, particularly in a landscape dominated by arable lands and meadows?

2. Materials and Methods

The study site is a 3 ha mixed oak stand in Pusztahencse (Tolna region, Hungary, geographical location: 46.575940° N, 18.771783° E; 136–150 m a.s.l.) The stand, managed by the Gyulaj State Forest Corporation, is composed dominantly of *Q. virgiliana* and its hybrids, *Q. petraea* s.l., *Q. robur* s.l. and *Q. cerris* individuals, was selected. The stand is surrounded by agricultural lands where the ecological conditions are not optimal for forest populations (forest-steppe climate, mean temperature of 20–21 °C in July and –1–0 °C in January, annual precipitation of 500–550 mm, admixture of calcareous sand and loess soil). Further climate information based on recent weather measurements is available at <http://www.ertgis.hu> (accessed on 28 May 2025).

The forest compartment (Pusztahencse 8/D) was registered as an *in situ* *Q. virgiliana* gene reserve, and a plus tree selection for *ex situ* purposes was also carried out. The stand is 85 years old (recorded), but due to the high proportion of coppiced trees, the estimated age might be at least 150–200 years or more. Based on the forestry management plan data, the stand was clear-cut 85 years ago and regenerated by seedlings of natural regeneration and partly by coppicing. During the regeneration and replacement period, *Q. robur* seedlings were artificially planted in a few patches within the stand, identified as Slavonian oak based on its phenotypic traits and specific habitus [48]. The presumably natural origin and

the history of the stand can be proved based on the following historical military maps and the recent forestry database:

- First Military Land Survey of the Hungarian Kingdom, 1782–1785 [<https://maps.arcanum.com/en/map/firstsurvey-hungary/?layers=147&bbox=2075541.607849373,6000935.425817063,2221001.2726760632,6054059.1604752615>] (accessed on 29 March 2025)]
- Second Military Land Survey of the Hungarian Kingdom, 1819–1869 [<https://maps.arcanum.com/hu/map/secondsurvey-hungary/?layers=5&bbox=2112256.0623183693,6020245.231900737,2130457.629678777,6027076.791303724>] (accessed on 29 March 2025)]
- Military Land Survey of Hungary, 1941 [<https://maps.arcanum.com/hu/map/hungary1941/?layers=29&bbox=2114166.9880255754,6020471.733854797,2132368.555385983,6027303.293257784>] (accessed on 29 March 2025)]
- Forestry Map of Hungary, 2025 [<https://erdoterkep.nebih.gov.hu/>] (accessed on 28 March 2025)].

According to military maps, the area was part of a forest block as early as 1782. Over the past nearly 250 years, the area has been clear-cut and naturally regenerated (coppiced) several times, according to the data of forestry management plans. The historical maps record the area with the name “Hegyesi” forest (Figure 1).

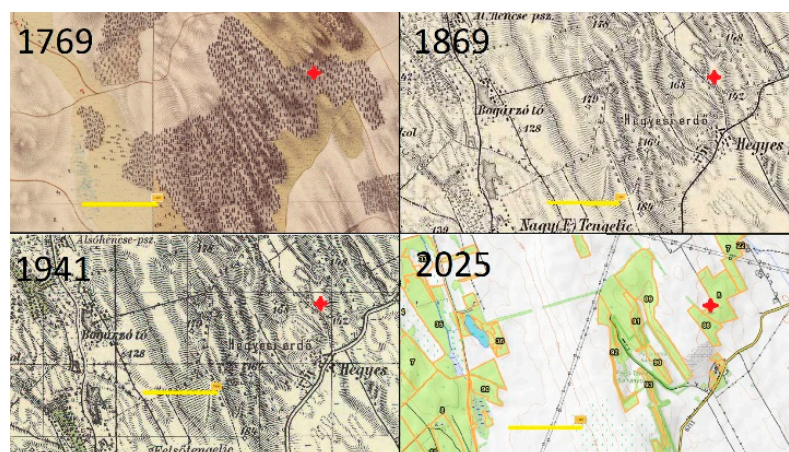


Figure 1. Military maps from 1782, 1869, and 1941 compared with the 2025 Forest Map of Pusztahencse 8/D (marked with a red asterisk). The yellow line is a standard one km scale.

2.1. Sampling of Plant Material, Analyses of Morphological Traits

A total of 138 trees were described by morphological traits, from which 97 were coppiced (vegetative origin), and 41 were saplings (seed origin). In 2019, 39 trees (IDs 21–59) were selected based on their pubescent phenotype and described as *Q. virgiliana* and dominantly *Q. virgiliana* hybrid plus trees. In 2022, 99 trees were additionally selected (IDs 61–159) and described as pure species and hybrids from the *Quercus* section using a standardized plus tree description sheet. The georeferences and origin (by seed vs. coppiced) were recorded (Figure 2 and Table S1). *Quercus cerris* individuals were excluded from the analyses as they belong to the *Cerris* section and do not hybridize with the taxa of the *Quercus* section.

When field surveys were carried out, some *Q. robur* trees were clearly distinct from the local genotypes and looked like Slavonian oak-type trees, based on their specific growth habitus [48]—straight trunk, spreading root, crown branches upright, etc.—described at the field survey and recorded on the description sheet.

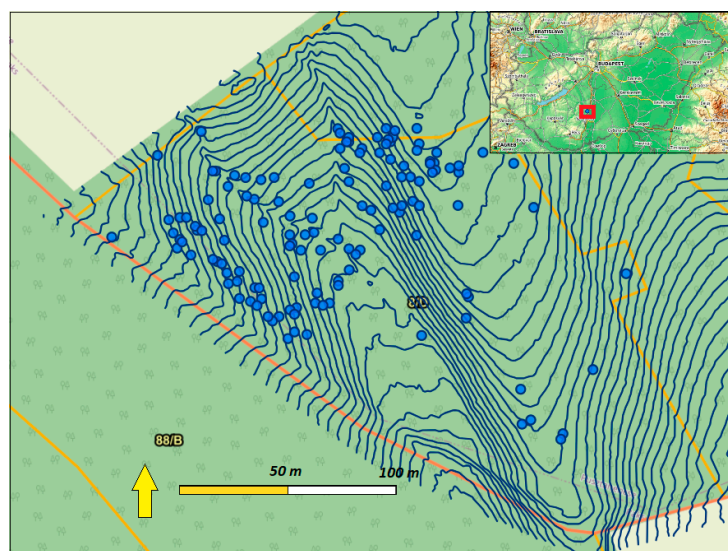


Figure 2. The study site with the analyzed trees in Pusztahencse 8/D (without *Q. cerris*). The red frame on the map of Hungary (right up) shows the location of the studied plot within the country (the yellow arrow marks north).

