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Genetic Characterization of Putative Sources of Ash Dieback Tolerance in Hungary

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Abstract

Ash dieback is an often-fatal disease caused by the fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya. It emerged in Europe during the 1990s and poses a substantial threat to ash populations. In Hungary, symptoms were first detected on common ash (*Fraxinus excelsior* L.) in 2008. The disease also severely impacts another native species, the narrow-leaved ash (*Fraxinus angustifolia* Vahl). An effective strategy for counteracting ash decline is to identify and utilize sources of tolerance. We are monitoring the health status of the selected trees that demonstrate low susceptibility (plus trees) and conducting molecular genetic studies to enable their genetic characterization and individual identification using 16 nuclear microsatellite (nSSR) markers. The PCoA (Principal Coordinates Analysis) separated the eight assessed groups into two distinct clusters based on the taxonomic traits. Based on the Structure analysis results, K = 2 was the most probable cluster number. Hybridization was also indicated in the case of several individuals across various groups. We intend to incorporate the results in the establishment of seed orchards using the selected plus trees, considering the taxonomical, geographical, and genetic distinctiveness of the different groups.

Keywords: *Fraxinus*; ash dieback; tolerance; nuclear SSRs; genetic structure; plus tree

1. Introduction

The incidence of the introduction of invasive forest pathogens, especially ascomycetes, has increased sharply in recent decades [1], with the spread of these pathogens being particularly concerning given their potential to disrupt ecosystems. Over the past three decades, the persistence of common ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*Fraxinus angustifolia* Vahl) has been severely threatened by ash dieback (ADB), caused by the invasive ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya [2,3]. First observed in Poland in the early 1990s [4], the disease has swept through the continent [5] and led to extensive decline, raising concerns about the long-term viability of ash populations [6]. In Hungary, it was first isolated from *F. excelsior* shoots in 2008 [7]. Several years later, the disease was observed in ash stands across the entire country, regardless of their age, structure or isolation, causing crown degradation and, ultimately, severe mortality [8].

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Although strong natural selection is acting on affected populations [9,10], the current rate of evolutionary change may be insufficient to ensure evolutionary rescue under ongoing disease pressure [11]. In this context, genetic variability within tree populations is a critical prerequisite for adaptation to novel biotic stressors.

Considerable phenotypic and genetic variation in susceptibility among ash individuals has generally been reported (for a review, see [12]). Notably, some trees in heavily affected stands remain largely symptom-free, suggesting the presence of heritable tolerance and/or resistance mechanisms. Quantitative genetic studies using clonal and progeny trials have consistently reported moderate to high broad (H^2 = 0.1–0.57)—and narrow-sense (h^2 = 0.37–0.53) heritability for ADB resistance traits [13]. On the contrary, the lack of a significant relationship in susceptibility between parents and their offspring was reported in natural populations [14], pinpointing that environmental effects should also be considered. These findings indicate that a substantial proportion of the observed variation in susceptibility is genetically determined and can be transmitted across generations, enabling breeding and conservation programmes capitalizing on tolerant sources selected from disease-affected stands.

Genetic methods are used to assess the relationship between the resistance of identified parent–offspring pairs, to demonstrate a correlation [9]. Significant difference in the tolerance of nine *F. angustifolia* clones from two clonal seed orchards in Croatia was reported [15]. Differences in necrosis size occurred both within and between *F. angustifolia* and *F. excelsior* clones in a Slovakian experiment [16]. Complete resistance to fungal infection was absent in all examined clones. As in the previously mentioned clone experiment, a highly significant variance in susceptibility was detected among *F. excelsior* clones of Danish and Austrian origin [17]. However, in some cases, no correlation was observed between the susceptibility of mother trees and their open-pollinated offspring. Nevertheless, the resistance levels of the pollinator trees (fathers) were unknown during the study, in which 35 clones and 30 families were tested. Exceptionally high resistance was found in only 2% of the tested genotypes, but individuals with a certain degree of resistance were noticed in all families [13]. Accordingly, only a few of 50 preselected clones in a Lithuanian experiment displayed elevated disease resistance by the end of the study [18].

The aim of this study was to genetically characterize the selected tolerance sources in order to:

(1) Enable plus tree identification;
(2) Verify the taxonomic classification based on morphological traits;
(3) Assess the genetic structure of the selected sources;
(4) Verify whether these sources adequately represent the current genetic composition of existing ash stands.

This information is crucial for designing and establishing artificial populations with improved tolerance, which can be used to source forest reproductive material for conservation and habitat restoration activities, as well as for production forestry.

In this publication, to emphasize that the study was not focused on natural populations of ash species affected by ADB, we consistently use ‘group’ to refer to individuals (plus trees) selected from the same region and ‘cluster’ to refer to any aggregation of these groups.

2. Materials and Methods

2.1. Material Collection

During the selection of plus trees, we focused on individuals that appeared healthy within infected and strongly declining stands (Figure 1). Their condition, compared with that of the surrounding diseased trees, suggests that they may be tolerant or only slightly susceptible to the pathogen. The ICP manual was applied for the health state assessment [19]. The following criteria were used to designate plus trees: defoliation of less than 10%, with no visible twig dieback. During field monitoring, the condition of each tree is checked annually to determine whether its status has changed, specifically whether any crown symptoms indicative of *Hymenoscyphus* infection have developed.


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

Figure 1. Ash trees with varying levels of tolerance. (**a**) On the left, *Fraxinus angustifolia* Vahl trees displaying infection by *Hymenoscyphus fraxineus* fungus. On the right, mature individual that is presumed to be tolerant. Photo by András Koltay. (**b**) Variation in susceptibility among individuals of *Fraxinus excelsior* L. trees. Photo by Csilla Éva Molnár.

