Genetic diversity in a historic lime tree allée of Széchenyi Castle in Nagycenk, Hungary

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Abstract Trees are key components of historic gardens and are valuable sources of genetic diversity that need preservation to enrich native populations' genetic basis in order to enhance long-term viability, and facilitate the species' adaptation to changing climatic and environmental conditions. The primary objective of our study was to assess the genetic diversity, structure and relationships of the *Tilia cordata* Mill. allée at Széchenyi Castle in Nagycenk (UNESCO heritage site), Hungary, to infer the late-Baroque garden's rich genetic heritage and conservation importance. The small leaved lime individuals at Nagycenk, with an age of 200 years, maintained high genetic diversity with non-significant inbreeding and high allelic richness and number of private alleles. Genetic diversity was similar to Central European

natural populations and slightly higher compared to other plant collections, e.g. Eszterházy Castle garden at Fertőd. The surveyed trees exhibited two age groups unrelated with shallow genetic differentiation indicating the potential seed source origin from the local geographic area. Bayesian clustering of individuals showed significant differences from the individuals growing at Fertőd-Esterházy gardens (approx. in a 15 km distance) and other *T. cordata* cultivars. We concluded that the *T. cordata* collection of Nagycenk is a unique genetic heritage of the former natural vegetation due to its genetic variability and divergence, which would be a valuable source of future breeding programs and conservation.

Keywords Lime tree · Microsatellite · Polymorphism · *Tilia cordata* · Genetic heritage · Late-Baroque lime tree allée

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Introduction

The importance of historic gardens in preserving genetic diversity is widely recognised. Consequently, the cultivation of living plants in ex-situ collections has been going on for a long time (Miller 2004; Wolff et al. 2019). Plant collections can avoid the harmful effects of human population growth (anthropogenic activities), adverse industrial effects, ecological/biological threats and natural disasters (Edwards and Jackson 2019).

Artificial populations are capable of maintaining genetic diversity for the long term, preserving the genetic fitness of individuals and populations to maintain population size, especially in case of species experiencing a decline in genetic variation and increasing inbreeding as well as the risk of extinction (Frankham and Ralls 1998; Frankham 2005). Also, they represent genetic variability of current or former natural populations while preserving alleles specific to the geographic region (Potter et al. 2017). Plant collections in historical gardens consist of not only herbaceous plants but also forest tree species. In particular, ancient or veteran forest trees are of biological, aesthetic and cultural interest due to their age and size (Ciaffi et al. 2018). Such species and their individuals require a high level of genetic variation to adapt to changing environmental effects because they are long-lived, stationary, and often widespread life forms compared to other plants (Potter et al. 2017).

Small-leaved lime (*Tilia cordata* Mill.) is a long-lived forest tree native to most parts of Europe, and it is the most widespread among the other *Tilia* species (Eaton et al. 2016). The species is a lowland tree with an extensive geographical range; it tolerates a vast range of climatic conditions (Pigott 2012). Nowadays, its occurrence in natural sites is rare and scattered, mostly found in mixed stands. However, lime is frequently planted in urban areas as shade and ornamental and street trees (Eaton et al. 2016; Fineschi et al. 2003). Regeneration can either happen from generative or vegetative reproduction, though many factors hamper sexual reproduction. For example, fertilisation is very sensitive to temperature, while seed ripening is more exposed to nutrient availability. However, *T. cordata* regenerates from vegetative reproduction, which is a life strategy of the genus *Tilia*. The species produces clump shoots and sprouts and thus ensures a much longer lifetime as sprouts can replace the old, dying parent tree (De Jaegere et al. 2016).

The lime tree allée of Nagycenk, which belongs to the Széchenyi Castle, was planted between 1754 and 1760. Today the area is part of the Fertő/Neusiedlersee Cultural Landscape UNESCO World Heritage Site (Harlov-Csörtán, 2017) and is managed by the Eszterháza Cultural Centre. In addition, it is a nature conservation area and a landscape value protected by the Fertő-Hanság National Park, Hungary (Gerzson et al. 2012).