Branches were collected from the light crown of the trees in June–July 2019 and 2022. Acorns with cups, where possible, were also collected in 2019 and 2024 for morphological description and evaluation. The taxonomic status of the trees was described based on morphological traits evaluated on-site, such as characteristics of bark, twigs, buds, leaves, flowers and fruit described for all the autochthonous oak taxa [49–53]. The values and rates were recorded on a field description sheet used in general for the gene conservation program [7]. All the characteristics (measured and rated) were evaluated at the same level of statistical weighting. The trees were assigned into six putative taxonomic groups (*virgiliana*, *dalechampii*, *petraea*, *polycarpa*, *pedunculiflora* and *robur*) based on aggregated values of each taxon-specific characteristic. Trees with simple taxon-specific aggregated values were classified into a single taxon group (e.g., V = *virgiliana*), and the trees with intermediate or mixed characteristics were clustered into a hybrid group of two species, depending on the dominance of the taxa by each (e.g., VPET = *virgiliana* × *petraea*). The trees, which were admixed of three or more taxa, were clustered into a ‘hybrid’ group, depending on the dominant taxon characteristics (e.g., DH = *dalechampii* × *virgiliana* × *robur*).

In addition, the collected herbarium material was measured from several points of view, and all the characteristics (on-site and laboratory measurements) were merged and synthesized. Hypothetically, six taxa were expected to occur on the site. The factors studied are listed in Figure 3; for the rated characteristics we used specific value scales: 1–2 = yes/no, 1–3 = yes/somewhat/no, 1–4 = firmly/moderately/weakly/no, 1–5 = acorn color: copper/green/ochre/brown/dense multicolored and 1–5 = acorn widest part: lower third/lower third-medium/medium/medium-top third/top third, 1–6 = taxon classification: 1 = *Q. virgiliana* (V), 2 = *Q. dalechampii* (D), 3 = *Q. petraea* (PET), 4 = *Q. polycarpa* (POL), 5 = *Q. pedunculiflora* (PED), 6 = *Q. robur* (R). The field description sheets and the herbarium are available at the MATE Department of Botany.

Morphological taxon groups (138 trees) were plotted using QGIS (v 3.36.0) to examine spatial patterns, and a map was created to separate native and non-native lineages of seed and vegetative origin.

origin of tree gen/veg	bark	fruit			flower male/female	branch		leaf
		acorn	cupula	peduncle		twig	bud	
origin	taxon classification	length (mm)	length (mm)	length (mm)	length of petiole (mm)	thickness (mm)	length (mm)	number of the lobe (pcs)
		diameter (mm)	diameter (mm)	acorn pcs/petiole	flower pcs/petiolae	color	hairiness	lamina length (cm)
		cross-striped	hairiness	hairiness	hairiness	lenticel	palm	petiole length (mm)
		germinating	taxon classification	taxon classification		hairiness	taxon classification	adaxial brightness
		oil stripe				taxon classification		adaxial color
		colour						indentation of blade
		widest part						sub lobe
		shape						shape of lobe
								adaxial hairiness
								abaxial hairiness
								taxon classification

Figure 3. Morphological traits evaluated for taxonomic classification. The measured (mm, cm) or counted (pcs) traits are indicated in blue in the table, and rated traits are indicated by shades of brown (1–2 scale, 1–3 scale, 1–4 scale, 1–5 scale, 1–6 scale).

2.2. Statistical Evaluation of Taxonomic Descriptors

The dataset of morphological traits was analyzed to test the validity of taxonomic descriptors used on description sheets. A cluster analysis was performed using Ward’s hierarchical method based on calculating Euclidean distances between the individuals from 16 generative and 17 vegetative variables. The nominal variables were first encoded to binary variables, and all were scaled. After data preprocessing, statistical analyses were conducted in R using the packages (Version R 4.4.2) ‘cluster’, ‘factoextra’ [54], ‘dendextend’ [55], and ‘ggplot2’ [56]. The morphotype groups classified in the field were then visualized using a dendrogram. The resulting morphological groups, generated from the field and statistical data, were compared to verify the “visual”-based taxon descriptors.

2.3. Microsatellite Analyses

Total genomic DNA was isolated from fresh leaves following the modified CTAB procedure [57,58]. DNA concentration was measured by Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and standardized to 10 ng/μL. Sixteen SSRs were used for amplification of nuclear microsatellite loci, namely QpZAG 15, QpZAG 20, QpZAG 110, QpZAG 1/5, QpZAG 36, QrZAG 7, QpZAG 16, QpZAG 9 [59], QrZAG 11, QrZAG 112, QrZAG 87, QrZAG 96, QrZAG 30, QrZAG 101 [60], MSQ 13 and MSQ 4 [61]. Among them, for six primer pairs (QpZAG9, QpZAG1/5, QpZAG16, QrZAG7, MSQ4, QpZAG36), the universal M13 (-21) fluorescent dye label method by Schuelke [62] was applied. The GoTaq G2 Flexi (Promega, Madison, WI, USA) polymerase kit was used for the PCR. PCR protocols are summarized in Table S2. PCR fragments were separated on a 1.75% agarose gel in 1 × TAE buffer, stained with GelRed (Biotium, Fremont, CA, USA). Then, fragments were diluted (up to 20-fold) for capillary electrophoresis to obtain an appropriate concentration and multiplexed by dye and size in formamide (Hi-Di, Applied Biosystems, Waltham, MA, USA) using GeneScan LIZ 500 (Applied Biosystems) internal size standard. SSR genotyping was performed on an ABI 3730 DNA Analyzer (Applied Biosystems) by BIOMI Ltd. (Gödöllő, Hungary).

2.4. Population Genetic Analyses

Following the fragment analysis, allele variants were scored by the Osiris 2.16 software (<https://www.ncbi.nlm.nih.gov/osiris/overview/>, accessed on 28 May 2025). The software GenAlEx 6.5 [63,64] was used to evaluate the genotypic data. The different fragments were used as allele variants to specify the unique genotypes for each sample. Seventeen samples out of the total 138 were deleted from further analysis due to missing values on more than two loci. Therefore, for the whole genetic evaluation, 121 samples were used.

First, matching genotypes were checked, searching for putative clones. The index probability of identity (P_{ID}) was calculated in GenAlEx for the applied 16 SSR markers to check the resolution of the marker set.

Population genetic analyses were carried out, applying different approaches both on the individual level and on the level of taxonomic groups.

The following main population genetic parameters were calculated in GenAlEx in the mean of the 16 markers based on the frequency values of the allele variants: number of alleles (N_a), number of effective alleles (N_e), number of private alleles (N_p), Shannon diversity index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e), and the fixation index (F). The genetic distance matrix was calculated between the taxonomic groups by GenAlEx's option of Gdpop, and a principal coordinate analysis (PCoA) was derived based on the genetic distances. For the visualization of the PCoA plot, the R package ggplot2 [56] was used.