The genetic analyses were conducted on the previously selected 125 plus trees, originating from different regions of Hungary (Figure 2 and Table A1). During the fieldwork between 2021–2025, 37 *F. excelsior* and 88 *F. angustifolia* individuals were sampled from seven distinct regions (Ormánság, Bereg, Somogy, Tolna, Csepel, Bükk, Gemenc) across Hungary (Figure 2). In this study, *F. angustifolia* is represented solely by one of its subspecies, the Hungarian ash (*F. angustifolia* ssp. *danubialis* Pouzar), which is found in the Pannonian Basin [20]. The northern range of *F. angustifolia* overlaps with the distribution area of *F. excelsior*. Consequently, hybrid individuals exhibiting intermediate or mixed morphological traits may be present within these populations [21,22]. *F. angustifolia* stands characterize the floodplain forests of the Bereg, Ormánság, Somogy, Csepel, and Gemenc, whereas the mountainous region (Bükk) is dominated by *F. excelsior*. In the Tolna population, *F. excelsior* was the predominant species; however, a small number of individuals exhibited mixed traits according to traditional classification methods [23,24], indicating potential hybridization at this site. Species designation has been carried out on vegetative morphological traits [25], focused on discrete, binary characteristics (e.g., leaflet serration, bud colour) rather than continuous quantitative traits (e.g., leaflet number) to reduce ambiguity. Individuals exhibiting a combination of traits characteristic of both species were flagged as putative hybrids. All evaluations were conducted by a single evaluator.

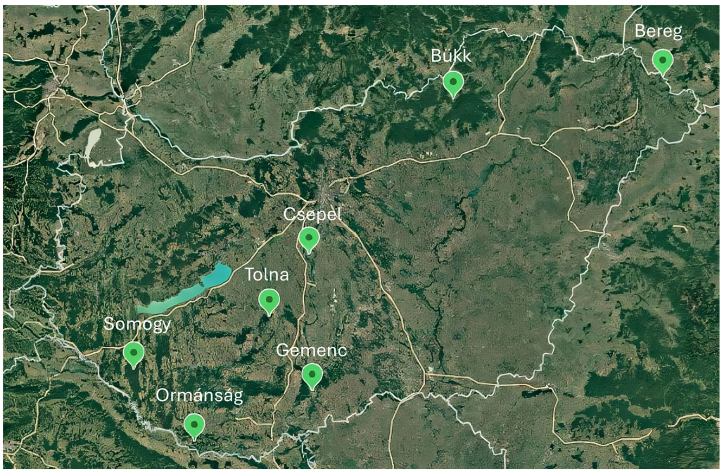

Figure 2. Origins of the selected genotypes/plus trees.

Moreover, in the Ormánság region, six obviously planted *F. excelsior* individuals were also selected as plus trees for further monitoring, even though their origin is unknown. These individuals were treated as a separate subgroup (Ormánság_B) in the genetic analyses.

2.2. DNA Extraction and PCR Protocol

Total genomic DNA was isolated from fresh leaves or wood core samples using a modified CTAB protocol [26,27]. To eliminate PCR inhibitors, DNA samples were purified using the NucleoSpin Inhibitor Removal Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. DNA concentration was quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to a final concentration of 10 ng/µL. Sixteen nuclear SSR markers were employed based on the literature [28–32]. Detailed marker information is available in Table A2. For the PCR, the Promega GoTaq G2 Flexi (Promega, Madison, WI, USA) polymerase kit was used. In the case of 12 primer pairs, the M13(-21) fluorescent dye-labeling method was applied [33], and the traditional method (forward primer with fluorescent label) was used in the case of four markers. Detailed PCR recipes and cycling conditions are provided in Table A3. PCR reactions were conducted in a Veriti Personal Thermocycler (Applied Biosystems, Foster City, CA, USA). PCR fragments were separated on a 1.75% agarose gel in 1× TAE buffer, stained with GelRed (Biotium, Fremont, CA, USA) to estimate concentration. Then, fragments were diluted (up to 20-fold) for capillary electrophoresis and multiplexed by dye and size in formamide (Hi-Di, Applied Biosystems, Waltham, MA, USA) using GeneScan LIZ 500 (Applied Biosystems, Waltham, MA, USA) internal size standard. SSR genotyping was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) by BIOMI Ltd. (Gödöllő, Hungary).

2.3. Data Analysis

The base-pair sizes of the amplified allelic variants were determined using the Osiris software (version 2.15.1004) [34]. The Micro-Checker software (version 2.2.3) was used to test for the presence of null alleles [35]. Missing data were validated with three independent PCR repetitions. A subset of samples (5–10 samples per locus) with unique allele composition was tested for adequate allele scoring with one PCR repetition.

The most important population genetic indicators (sample size—N, number of alleles—N_a, number of effective alleles—N_e, number of private alleles—N_{pr}, observed heterozygosity—H_o, expected heterozygosity—H_e, unbiased heterozygosity—uH_e, fixation index—F), along with a principal coordinate analysis (PCoA), were generated in GenAlEx (version 6.51b2) [36] using a Pairwise Population Matrix of Mean Population Codominant Genotypic Genetic Distance. For the visualization of the PCoA plot, the R package 'ggplot2' (version 4.0.1) [37] was used.

The partitioning of the molecular variance was evaluated by AMOVA in GenAlEx with 9999 permutations. Pairwise F_{ST} values were estimated using the Codom-Allelic distance option. The pairwise F_{ST} values and the significance level were interpreted in a heat map matrix format using the R package 'ggplot2'. In parallel, a UPGMA dendrogram was generated from the F_{ST} matrix using the 'aboot' function of the 'poppr' [38] package, with 1000 bootstrap replicates to assess node accuracy.