Initially, around 600 individuals, mostly *Tilia cordata*, were planted from propagating material of unknown origin, most probably seedlings from the surrounding forests. However, today this number has decreased significantly; the allée consists of 499 individuals, including many replacements as well. According to previous surveys (Gerzson and Szilágyi 2011), about 20% of today's individuals are still from the original planting or a very early replantation. Nowadays replanting and full allée renewal are becoming more and more urgent due to the deteriorating health of the trees. According to the assessment performed in 2018, the deterioration process is rapidly proceeding. Even though the life span of *T. cordata* can reach up to 1000 years, the first signs of rot inside the trunk may occur at around 250 years (De Jaegere et al. 2016). On the other hand, the original stem can be retained for approximately 300 years (Pigott 1989). Therefore, it will be necessary to renew the allée in the near future. Consequently, it is crucial to be aware of the genetic composition of the current and the original stock, as this knowledge may affect the renewal's planting strategy and especially the propagating material for replantation.

Genetic diversity within the genus *Tilia* has been assessed only in a few studies using different molecular markers. The first studies aimed at determining parents of hybrids, differentiation within and among populations. The development of molecular markers is apparent, just as in the case of any other plant species. The early studies have been performed by RFLP (Fineschi et al. 2003) and allozymes (Fromm and Hattemar 2003). The next choice of marker system was RAPD (Liesebach and Sinkó 2008; Hosseinzadeh Colagar et al. 2013). Nuclear rDNA ITS was used for species differentiation by Yousefzadeh et al. (2012). The first set of microsatellite markers have been developed for *T. platyphyllos* by Phuekvilai and Wolff (2013); they also reported the markers' transferability to other *Tilia* species. Later the same research group used these SSR markers successfully for assessing genetic diversity in populations of *T. platyphyllos* and *T. cordata* (Logan et al. 2015). Mylett (2015) developed SSR markers for *T. cordata*, and also in that study, transferability to *T. platyphyllos* was reported.

According to our hypothesis, in addition to the cultural and historical significance of the lime tree allée of Nagycenk, Hungary, it can also represent an outstanding value from the gene conservation point of

view, being an ex-situ gene reserve for an essential European tree species. Therefore we aimed to assess the genetic diversity of the allée with SSR markers.

Material and methods

Plant material and sampling strategy

In 2018, a detailed visual and instrumental tree assessment of the Nagycenk allée created the scientific fundamentals for the allée renewal (Szilágyi and Szabó 2020). Based on the tree survey, 72 old lime trees have been selected in the present study. The trees chosen have been estimated to be around 150 to 200 years, based on the International Society of Arboriculture (Lukaszewicz and Kosmala 2008). Some trees might be even older, but as the trunk was already split or the sprouts grew close to the original trunk, it was hard to measure the parameters of the original trunk. The allée contained few *T. platyphyllos* individuals, of which four were also included in our study to confirm their identity.

The individuals from Nagycenk have been divided into two groups, “Nagycenk” and “Nagycenk old individuals”. This partition was done based on the trunk diameter, with the boundary between the two groups at 100 cm. In addition, twenty-one *T. cordata* individuals from the Fertőd Eszterházy Castle (located 15 km away) were also included in our study. Trees with similar age and growth parameters stand here with allée replantation documentation dating to the very beginning of the 20th century. Hence, those trees are approx. 120 years old.

According to the trunk diameter based age estimation for both the Nagycenk and Fertőd we can state that trees with a diameter of 1 m or more are at least 200 years old; this age estimation refers to 20 individuals of the study (“Nagycenk old individuals”). The other 52 trees are younger, but still many are around 150 years. This is because the even younger trees have been omitted already at the sampling time.

As a control group, we used six, nowadays popular small-leaved lime cultivars within the study (five *T. cordata* cultivars. ‘Rancho’, ‘Savaria’, ‘Roelvo’, ‘Greenspire’, ‘Winter Orange’, and a *Tilia* hybrid ‘Szent István’). Their leaves were collected in the Soroksár Experimental Field of the Hungarian University of Agriculture and Life Sciences (MATE). All

leaf samples’ collection happened at the end of the summer of 2018. The leaves were stored at $-20\text{ }^{\circ}\text{C}$ until used.

DNA extraction and fragment sizing

DNA extraction from the frozen leaves was performed with SP Plant Mini Kit (Omega, VWR International Kft, Budapest). The DNA concentration and quality were assessed using NanoDrop (BioScience, Hungary) and visually checked on 1% agarose gel.