The partitioning of the molecular variance was evaluated by AMOVA in GenAlEx with 999 repetitions. The pairwise F_{st} values and the significance level were interpreted in a heat map matrix format using the R package 'ggplot2'.

Individual genotypes were also evaluated using the Bayesian clustering method by STRUCTURE [65]. The cluster numbers 1–6 were tested, and the Evanno method was applied to evaluate the most probable K cluster [66] using the R package 'pophelper' [67]. The ancestry of the individuals was plotted using QGIS (v 3.36.0) to examine spatial genetic patterns of the stand (121 trees). Population genetic indices were also calculated for the defined clusters.

Finally, the genetic structure of the stand was also evaluated based on the reproductive origin of the individuals, dividing the samples into two main groups: autochthonous seed origin (age 85 yrs) and autochthonous vegetative origin (age min. 150–200 yrs). Four leading indices were compared in the different genetic clusters of the two main groups: the number of private alleles (N_p), the observed heterozygosity (H_o), unbiased expected heterozygosity (uH_e), and the fixation index (F).

2.5. Test of Autochtony

A chloroplast microsatellite analysis was performed to verify the origin (autochthony) of some selected trees. The minimum sample number was decided based on the conceptualization of a previous haplotype study [11] to sample at least 10 trees within a stand for chloroplast haplotype analyses. According to this protocol, four putative non-autochthonous (Slavonian oak) trees (ID 92, 97, 99, 147,) and seven others (ID 24, 28, 32, 80, 100, 125, 150) were chosen, considered native, containing both seedling and coppiced trees, and classified into different taxon groups. Ten chloroplast SSR markers were selected and arranged in two multiplex mixes from the cmcs-series [68], the ccmp-series [69] and the μ dt/ μ kk-series [70]: Mix1) cmcs9 (VIC), cmcs1 (6FAM), ccmp6 (PET), cmcs8 (NED), cmcs6 (VIC), cmcs7 (6FAM), Mix2) μ dt4 (PET), μ dt3 (6FAM), μ dt1 (VIC), μ kk4 (NED). Primer pairs were synthesized with a universal M13(-21) tail on the 5' end of the Forward primer following the description of Schulke [62] by IDT (Integrated DNA Technologies Inc., Coralville, IA, USA). Fluorescent dyes compatible with the Applied Biosystems G5

Matrix were used on the universal M13 (-21) primer 5' end. For the PCR, the GoTaq G2 Flexi (Promega, Madison, WI, USA) polymerase kit was used, and the protocol described by Schulke [62] was applied. PCR fragments were separated on a 1.75% agarose gel in 1× TAE buffer, stained with GelRed (Biotium, Fremont, CA, USA). Then, fragments were diluted (up to 20-fold) for capillary electrophoresis to obtain an appropriate concentration and multiplexed by dye and size in formamide (Hi-Di, Applied Biosystems, Waltham, MA, USA) using GeneScan LIZ 500 (Applied Biosystems) internal size standard. SSR genotyping was performed on an ABI 3730 DNA Analyzer (Applied Biosystems) by BIOMI Ltd. (Gödöllő, Hungary).

Following the fragment analysis, allele variants were scored by the Osiris 2.16 software (<https://www.ncbi.nlm.nih.gov/osiris/overview/>, accessed on 28 May 2025). The software GenAlEx 6.5 [64] was used for the data analysis. The different fragments and their frequencies for each marker region were detected, followed by the specification of the unique haplotypes for each sample. A genetic distance matrix was calculated based on the stepwise mutation model for each haplotype by GenAlEx, and a principal coordinate analysis was conducted based on this matrix to represent the genetic relatedness between the detected haplotypes.

The spatial distribution of native and non-native individuals within the stand was plotted on a map.

3. Results

3.1. Phenotype Classification Based on Morphological Traits

Following a fine-scale approach, 138 trees were selected. For the on-site description, a set of morphological traits was used, focusing on the characteristics of bark, leaf, twig, bud, flowers, and fruit (if available). The characteristics of all the trees were recorded on the plus tree description sheets. Hypothetically, six taxa, such as *Q. virgiliana* (V), *Q. dalechampii* (D), *Q. petraea* (PET), *Q. polycarpa* (POL), *Q. pedunculiflora* (PED), and *Q. robur* (R) were expected to be occurring. The specified classification method, listed in Figure 3, was used for taxonomic descriptions of all the trees on-site. In the case of admixed traits, groups of transitional taxon variants were formed based on the dominance of each characteristic. The following 12 morphotype groups were determined: uniform: V(8), R(9) and transitional: DH(7), PETH(14), PETV(7), RPED(14), VH(11), VD(13), VDPET(17), VPET(19), VPETD(15), VR(4) (number of individuals per groups in brackets). Trees with any characteristics of *Q. polycarpa* were not detected and described on the study site, so this category was excluded from further statistical analyses.

Due to a lack of acorns during the 5-year study period, acorns could be sampled only on 75 trees out of the 138. Therefore, the characteristics of acorns were excluded from further statistical analysis. Generative traits were used only for the taxonomic classification in the field. The dataset of morphological traits, both measured and rated on all 138 trees (phenotypes), was analyzed and clustered by Ward's hierarchical method. The multivariate morphology statistic evaluation, specifically Ward's clustering, was a tool used to assess the effectiveness and accuracy of the original on-site description. For the clusters, a distance matrix was constructed and plotted as a dendrogram (Figure 4).

The dendrogram structure and colors were based on statistical measurements and dissections, while the labels were applied according to 12 morphotype groups classified on-site. Two small groups (incl. only four trees) were merged for statistical analysis (VR virg × rob and RV rob × virg = VR group). As a result, the 12 morphotype groups described on-site are clearly distinguished on the dendrogram.

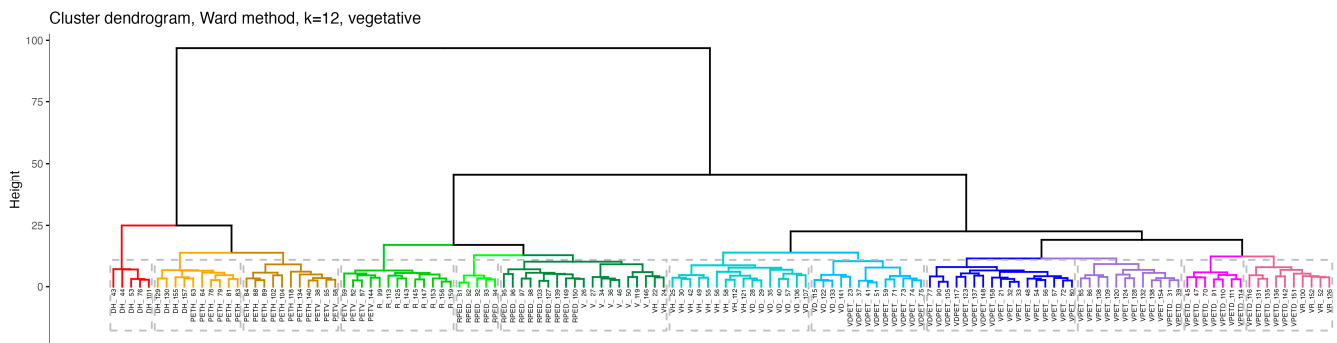


Figure 4. The dendrogram was constructed applying Ward’s hierarchical method based on the calculation of the Euclidean distance matrix from measured and rated morphological traits. The labels are according to the 12 on-site morphotype groups. Morphotype groups on the dendrogram from left to right: DH, PETH, PETV, R, RPED, V, VH, VD, VDPET, VPET, VPETD, VR.