Individual genotypes were also evaluated using the Bayesian clustering method by STRUCTURE [39] with the following parameter settings: 500,000 repetitions in the burn-in period, followed by 800,000 MCMC repetitions, with K set 1–10, and 10 iterations in each K. The Admixture Model was used, without the LOCPRIOR option. The cluster numbers 1–10 were tested using both the Puechmaille [40] and Evanno methods [41] to evaluate the most probable K cluster. The results were generated through the StructureSelector platform (https://lmme.ac.cn/StructureSelector/) (15 December 2025) [42]. Visual representation was generated using the R package 'pophelper' (version 2.3.1) [43]. The proportions of the defined clusters for each group were plotted in QGIS (v 3.36.0) [44] using a pie chart.

3. Results

3.1. Analysis of Population Genetic Diversity

The 16 nSSR markers used in the study exhibited adequate polymorphism. During the laboratory process, all missing values were verified by performing three independent PCR reactions. Of the 125 analyzed samples, only nine had missing data in the final dataset for one locus each (Femsatl 10: 1/Gemenc; Fp14665: 1/Csepel, 4/Tolna; Fp18437: 1/Gemenc, 1/Bükk; FREX14: 1/Bükk). The probability of identity (P_{ID}) for increasing combinations of the 16 loci was calculated as 1.5×10^{-20} ($P_{ID} < 10^{-6}$), confirming the high discriminatory power of the selected markers. Furthermore, no matching genotypes were detected, underscoring the method's accuracy and its alignment with the species' reproductive characteristics. The sample set was tested separately for the two species using Micro-Checker to detect putative null alleles. Overall, the tested populations were in Hardy–Weinberg equilibrium, and there were no signs of large allele dropout. In the *F. excelsior* subgroup, null alleles were suggested in 5 of 48 locus × population combinations; five loci showed (FEMSATL10, FEMSATL19, FREX1, FREX14, FREX10) putative null alleles due to a general excess of homozygotes for most allele size classes. On the other hand, in the case of the *F. angustifolia* subgroup, null alleles appeared possibly in 13 of 80 locus × population combinations. In this case, 10 loci (FEMSATL04, FEMSATL10, Fp14665, Fp18437, Fp20456, FREX11, FREX14, FREX18, FREX31, M2-30(a)) showed putative null alleles; four loci out of these (FEMSATL04, FREX11, FREX14, FREX18) represented alleles of one repeat unit difference, as well.

The average number of alleles per locus (N_a) and effective alleles (N_e) reached the highest average values per locus in the groups that represented the highest sample size, namely the Bereg group ($N = 27$; $N_a = 11.563$; $N_e = 5.762$), the subgroup Ormánság_A ($N = 25$; $N_a = 10.625$; $N_e = 5.010$) and the Gemenc group ($N = 23$; $N_a = 10.125$; $N_e = 4.282$) (Table 1). These groups were all dominated by *F. angustifolia* individuals. The Bükk Mountain group also showed a high number of alleles and the highest number of effective alleles ($N = 17$; $N_a = 9.688$; $N_e = 5.776$). In line with this pattern, the number of private alleles was also higher in these sampling areas in the following order: Bereg, Bükk, Gemenc, and Ormánság_A ($N_{pr} = 1.438$, 1.375, 1.125, and 0.938, respectively). The three regions with the lowest allele number values were the Ormánság_B, Csepel, and Somogy, all with the smallest sample sets (six, nine, and four individuals, respectively). The expected heterozygosity exceeded observed heterozygosity in all groups (except for the Somogy and Ormánság_B groups), indicating a moderate heterozygote deficit. Consistent with this pattern, fixation indices (F) were positive across all analyzed populations, ranging from low (0.04–0.07) to moderate values (0.11–0.17). The Somogy and Ormánság_B groups were an exception, exhibiting a minor heterozygote excess resulting in a slightly negative fixation index ($F = -0.078$; $F = -0.012$), probably due to the limited sample sizes. In general, the *F. excelsior*-dominated groups represented higher expected heterozygosity values. The sample sets from the Bükk Mountains and the Csepel region showed the closest fixation indices to zero ($F = 0.007$ and 0.057, respectively), suggesting approximate Hardy–Weinberg equilibrium. On the other hand, the highest positive fixation index values were observed in the selected Hungarian ash samples of the Ormánság_A and Bereg regions ($F = 0.138$ and 0.159, respectively), indicating a moderate heterozygote deficit in these sample sets.

Table 1. Population genetic indices averaged across 16 SSR loci for the analyzed groups.

Region	Main Species	N [1]	N_a [2]	N_e [3]	N_{pr} [4]	H_o [5]	H_e [6]	uH_e [7]	F [8]
Bereg	*F. angustifolia*	27.000 (27)	11.563	5.762	1.438	0.574	0.676	0.688	0.159
Gemenc	*F. angustifolia*	22.875 (23)	10.125	4.282	1.125	0.586	0.656	0.671	0.110
Somogy	*F. angustifolia*	4.000 (4)	4.125	3.183	0.125	0.641	0.594	0.679	−0.078
Csepel	*F. angustifolia*	8.938 (9)	6.063	4.123	0.563	0.598	0.650	0.688	0.057
Ormánság_A	*F. angustifolia*	25.000 (25)	10.625	5.010	0.938	0.598	0.695	0.709	0.138
Ormánság_B	*F. excelsior*	6.000 (6)	5.500	4.114	0.500	0.688	0.676	0.738	−0.012
Tolna	*F. excelsior* (with putative hybrids)	13.750 (14)	7.875	4.902	0.625	0.618	0.699	0.726	0.119
Bükk	*F. excelsior*	16.875 (17)	9.688	5.776	1.375	0.703	0.717	0.738	0.007

[1] average sample size (original sample size), [2] number of alleles per locus, [3] number of effective alleles, [4] number of private alleles, [5] observed heterozygosity, [6] expected heterozygosity, [7] unbiased expected heterozygosity, [8] fixation index.