3.2. Genetic Analyses

3.2.1. Microsatellite Analyses

All 16 nSSR markers used were well amplified and polymorphic in the sample set tested. Out of 138 trees, 121 individuals were genotyped and analyzed. Only two clone pairs were found: 110–111 (VPETD) and 149–150 (R), based on nSSR-genotyping. The low P_{ID} value ($P_{ID} = 3.19 \times 10^{-26}$) indicates that the probability of random matching is negligible, so individuals with the same genotype detected can be considered as actual clones of vegetative origin. Based on the microsatellite genotyping, trees ID 97 and 99 were found to be siblings, and they were described on-site as planted non-autochthonous trees.

Using principal coordinate analysis (PCoA) derived from the genetic distance matrix of the morphotype groups, we visualized the genetic relationship and separation between the groups by considering the principal coordinates PC1 (80.33%) and PC2 (7.77%). Figure 5 shows that the R and RPED groups are well separated, with the groups that also show PET characters being relatively close to each other in the lower left quadrant and the groups that also show V and D characters being in the middle and upper left quadrants. The separation of the small groups DH(7) and PETV(5) can be a spurious result of the low sample size of the groups. The gene pools of the autochthonous ancestry genotypes are similar despite differences in phenotypic appearance, as shown by their positioning to the left of the PC1 axis.

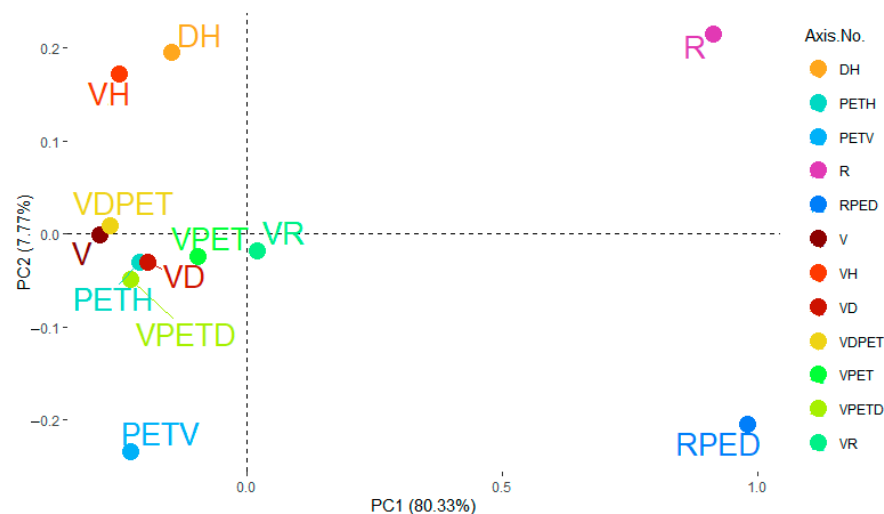


Figure 5. PCoA plot derived from the genetic distance between morphotype taxon groups.

The main diversity indices calculated from the allele frequency values were first evaluated for the field morphological groups (Table 1). Since three of the morphotype groups (PETV, R, VR) had critically low sample numbers, and two other groups (DH, V) had moderately low sample numbers, their values should be treated with caution in the comparison.

Table 1. Population genetic indices of the 12 on-site morphological groups (121 samples) based on the mean of the applied 16 nSSR markers.

Groups	N	N _a	N _e	N _p	I	H _o	H _e	uH _e	F
DH	7	7.563	5.765	0.313	1.823	0.768	0.800	0.862	0.047
PETH	13	10.000	6.491	0.625	1.980	0.750	0.807	0.839	0.088
PETV	5	5.438	4.254	0.188	1.497	0.700	0.728	0.808	0.064
R	4	5.188	4.272	0.500	1.493	0.781	0.729	0.833	−0.079
RPED	14	10.313	6.636	1.688	1.956	0.763	0.787	0.816	0.032
V	8	7.875	5.742	0.375	1.820	0.680	0.789	0.841	0.160
VH	11	9.375	5.862	0.375	1.935	0.761	0.801	0.839	0.062
VD	10	8.563	6.032	0.250	1.910	0.813	0.813	0.856	0.007
VDPET	15	10.813	6.632	0.438	2.032	0.721	0.814	0.842	0.128
VPET	16	11.625	7.106	0.438	2.115	0.727	0.838	0.865	0.139
VPETD	14	10.688	6.652	0.625	2.032	0.781	0.817	0.848	0.040
VR	4	5.750	4.918	0.125	1.632	0.813	0.773	0.884	−0.050

Regarding allele structure, the VPET group had the highest number of alleles observed (N_a), followed by the VDPET and VPETD groups. The VPET, VPETD, and VDPET groups also had the highest values for the effective allele number (N_e), with the addition of the RPED group. Unique alleles (N_p) were observed in all groups, partly due to the large marker size and diversity. However, the RPED group had an outlying (1.688) value for unique alleles. The following two groups with the highest values were PETH and VPETD (with a value of 0.625), but they were well below the value of RPED. The groups with the highest diversity expressed by the Shannon index (I) were VPET, VPETD, and VDPET.

Regarding the observed heterozygosity (H_o) indices, the highest values were found in the VD and VR groups, and the lowest were in the V and PETV groups. In terms of expected heterozygosity (H_e), VPET, VPETD, VDPET had the highest values. The PETV group had the lowest values for both observed and expected heterozygosity. However, heterozygosity values were balanced between groups and were high with the markers used.

Concerning the fixation index (F), a slight shift in the positive direction was observed in three cases compared to the equilibrium state around 0, indicating a homozygous surplus (i.e., fixation of some alleles) in groups V, VPET, and VDPET.

Molecular analysis of variance (AMOVA) was performed to further investigate the genetic segregation of morphological taxon groups. The variance distribution was as follows: 2% among populations, 11% among individuals, and 87% within individuals. The pairwise F_{st} matrix derived from the AMOVA, showing the differentiation between taxon groups, was plotted on a heatmap (Figure 6). The R and RPED groups are significantly distinct. At the same time, no significant differences can be observed between the other groups.



Figure 6. Pairwise F_{st} values derived from the AMOVA analysis (F_{st} Values below diagonal, p values above diagonal).

3.2.2. Analyses of Individual Genotypes

In the STRUCTURE analysis, the most probable cluster number was K3 according to the Evanno method out of the tested K1-6 clusters. A hidden subcluster can also be observed in the K4 clustering. Therefore, both clustering results are presented in Figure 7. Regarding the morphological classification of the samples in each cluster, cluster 1 (dark blue) group typically contains R and RPED samples, and cluster 4 (yellow) group, which appears as the fourth subcluster, mainly contains VD-type individuals. The other two large clusters, cluster 2 (light blue) and cluster 3 (red) are more heterogeneous regarding species composition. Still, the *petraea* type typically dominates the light blue, while the red group mainly contains *virgiliana*-type samples.

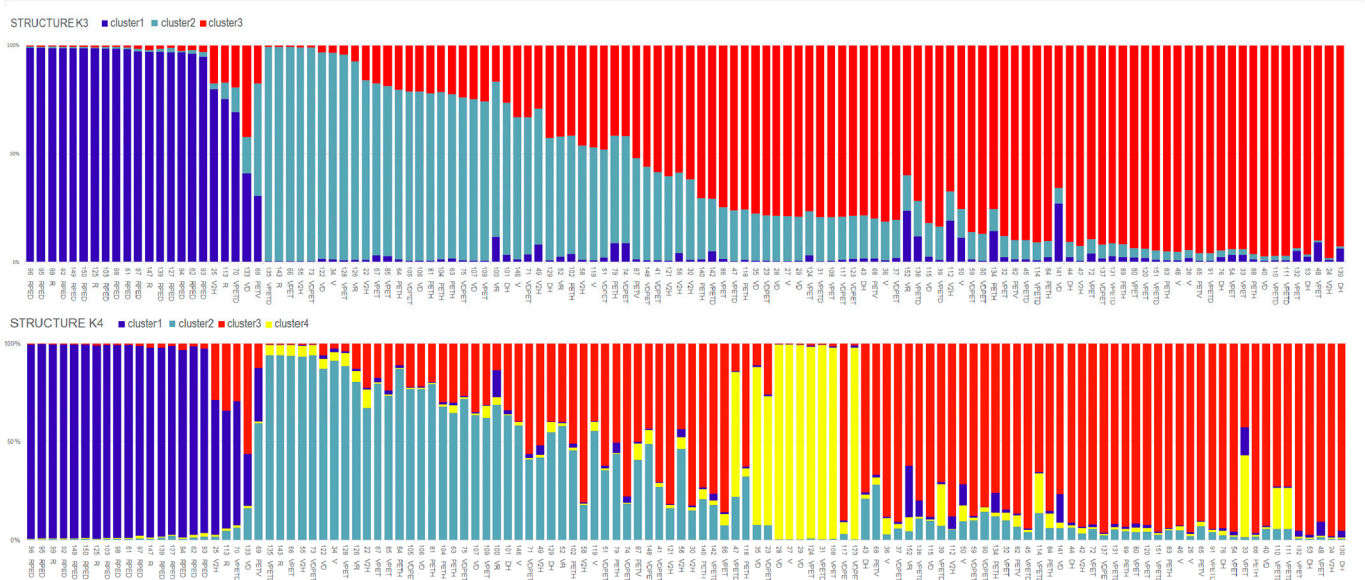


Figure 7. Results of STRUCTURE K3 and K4 analysis. The coloring of the bars indicates the probability of individuals belonging to a given genetic cluster.

To gain insight into the genetic patterns of the clusters separated by STRUCTURE and to explore their differences, we also calculated the main population genetic indices along the K4 clusters (Table 2). We considered one genotype belonging to a given cluster if the value of the posterior probabilities of individual assignment reached 80%. Below this threshold, they were assigned to the mixed group (introgressed forms or true hybrids). The

allele number (N_a) was partly explained by the sample size of the groups, highest in the mixed group (individuals with ambiguous ancestries) and cluster 3 and lowest in clusters 4 and 2. The effective allele number (N_e) was highest in the mixed group and in clusters 3 and 1, which were also the most diverse in terms of the Shannon index (I). The unique allele number (N_p) was the highest in the mixed group, followed by clusters 1 and 3. The observed heterozygosity (H_o) was the highest in cluster 1 and in the mixed group, while the expected heterozygosity (H_e and uH_e) indices were the highest in the mixed group and cluster 3. The fixation index (F) values indicated an approximately equilibria state, with values close to 0 in the cases of clusters 1 and 2. A shift towards homozygote excess was observed in cluster 3, while a slight heterozygote excess appeared in cluster 4.

Table 2. Main population genetic indices of the STRUCTURE K4 clusters (121 samples).

Cluster	N	N_a	N_e	N_p	I	H_o	H_e	uH_e	F
cluster 1	17	11.375	6.991	2.313	2.023	0.768	0.796	0.820	0.034
cluster 2	9	6.438	4.160	0.438	1.507	0.736	0.695	0.736	−0.064
cluster 3	39	15.000	7.005	2.250	2.193	0.731	0.832	0.842	0.128
cluster 4	8	4.438	3.239	0.313	1.236	0.758	0.650	0.693	−0.157
mixed	48	16.938	8.009	2.438	2.297	0.766	0.844	0.853	0.098

3.2.3. Genetic Structure of Coppiced vs. Seed-Originated Cohorts

We also analyzed the gene pool of the putatively parental (coppiced trees) and offspring (generative saplings) generation (Table 3). The two groups (seed/vegetative) were examined according to STRUCTURE K4 clustering, and the values of unique alleles (N_p), observed heterozygosity (H_o), unbiased expected heterozygosity (uH_e), and the fixation index (F) were compared. The number of unique alleles (N_p) is almost equal in the clusters of the two groups, except for clusters 1 and 3. Cluster 1 has an outstandingly high number of private alleles in the seed-originated group. The observed heterozygosity (H_o) values were higher in the clusters of the vegetative group, except in the mixed group. The unbiased expected heterozygosity (uH_e) exhibited different tendencies and values among groups compared to the observed heterozygosity. The fixation index, derived from the former two indices, showed a considerable heterozygote excess in the vegetative group of cluster 1, and in clusters 2 and 4 overall. The vegetative group of cluster 3 and the mixed group represented a slight homozygote excess. On the other hand, in the seed-derived group of clusters 1 and 3, as well as the mixed, a near equilibria state could be observed. Since the sample number is very limited in the case of clusters 2 and 4, as well as the vegetative group in cluster 1, we should be cautious in drawing any far-reaching conclusions. Comparison of genetic indicators between coppiced and seed-originated cohorts following the STRUCTURE K4 clustering.

Table 3. Comparison of genetic indicators between coppiced and seed-originated cohorts following the STRUCTURE K4 clustering (n.d.—data not available).