3.2. Patterns of Population Clustering

The molecular variance analysis (AMOVA) revealed that the majority of genetic variation (80%) resides within individuals relative to the total. The level of population structuring is 20%, partitioned into a 12% variance component attributable to differences among individuals within groups (corresponding F-statistic: F_{IS} = 0.139) and an 8% component attributable to differences among groups (F_{ST} = 0.075).

Pairwise F_{ST} values derived from the AMOVA showed generally low to moderate genetic differentiation among populations, with clear taxonomic structuring (Figure 3). The highest levels of differentiation were observed between the Bükk and most of the other groups (F_{ST} = 0.147–0.173), except for the Tolna and Ormánság_B. Among these, the separation of Bükk–Somogy was the most decisive (F_{ST} = 0.173). The lowest, non-significant F_{ST} value was observed between the Csepel and Ormánság_A groups (F_{ST} = 0.012). The low differentiation was also apparent between the Ormánság_A–Bereg (F_{ST} = 0.013), the Ormánság_A–Gemenc (F_{ST} = 0.016), followed by the Bereg–Csepel (F_{ST} = 0.02) and Bereg–Gemenc (F_{ST} = 0.026) groups. The UPGMA dendrogram highlights the separation of the Ormánság_B, Bükk and Tolna groups, indicating a *F. excelsior* branch. In the other branch, clustering of the *F. angustifolia*-dominated groups is evident, with a slight separation of the Somogy sample set. Detailed AMOVA statistics and the original pairwise F_{ST} matrix, with the corresponding *p*-values, are provided in Table A3.

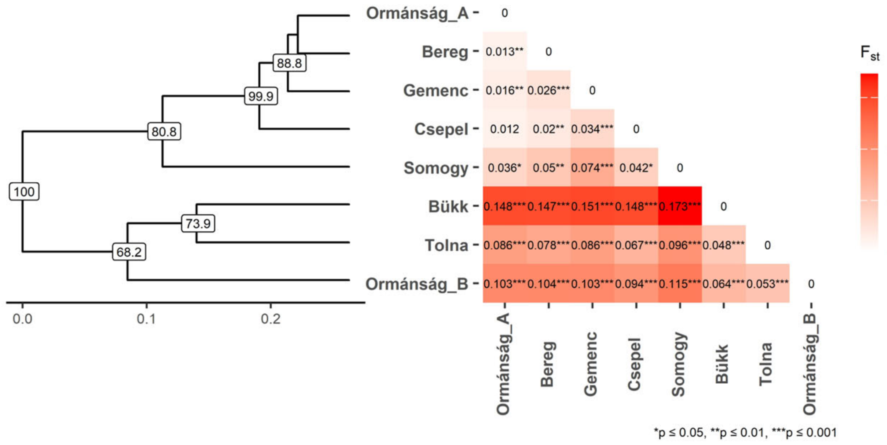

Figure 3. Heatmap of the pairwise population F_{ST} values with significance level and the corresponding UPGMA dendrogram.

The *F. angustifolia* sample subset was also analyzed alone. The overall partitioning of molecular variance revealed only 2% population differentiation (F_{ST} = 0.023), whereas

15% of the variance was attributable to within-group variation (F_{IS} = 0.153). 83% of the variance can be described within the individual level relative to the total. Pairwise F_{ST} values and significance levels were highly correlated with those from the overall analysis; therefore, they can be evaluated based on the upper part of Figure 3.

The genetic differences among the selected plus tree subsets were visualized using a PCoA, based on pairwise genetic distances among individuals (Figure 4). The separation of the Bükk samples is apparent in the first coordinate; individuals from the Tolna region occupied a transitional position, whereas the other groups formed distinct clusters, with mostly overlapping patterns. The Somogy group, despite being the smallest group in the analysis with only four samples, formed a clear, distinct group, rather than a scattered one. The slight separation of these samples could also be observed.

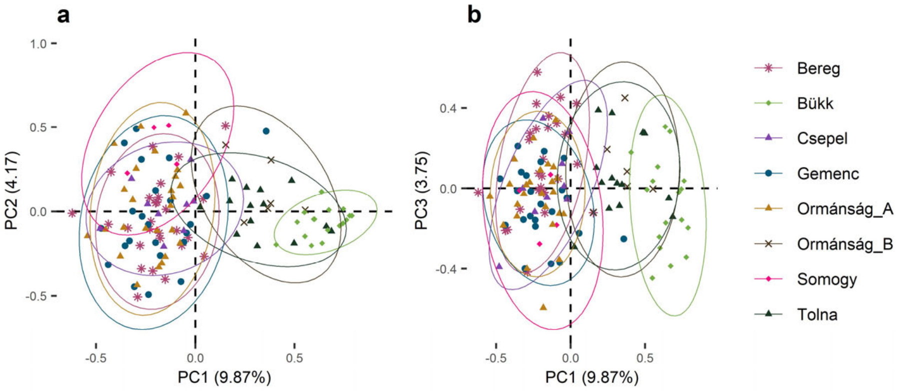

Figure 4. Individual-level PCoA analysis along (**a**) the first and second coordinates (PC1 and PC2), (**b**) the first and third coordinates (PC1 and PC3).

To reveal the genetic relationships among the seven sample groups, a population-level PCoA was also conducted. The first two axes explained 76.11% of the genetic variation (PC1 = 61.97%, PC2 = 14.14%) (Figure 5). Similar to the individual-based approach, the Bükk, Tolna, and Ormánság_B groups separated distinctly along PC1, indicating substantial genetic differentiation relative to the other groups; the Somogy group showed a slightly different pattern. Along the third coordinate (PC3 = 10.99%), a further component of the genetic variation can be observed: a distinction between the *F. excelsior* group of the Ormánság_B from all other groups.

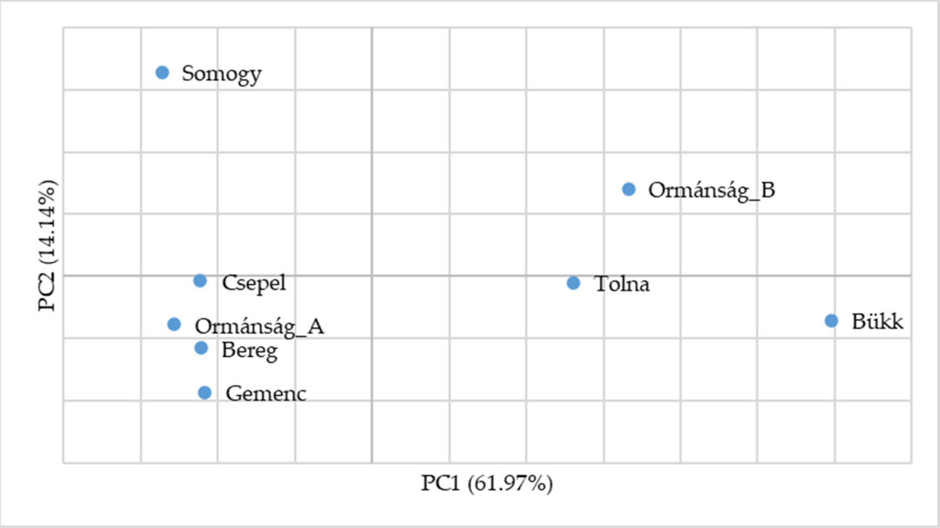

Figure 5. PCoA results based on genetic distances among the analysed populations plotted along the first and second coordinates (PC1 and PC2).

3.3. Genetic Structure Inferred by STRUCTURE

The Bayesian clustering analysis using STRUCTURE revealed K = 3 as the most likely number of clusters, with two main clusters (also confirmed by the K = 2 scenario): Cluster1 (blue) comprising the *F. excelsior* lineages, and Cluster2 (yellow) the *F. angustifolia* lineage. A third subcluster (Cluster3—red) emerged within the *F. angustifolia* group. This third Cluster was observed across all *F. angustifolia* groups; it contributed a higher proportion to the genetic composition of individuals in the Gemenc group. K = 3 was confirmed by the Puechmaille method, and as a sub-cluster by the Evanno method. An admixed assignment was observed in two individuals in the Bereg group, six individuals in the Gemenc group, and one individual in the Ormánság_B subgroup, mixed with the *F. angustifolia* samples. Signs of former hybridization (introgression) can be seen in the Tolna group among *F. excelsior* individuals (Figure 6).

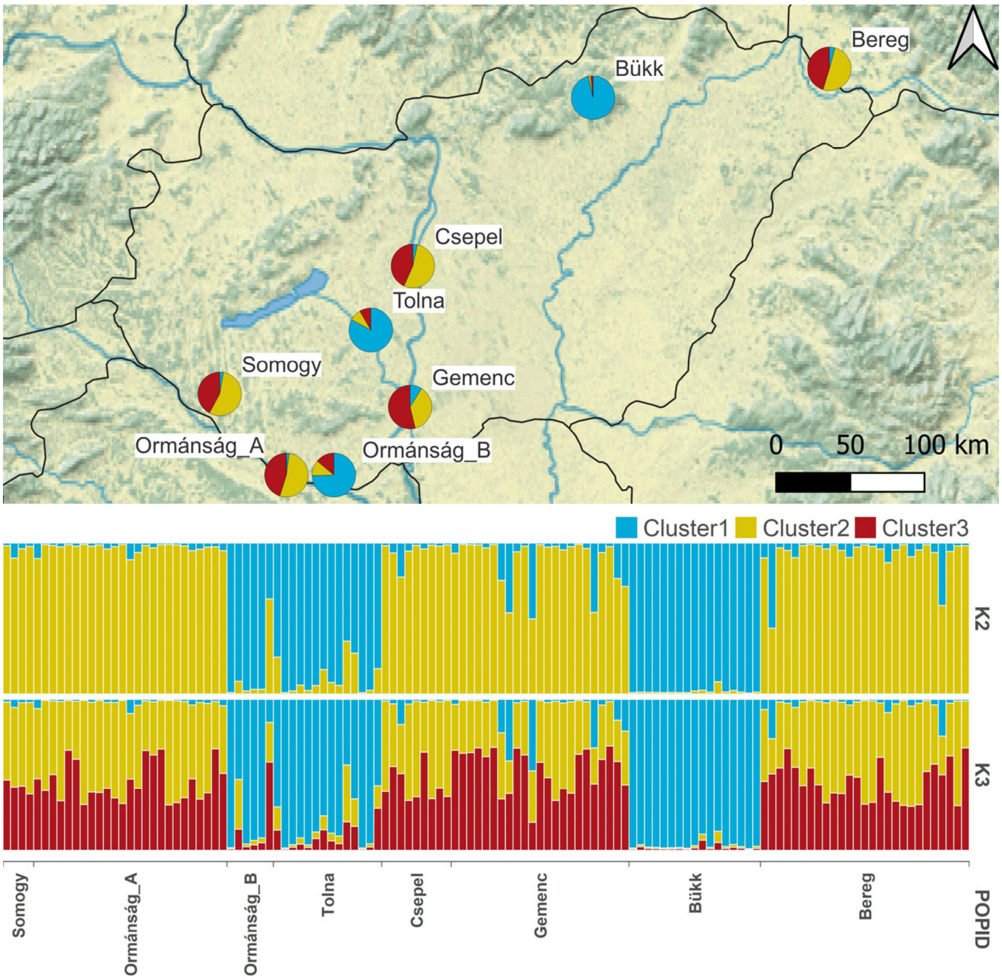

Figure 6. The population genetic clustering of the selected plus trees by the STRUCTURE analysis.