Cluster/Origin	N		N_p		H_o		uH_e		F	
	Seed	Veg	Seed	Veg	Seed	Veg	Seed	Veg	Seed	Veg
cluster 1	14	3	4.563	0.375	0.763	0.792	0.811	0.750	0.027	−0.259
cluster 2	2	7	0.375	0.313	0.688	0.750	0.750	0.727	−0.191	−0.110
cluster 3	7	32	1.688	2.938	0.714	0.734	0.831	0.840	0.075	0.122
cluster 4	2	6	0.438	0.375	0.719	0.771	0.667	0.715	−0.431	−0.164
mixed	7	41	2.313	3.250	0.795	0.761	0.864	0.849	0.010	0.099

3.3. Spatial Structure

The spatial position of the trees was represented on a map using the geographic coordinates in QGIS 3.36 software. Each tree was plotted with a unique pie chart corresponding to the STRUCTURE K4 grouping (Figure 8). It can be observed that cluster 1 and cluster 4 are spatially separated on the site. The trees belonging predominantly to cluster 2 and cluster 3 also show a small degree of spatial clustering.

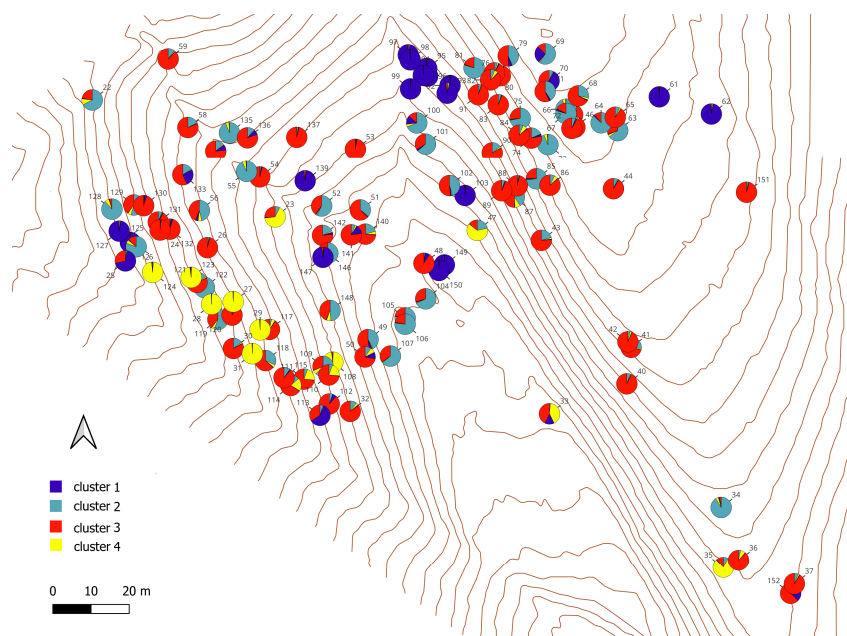


Figure 8. Map representation of STRUCTURE K4 clustered genotypes with pie charts of the studied trees (121 individuals).

The morphotype taxon groups were also plotted to examine spatial patterns (Figure 9).

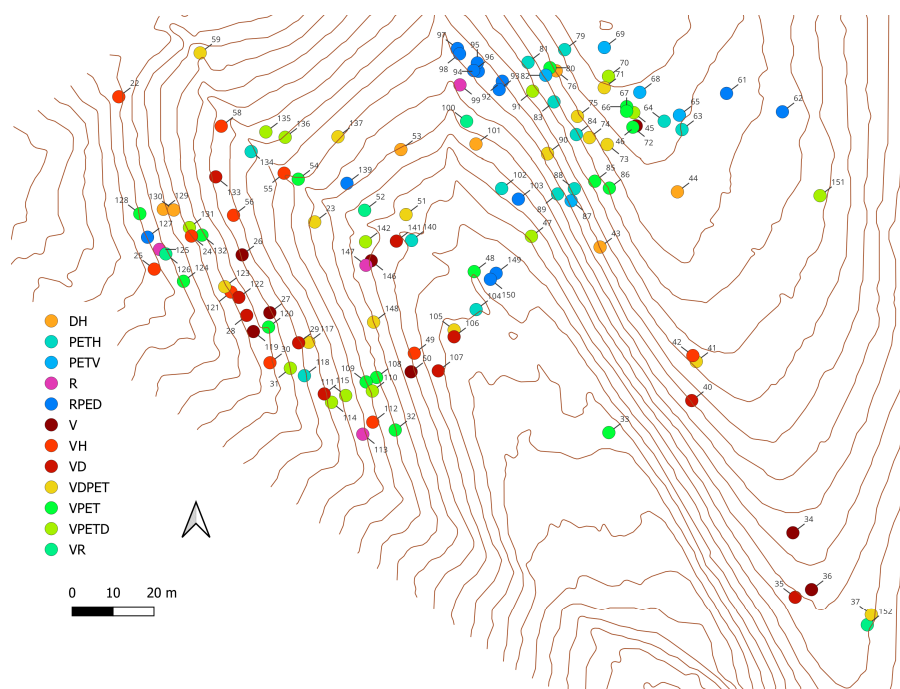


Figure 9. The twelve morphological groups on the topography (138 individuals).

3.4. Verification of Autochthony

Some of the seed-originated *robur* trees showed an apparently distinct morphotype typical for the Slavonian type. Furthermore, the nSSR markers also exhibited a remarkable difference between these two *robur* groups based on the private allele composition; a test of autochthony was also conducted by applying chloroplast SSR markers. The chloroplast genotypes of 11 trees included in the chloroplast DNA analysis were compared, and haplotype analysis confirmed the non-native origin of the planted *Q. robur* individuals. The haplotypes of these individuals differed significantly from the allele pool of the native stand, where the analyzed individuals shared identical haplotypes. Figure 10 represents the genetic distance of observed haplotypes based on principal coordinate analysis (PCoA).

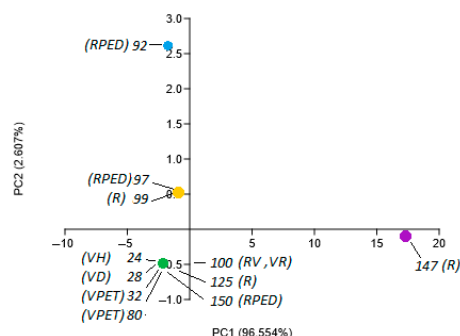


Figure 10. Genetic distance of the chloroplast haplotypes applying a PCoA plot.

Haplotypes of the individual ID 92, 97, 99, and 147 are clearly separated from the native group. Based on this result and the identical phenotype of the nine individuals of the 25 trees in the R and RPED groups, described as Slavonian oak on-site, we can conclude that these seed-originated trees are certainly non-autochthonous. Table S3 summarizes the most important differentiating nSSR markers with the private alleles, as well as the haplotype composition of the studied *robur* trees in the stand. The markedly different gene pool of these genotypes may also influence the strong separation of the two *robur*-type groups.