4. Discussion

The applied method was suitable for both the identification of selected plus trees and the taxonomic distinction between the two ash species. As expected, no matching genotypes due to clonality were found; all selected plus trees represented unique genotypes. However, from a technical aspect, null alleles should be considered with caution in subsequent population genetic studies.

Since *F. excelsior* and *F. angustifolia* have overlapping distributions in the eastern-central part of Europe, and moreover, these two species are taxonomically close relatives with a weak or absent reproductive barrier, the question of precise classification is crucial. One of the main aims of this study was to assess the suitability of nuclear SSR markers for taxonomic identification. Based on the results, we can conclude that the resolution of the 16 applied nSSR markers was sufficient to distinguish the two closely related ash species at the individual level, as well as their hybrids and introgressed forms due to former hybridization in their ancestry. Likewise, Fernández-Manjarrés et al. [45] successfully characterized introgression patterns in French hybrid populations using five microsatellite markers (FEMSATL 4, FEMSATL 11, FEMSATL 16, FEMSATL 19, and M2–30) in combination with morphometric data. In the context of genetic conservation of tolerant ash trees, this is an essential step. Outliers should be considered with caution when designing an artificial population, and hybrids are among the most apparent forms of outlying genotypes.

The second important question concerns the proper handling of the two species, particularly when they are selected from mixed stands or regions where they tend to occur together. Classical taxonomic approaches based on morphological traits of mature individuals are not always reliable and/or require highly specialized professional expertise. For this reason, molecular genetic techniques may serve as an objective and efficient tool for taxonomic classification. Thomasset et al. [46] conducted morphological and molecular characterization of reciprocal F1 hybrids between the two ash species. The study revealed that first-generation hybrids tend to exhibit intermediate morphological characteristics; however, some trait asymmetry can be explained by parental effects (e.g., stomatal traits). In this study, the combined use of molecular (SSR) markers and multivariate descriptors was a more effective tool for hybrid identification.

In the analyzed sample set, it was apparent that all the selected groups originated from different lowland areas in Hungary and contained some amount of *F. excelsior* individuals or ancestry. Despite the fact and preliminary concept that these, mainly riparian ecotypes, are dominated by *F. angustifolia* individuals. On the other hand, in mountainous regions, the dominance of *F. excelsior* may be more pronounced. However, in our study, we focused mainly on the *F. angustifolia* sites and only one mountainous region (Bükk Mt.) was involved in the research; yet, the dominance and taxonomic uniqueness of this sample set were apparent based on the genetic analysis.

Nuclear microsatellites are also an appropriate tool for evaluating genetic patterns and population diversity indices at the population level, considering these populations, whether natural or artificially selected. Although we do not have preliminary information on the natural genetic patterns of local ash populations in the Carpathian Basin, the study presented here can also provide some details on this aspect. Regarding the natural genetic patterns of *F. angustifolia*, a comprehensive study by this time evaluated 38 populations across nearly the entire natural range of the species using six nSSR markers [47]. The study revealed that natural populations in the western Mediterranean region and the Carpathian Basin (Pannonian region) exhibited the highest levels of genetic diversity prior to the ADB cascading throughout Europe. From this perspective, these regions are the most important reservoirs for conserving the genetic resources of *F. angustifolia*. On the other hand, we should keep in mind that our sample sets cannot represent the natural genetic pattern of a single geographic region, as strong selection processes acted on the sampling strategy, namely the selection of low-susceptibility sub-groups of a population, in some cases with very limited sample numbers. A project in Germany aimed to establish a genetically diverse seed orchard by grafting selected *F. excelsior* plus trees [48]. Molecular assessment of 361 candidate trees using 16 microsatellite loci revealed no significant genetic differentiation among the ash stands examined within a single region of Germany (Mecklenburg-Vorpommern), thereby supporting the establishment of a single, large seed orchard comprising all selected genotypes. Similarly, in Denmark, microsatellite analysis of 39 native *F. excelsior* clones, derived from 14 populations, confirmed clonal identity, and

STRUCTURE analysis using four SSR markers (FEMSATL11, 12, 16, 19) revealed a single, homogeneous gene pool (K = 1) with no geographic sub-structuring [49]. However, an Austrian study found that the allelic profiles of clones in the two seed orchards differed and closely resembled those of local seed lots [50].

In the study of non-selected trees with varying vitality, moderate to high genetic diversity and low interpopulation differentiation were reported across five Polish stands [51]. In this case, the mixed genetic background detected among declining trees (STRUCTURE K = 3) indicated that certain genetic lineages may be more vulnerable, underscoring their relevance for identifying and selecting potentially resistant genotypes.

Nevertheless, we can conclude that the riparian sites supplied genetically similar resources across three different river corridors: the Danube (Csepel-Gemenc), the Drava (Ormánság), and the Tisza (Bereg). The slight distinctiveness of the Somogy group may resonate with an as-yet-undiscovered sub-pattern in native *F. angustifolia* populations. The main ecotype differences, namely, gallery high forest and flood plains along riversides versus mixed gallery forests along smaller rivers, may lead to differences in the genetic pattern as well. To prove or disprove this concept, a broader sampling is necessary at these specific sites located further from the vicinity of the main rivers. Since only four individuals have been identified as appropriate for plus tree selection at this time in the Somogy region, additional sampling is essential to capture additional features of this location. Temunović et al. [52] investigated genetic variation using nuclear SSR markers in natural populations of *F. angustifolia* across three biogeographical regions (Mediterranean, continental, and Alpine). Their results indicated that environmental variables shaping habitat conditions—particularly temperature, precipitation, and elevation—contribute more substantially to patterns of genetic differentiation among populations than geographic distance. In the continental region, gene flow among populations appears to be facilitated by major river systems and their associated floodplains, where pollen and seed dispersal are less constrained. Conversely, in the comparatively drier Mediterranean region, populations occupying suitable habitats tend to become more isolated, thereby restricting allele exchange and reducing overall gene flow.