Finally, the autochthonous trees, the coppices and saplings, and the putatively non-autochthonous individuals were also mapped in Figure 11 to visualize the spatial structure of the population.

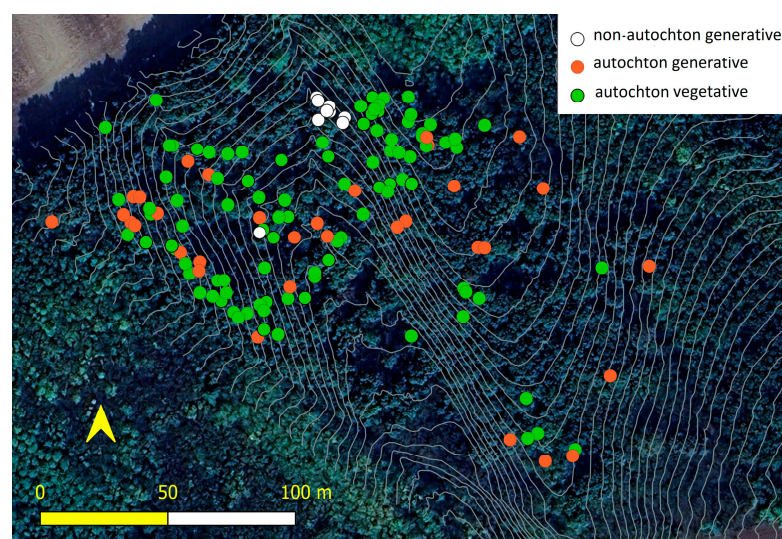


Figure 11. Spatial distribution of naturally regenerated trees (orange = autochthon generative saplings), (green = autochthon vegetative coppiced), and artificially planted trees (white = non-autochthon saplings).

4. Discussion

In the very first phase of the gene conservation program, trees with *Q. virgiliana* morphological characters (hairiness, bark pattern, etc.) were selected within the population, a total of 39 trees. Following the plus tree selection, the whole stand was registered as an *in situ* gene conservation stand, although other oak taxa were occurring within the stand. In addition, a further 99 trees were sampled, resulting in 138 samples altogether, which were mapped and analyzed. All the trees included in the study belonged to the *Quercus* section. In contrast, all the *Q. cerris* trees were excluded for further taxonomic and genetic analyses. There were nine trees classified as typical *Q. robur* and eight *Q. virgiliana* individuals based on morphological traits. Indeed, most of the trees (121 in total) were classified as a hybrid and clustered into one of the hybrid groups by both morphological and genetic analyses. No trees were found, clustered exclusively as a *petraea*, *dalechampii*, *polycarpa*, or *pedunculiflora* type.

All four *Q. robur* trees described previously as Slavonian oak type [48] were clearly distinguished from local genotypes by both nSSR and cpSSR markers, as well as based on their growth habitus. The results of genetic analyses supported our hypothesis that non-autochthonous seedlings were introduced and presumably planted into the stand as replacements during or after the reforestation. The differences both in the nuclear and chloroplast genomes were significant (Figures 9 and 10) and confirmed previous scientific evidence that chloroplast haplotypes might be a suitable tool for determining lineages of origin, even at the regional or local level [11,13]. Previous studies [13,48,71] reported that Slavonian oaks were widely planted in Hungary from the end of the 19th century up until the present. The Slavonian oak samples represented Haplotypes 4, 5, and 7, which were generally distinguishable from local native stands in Hungary [13]. Nevertheless, more detailed cp haplotype analysis and determination of the origin of the Slavonian oak trees were not part of the present study.

In the case of Slavonian oak types, the applicability of field descriptors used for plus tree selection was relatively high to distinguish the local *Q. robur* trees when they were surveyed. But in most cases, e.g., for *Q. petraea* or *Q. virgiliana* taxa, the applicability of field descriptors might be insufficient. Therefore, genetic analyses are definitely necessary to screen out non-autochthonous lineages within *in situ* gene conservation stands.

The results of both taxonomic classification and genetic analyses pointed out that *Q. virgiliana* forms a reproductive community with other related *Quercus* taxa. Most of the trees analyzed were in a continuous transition between groups of the taxa at the species level and formed hybrids. One reason for this might be a constant introgression, which can be explained by the fitness of various hybrid forms, which can use the microhabitats of the forest at this xeric limit more persistently and efficiently than the individuals of the parental species. An alternative explanation for the dominance of hybrid genotypes might be the secondary evolutionary processes specific but general to European white oak taxa [35,43].

A combined assessment of the morphological traits and genetic markers resulted in a partly similar clustering for the *virgiliana* and hybrid cohorts. However, the *petraea* and *dalechampii* type trees were classified into one separate cluster by morphological traits (Figure 4), while the *Q. robur* s.l. cohort was clearly separated by the structuring of nSSR markers (Figure 7). The *robur* group, including the Slavonian oak trees planted and the *pedunculiflora* hybrid types, was separated relatively clearly from the other taxa. This was also confirmed by the result of the principal coordinate analysis (Figure 5), where the separation of the *robur* group was apparent. In both cases (morphological traits and nSSR markers), the *virgiliana* types and their hybrids were classified into one group and formed a continuous transitory but dominant cluster of the trees. However, it was still not answered

whether the hybrids were dominating within the population due to ‘optimal hybrid fitness’ or if that was a result of ongoing secondary evolutionary processes.

Based on the field descriptors’ dataset, both generative (41 trees, 30%) and vegetative regeneration (97 trees, 70%) represent a significant proportion within the stand. Nevertheless, the individuals have a low tendency to vegetative reproduction, i.e., spreading from the rootstock (increase in number of individuals or clonal reproduction) is not typical for the trees of the stand, with only two clone pairs identified. However, both strategies might have a significant role in the mating system of oak populations at the xeric limit. This is partly due to historical reasons (extensive succession in the region’s forests) and partly due to the good vegetative regeneration ability of *Q. petraea* s.l. and *Q. pubescens* s.l. taxa. Recently, the stand is recorded as 85 years old, i.e., the previous stand was clear-cut during World War II and then regenerated partly by coppices, which was a standard management method at that time. Besides the seed-originated saplings, the stand is characterized by stump sprouts and root suckers in a roughly even but completely random way (Figure 11). And the presumably seedling-deficient patches were planted with Slavonian oak individuals. Some of these were found in the valley in areas with better water supply, but also in the southwest-facing hillside part of the site, where we found Slavonian oak trees. Genetic analysis (nSSR markers) revealed a relatively low number of clones. This is in contrast to a previous study [45], where, due to long-term intensive human impacts, the proportion of clones was significantly higher, where the studied 59 *Q. robur* and 21 *Q. petraea* tree individuals shared only 14 genotypes. Based on our results, vegetative reproduction by coppicing did not radically affect the genetic structure of the population. Comparing the seed vs. vegetative origin genotypes (allele frequency, allele number, heterozygosity, or fixation index), only slight differences were found between the vegetative cohort (i.e., the parent generation) and the seed origin cohort (i.e., the offspring generation). The evolution of the number of unique alleles in two groups indicates a difference between vegetative and seed-origin individuals, with a significantly high number of unique alleles within the seed-origin path in the case of cluster 1. In contrast, for cluster 3, the opposite is true, where the vegetative origin individuals hold more unique alleles. There are no significant differences in the observed heterozygosity, but in cluster 2, cluster 3 and cluster 4, individuals of vegetative origin have higher observed heterozygosity. The fixation index indicates a slight heterozygote excess in individuals of seed origin in cluster 2 compared to vegetative origin. There is no significant difference between the seed and vegetative cohorts for the other clusters, but a shift from the homozygote excess can be observed to the equilibrium in the seed-derived generation.