The forests in the Carpathian Basin have been shaped by a long history of anthropogenic pressure. Uncontrolled seed transfer and planting of non-local material have disrupted, or at least seriously affected, the natural stands, diversity patterns, and local gene pools of the major tree species. While resistant trees can be found in non-autochthonous stands, careful consideration should be given to the role and use of outlier genotypes when establishing artificial populations that should match the existing local genetic diversity and structures. Consequently, identifying these outliers is essential, too.

In the Ormánság region, ten plus trees were selected from artificial stands or non-forest land (e.g., rural areas, alleys, amenity plantings). The analyses have clearly clustered six trees exhibiting obvious *F. excelsior* morphology (Ormánság B subgroup), while the other *F. angustifolia* genotypes resemble the local natural stands. The applied markers were suitable for both identifying the appropriate taxon and for characterizing the artificial subgroup.

Presumably, the Tolna group also bears traces of anthropogenic influence. This hilly region is characterized mainly by arid climatic conditions and can therefore be considered the xeric edge for both ash species. The local *F. excelsior* population here is possibly non-autochthonous; however, no information of artificial origin is available, either. The selected plus-tree individuals from the Tolna stand exhibited substantial mixed traits in morphology, and the genetic pattern of this group was remarkably transitional between the two ash species' point clouds in the PCoA plot. On the other hand, the individual-based Structure analysis did not confirm the presence of first-generation (F1) hybrids; only a hint of introgression could be detected in individual genotypes. However, a detailed morphological description was not provided in this study; this finding again draws our attention to the locally specific, non-typical sites and the selection effects they may entail.

The third main objective of our study was to evaluate the genetic diversity of the selected plant material and, if necessary, delineate any clusters from the analyzed groups. Overall, the diversity indices indicate that all groups exhibited high genetic variability, regardless of sample size. In general, the highest heterozygosity values were observed in the *F. excelsior* groups. Genetic analysis of two approved and three non-approved *F. excelsior* seed stands in Slovenia using five nuclear microsatellites revealed high diversity ($H_E = 0.80$) and low differentiation ($F_{ST} = 0.018$) [53]. By contrast, the more extensively evaluated *F. angustifolia* groups appeared relatively similar from a genetic perspective. Similar results were obtained in a study of *F. angustifolia* populations in Croatia, where the studied stands exhibited high within-population polymorphism but low among-population differentiation [52]. Groups from the two main floodplain areas (Bereg, Csepel-Gemenc), including the Ormánság region, exhibited the most similar genetic patterns. All three were represented with the highest sample size. Designing an artificial plantation, these three groups can probably be clustered together; however, verification of the local population genetic pattern is still needed. On the other hand, the potential uniqueness of the Somogy region should be further explored by selecting and evaluating additional plus trees.

5. Conclusions

The nuclear SSR approach reliably distinguished both individual plus trees and the two ash species. The absence of clonal duplicates confirms the genetic uniqueness of the selected trees.

The results indicate that riparian forest populations along the Danube, Drava, and Tisza River corridors showed considerable genetic similarity, suggesting substantial gene flow within these floodplain systems. Suspected introgression (Tolna) and anthropogenic influence (Ormánság_B) in some stands highlight the importance of careful taxonomic verification and the identification of outlier genotypes when establishing artificial populations or seed orchards. Molecular tools provide an objective basis for such decisions, particularly in regions where morphological identification is ambiguous due to hybridization or site-specific selection effects.

This study contributes insights into the genetic structure and diversity of selected ash material in the Carpathian Basin and provides information for conservation, breeding, and restoration strategies aimed at maintaining adaptive potential and local genetic integrity in the face of ongoing environmental pressures.

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Appendix A

Table A1. Detailed geographic data of the selected plus trees.

Geographic Region	Forestry Subcompartment ID	Number of Selected Plus Trees	Number of Observation Years	Latitude	Longitude	Elevation (a.m.s.l.)
Bereg	Beregdaróc 6/C	4	5	48.22836000	22.51054000	114
	Beregdaróc 7/F	5	5	48.22723000	22.50767000	114
	Darnó 1/C	4	5	47.95419000	22.67740000	119
	Gelénes 6/A	5	5	48.18098000	22.40102000	115
	Jánkmajtis 9/C	4	5	47.93594000	22.68354000	124
	Kömörő 5/F	2	5	48.02933000	22.61277000	116
	Kömörő 7/D	1	5	48.02891000	22.61290000	114
	Kömörő 8/A	2	5	48.02876000	22.61369000	115
Gemenc	Baja 48/I	5	1	46.20144000	18.88310000	94
	Dunaszentbenedek 1/E	6	1	46.59380000	18.88005000	96
	Hercegszántó 3EY1&2	5	1	45.94092000	18.86528000	93
	Homorúd 36/C	1	1	45.94145000	18.85423000	89
	Homorúd, non-forest land	1	1	45.94128000	18.85425000	88
	Őcsény 39/K	5	1	46.31946000	18.86584000	98
Somogy	Kaszó 26/F	4	5	46.31924809	17.23034478	153
Csepel	Makád 5/H	9	3	47.08240000	18.90227000	103
Ormánság_A	Bogdása, non-forest land	1	5	45.87831000	17.78990000	100
	Bogdása, non-forest land	2	5	45.89869000	17.79471000	97
	Drávaiványi 10/A	1	4	45.83780000	17.81162000	116
	Drávaiványi 8/C	5	4	45.83535000	17.80819000	115
	Lakócsa, non-forest land	1	5	45.90330000	17.68387000	102
	Lakócsa 33/A	3	5	45.90455000	17.68174000	102
	Lugi Forest	13	5	45.91425000	17.65435000	105
Ormánság_B	Bogdása 24/D	6	5	45.89840000	17.79606000	97
Tolna	Kisszékely 6/G	3	3	46.69996000	18.54032000	161
	Kisszékely 7/I2	5	3	46.69978000	18.53861000	165
	Simontornya 3/B	6	3	46.72527000	18.53865000	174
Bükk	Szilvásvárad 58/B	3	1	48.06238000	20.46820000	917
	Szilvásvárad 67/F&G	10	1	48.06211000	20.46782000	916
	Szilvásvárad 74/D&E	7	1	48.05792000	20.42849000	864

Table A2. Summary of Marker Sets Information.

Marker Name	Fluorescent Label	Marker Set	PCR Method	Reference
Fp14665	VIC	Set1	M13 [33]	Noakes et al. 2014 [28]
Fp18437	NED	Set1	M13 [33]	Noakes et al. 2014 [28]
Fp21064	PET	Set1	M13 [33]	Noakes et al. 2014 [28]
Fp20456	NED	Set1	M13 [33]	Noakes et al. 2014 [28]
FEMSATL11	PET	Set1	Traditional	Lefort et al. 1999 [29]

Table A2. *Cont.*

Marker Name	Fluorescent Label	Marker Set	PCR Method	Reference
FEMSATL04	VIC	Set1	Traditional	Lefort et al. 1999 [29]
FEMSATL19	NED	Set1	Traditional	Lefort et al. 1999 [29]
FEMSATL10	6-FAM	Set1	Traditional	Lefort et al. 1999 [29]
FREX11	HEX	Set2	M13 [33]	Hartung et al. 2022 [30]
FREX14	ATTO550	Set2	M13 [33]	Hartung et al. 2022 [30]
FREX10	HEX	Set2	M13 [33]	Hartung et al. 2022 [30]
FREX18	56-FAM	Set2	M13 [33]	Hartung et al. 2022 [30]
FREX31	56-FAM	Set2	M13 [33]	Hartung et al. 2022 [30]
FREX25	ATTO565	Set2	M13 [33]	Hartung et al. 2022 [30]
ASH7867	ATTO565	Set2	M13 [33]	Bai et al. 2011 [31]
M2-30 (a)	ATTO550	Set2	M13 [33]	Brachet et al. 1999 [32]

Table A3. PCR Cycling and Reaction Conditions.

Marker Name	PCR Method	PCR Recipes	PCR Conditions
Fp14665, Fp18437, Fp21064, Fp20456, FREX11, FREX14, FREX10, FREX18, FREX31, FREX25, M2-30 (a), ASH7867	M13 (-21) fluorescent dye-labeling [33]	1 × reaction buffer (Promega GoTaq G2 Flexi, 5× buffer without Mg^{2+}) 2 mM $MgCl_2$ (Promega, 25 mM stock) 0.2 mM of each dNTP (Promega dNTP mix, 10 mM each in stock) 0.05 µM for the forward primer (IDT 10 µM in stock) 0.2 µM each for the reverse and M13(-21) primers (IDT/ABI, 10 µM in stock) 0.375 U of polymerase (Promega GoTaq G2 Flexi, 5 U/µL) PCR clear ddH_2O in a total volume of 15 µL	An initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 45 s; an additional 8 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min.
FEMSATL11, FEMSATL04, FEMSATL19, FEMSATL10	Traditional	1 × reaction buffer (Promega GoTaq G2 Flexi, 5× buffer without Mg^{2+}) 2 mM $MgCl_2$ (Promega, 25 mM stock)/2.5 mM $MgCl_2$ for FEMSATL11 0.2 mM of each dNTP (Promega dNTP mix, 10 mM each in stock) 0.05 µM for the forward primer 0.2 µM each for the reverse and M13(-21) primers (ABI, 10 µM in stock) 0.375 U of polymerase (Promega GoTaq G2 Flexi, 5 U/µL) PCR clear ddH_2O in a total volume of 15 µL	An initial denaturation at 96 °C for 5 min, followed by 35 cycles consisting of 94 °C for 1 min, annealing at 52 °C (for FEMSATL11 and FEMSATL19) or 55 °C (for FEMSATL10) for 1 min, and 72 °C for 30 s, followed by a final elongation step at 72 °C for 10 min.

Table A4. Summary of the AMOVA analysis with the F-statistics.

Source	df	SS	MS	Est. Var.	%
Among Pops	7	141.003	20.143	0.456	7%
Among Indiv	117	755.081	6.454	0.787	13%
Within Indiv	125	610.000	4.88	4.88	80%
Total	249	1506.084		6.123	100%
F-Statistics	Value		p (rand >= data)		
F_{ST}	0.075		0.0001		
F_{IS}	0.139		0.0001		
F_{IT}	0.203		0.0001		

Table A5. The pairwise population F_{ST} matrix derived from AMOVA applying 9999 permutations (F_{ST} values below diagonal, corresponding *p*-values above).

	Ormánság_A	**Bereg**	**Gemenc**	**Csepel**	**Somogy**	**Bükk**	**Tolna**	**Ormánság_B**
Ormánság_A	0	0.0016	0.0014	0.0796	0.0163	0.0001	0.0001	0.0001
Bereg	0.013	0	0.0001	0.0065	0.0022	0.0001	0.0001	0.0001
Gemenc	0.016	0.025	0	0.0005	0.0008	0.0001	0.0001	0.0001
Csepel	0.010	0.019	0.033	0	0.0293	0.0001	0.0001	0.0001
Somogy	0.036	0.051	0.073	0.038	0	0.0001	0.0001	0.0001
Bükk	0.147	0.146	0.149	0.142	0.170	0	0.0001	0.0001
Tolna	0.089	0.082	0.091	0.067	0.097	0.048	0	0.0001
Ormánság_B	0.103	0.104	0.103	0.093	0.115	0.063	0.058	0

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