Analyzing the spatial structure of the different taxonomic groups (Figure 9), despite the relatively low number of sampled trees, we can see that the *virgiliana*-type trees (pure and hybrids together) can typically be found on the southeast-exposed ridges and sides of the hill. In contrast, the *petraea* (including hybrids) types were found in western exposures and on the sides of valleys, which are less affected by direct sunlight and where air humidity may be higher than in the more light-exposed southern microsites. Autochthonous *robur* trees (including their hybrids) are typically found in small valleys or lower hillsides where a notable water supply can increase the soil’s productivity and make the microsite patches wetter than on ridges or steeper hillsides. Nevertheless, no clear and striking spatial taxon structure was found, probably influenced by the small sample size and relatively small area. Further studies in this geographical region would be worthwhile, with a larger area and sample size, but in habitats with similar characteristics.

Our results supported our hypothesis that the gene pool of the mixed oak population, including a high number of trees with xeromorphic characteristics, has been adapted to the xeric ecological factors of the forest-steppe climate zone. Due to the high introgression rate

of *Q. virgiliana*, which was verified by both morphological traits and genetic markers, the local population might be tolerant to drought and heat, which are frequent climatic factors in the region [<http://www.ertigis.hu>, accessed on 28 May 2025]. A previous study [7] pointed out that *Q. virgiliana*-type oak trees on wood pastures can persist and regenerate in an arid landscape dominated by agriculture for centuries. Drought tolerance is a critical factor for the native forests distributed in the region, and native oak taxa are determinant in this context. The analyses of historical maps confirmed that there has been continuous agricultural activity in the area over several centuries, in which the forest patches, including this study area, have been determinatively shaping the landscape and probably maintaining optimal climatic conditions for arable farming, but not for forests.

Due to the expected direction and magnitude of climate change [1,3]—strong warming and drought—such mixed oak populations and their gene pool will be crucial genetic resources (high heterozygosity values, hybridization, continuous hybrid transition between basic taxa). In a large part of the Pannonian Basin, the current forest-steppe climate zone is predicted to be replaced mainly by a steppe climate. The lower region of the deciduous forest zones, dominated currently by *Q. cerris*—*Q. petraea* mixed forest communities, will be shifted in conditions typical of the forest-steppe climate the ± 200 m a.s.l. zone recently [1–3]. The studied population is located in this altitudinal belt, and the hybrid genotypes of *Q. virgiliana*, which are typical for this population, are likely to be able to adapt to further climatic changes. This mixed oak stand, with its current species composition and genetic and spatial structure, is expected to be able to fulfill all the requirements of an *in situ* gene conservation stand.

Within the framework of the gene conservation program, reproductive material (seed, seedling) has already been collected from most of the selected plus trees and preserved in *ex situ* conservation stands and seedling-seed orchards. The *ex situ* stands, and seed orchards were established in areas of similar habitat conditions in the region, but typically in higher altitudes, which are still in the xeric deciduous forest zone (200–300 m a.s.l.) and will probably be sustainable in the long term, despite the adverse climate change effects predicted.

5. Conclusions

As a consequence of our results related to the phenotypic traits, genetic analyses, and spatial structure of the oak trees, the following priorities are proposed for better forest management:

1. The non-autochthonous (planted) trees should be removed whenever the next thinning is carried out to preserve the adapted local gene pool as much as possible.
2. Preference should be given to trees with drought tolerance phenotypic traits of *Q. virgiliana* (e.g., hairiness, bark type, and oil-striped acorn) in silvicultural interventions (thinnings, natural regeneration).
3. The seed vs. vegetative origin should not be a thinning consideration for trees. Traditional practice favors trees of seed origin over coppiced trees. In addition to the criteria described in the first two points, selection should be based on each tree's general thinning aspects or health condition.
4. The slight allelic decline in seed-originated trees (offspring generation) underlined the importance that the *in situ* gene conservation stands should not be maintained as an even-aged stand. Natural regeneration techniques typically carried out over a short period (3–10 years) and based on seedlings of a single crop year increase the potential for allelic loss, which may be exacerbated by the isolated and fragmented pattern of the studied gene conservation stand, which may also reduce the potential for external pollen flow.

5. If natural regeneration by seed is not possible - in whole or in part -, the use of local reproductive material must be preferred in the case of both reforestation and replacement (assisted regeneration, assisted gene flow). However, the first two priority points should also be considered.

The success of dynamic gene conservation programs primarily depends on how the genetic diversity of the target species or population is preserved and how well the genotypes represent the gene pool of the original populations as recommended by the EUFGIS minimum requirements (<http://portal.eufgis.org/genetic-conservation-units/>, accessed on 28 May 2025). A further important factor is to consider all the ecological aspects, similar to natural circumstances, for the maintenance of the evolutionary capacity of the preserved population [8,44]. For example, the external pollen flow helps to maintain the adaptability of oak populations, basically by the consequences of hybrid effects [9,36,39–41]. Small, isolated oak populations within a large agriculture-dominated landscape, like this studied population, can interconnect larger woodlands located even at a wider distance to ensure pollen flow connection (flagstone population). Genetic information, therefore, helps both to maintain *in situ* reserves and to measure the effectiveness of *ex situ* conservation on an ongoing basis. By the use of this information, we have a chance to decide which phenotype is likely to be successful based on its underlying genotype, at present or in the future, under changing—typically warming and drying—climatic, water management, or even eroded soil conditions.

Supplementary Materials: The following are available online at: <https://www.mdpi.com/article/10.3390/f16060939/s1>. Table S1. Listed taxa, coordinates, on-site morphological status and origin of sampled trees, Table S2. Description of SSR markers and PCR protocols, Table S3. A complex assessment of nSSR and chloroplast haplotype pattern to analyse autochtony of robur type trees.

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