Nine SSR (Simple Sequence Repeat) primers (Tc963, Tc927, Tc937, Tc7, Tc31, Tc951, Tc5, Tc11, Tc915) were chosen based on the study of Phuekvilai et al. (2013). PCR was performed in 25 μl reaction volume containing 20–80 ng DNA, 10X PCR reaction buffer, 2.5 mM MgCl_2 , 0.02 mM dNTP mix, 2.5 μmol of primers (forward primers were fluorescently labelled with 6-FAM), 0.5 unit of DreamTaq DNA polymerase (Fermentas, Szeged, Hungary) 1% BSA and 2% DMSO and sterile distilled water. For the amplification of SSR fragments, the following program was used: initial denaturation at $94\text{ }^{\circ}\text{C}$ for 5 min; followed by 30 cycles of $94\text{ }^{\circ}\text{C}$ for 45 s, $50\text{ }^{\circ}\text{C}$ for 60 s, $72\text{ }^{\circ}\text{C}$ for 60 s; and a final synthesis at $72\text{ }^{\circ}\text{C}$ for 5 min. The PCR products were applied on a 1% (w/v) ethidium bromide-stained agarose gel in 1xTBE buffer with xylene cyanol loading buffer for verifying the amplification. The amplified SSR fragments were run on an automated sequencer ABI PRISM 3100 genetic analyser (Applied Biosystems, Budapest, Hungary). Fragment length analysis (FLA) was performed manually using Peak Scanner software 1.0 (Applied Biosystems 2006).

We used Micro-Checker (Van Oosterhout et al. 2004) to test all nine SSR loci for null alleles and possible scoring errors derived from significant allele dropout and the presence of microsatellite stutter bands.

Diversity estimates

GenAlEx v.6.5 (Peakall and Smouse 2006) was used to count the number of alleles per loci as well as to calculate the mean number of different alleles (N_a) and effective alleles (N_e) for each group and loci (Table 2).

Expected heterozygosity (H_e), observed heterozygosity (H_o), and inbreeding coefficient (F_{IS}) were

calculated in R (R Core Team 2013), using the *Adegenet* package (Jombart and Ahmed 2011). The significance of F_{IS} values was calculated separately with *Hierfstat* package (Goudet 2005), using 1000 permutations. Allelic richness (AR) was calculated using the *Popgenreport* package (Adamack and Gruber 2014). Private alleles were counted in each group using the *Poppr* package (Kamvar et al. 2014).

Differentiation, structure and relatedness

Genetic differentiation between groups was measured using F_{ST} (Nei 1973) by creating a pairwise distance matrix using the *Hierfstat* package and visualised as a heatmap. In addition, a UPGMA (unweighted pair group method with arithmetic mean) dendrogram was created, based on the F_{ST} distances, using the *Ggden-dro* package in R (de Vries and Ripley 2013).

To infer the group structure, sub-populations and relationship among groups, a model-based Bayesian analysis was carried out as implemented in Structure 2.3.4 software (Pritchard et al. 2000). The analysis was performed with an admixture model with correlated allele frequencies. K-value (the number of genetic groups) was set to 1–10 with a burn-in period of 100 000 steps followed by 500 000 repetitions of Markov chain Monte Carlo (MCMC), with 20 repetitions for each run. We applied the Evanno method (Evanno et al. 2005), as implemented in the *Pop-helper* package (Francis 2017), to detect the value of the optimal K that best fit the data. The 20 simulations were averaged using CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and represented in bar graphs using the *Pophelper* package.

Additionally, a Neighbor-Net network (Bryant and Moulton 2004) was constructed based on Cavalli-Sforza and Edwards Chord distance (Dch) by Cavalli-Sforza and Edwards (1967) and using a Neighbor-Joining (NJ) algorithm of *Phangorn* package (Schliep 2011).

We also performed a Principle Component Analysis (PCA) using the *Hierfstat* and *FactoMineR* (Lê et al. 2008) packages in R to compare genetic differentiation among individuals (genotypes) and populations. Population centroids with 95% confidence ellipses derived from the PCA were plotted for each genetic cluster using *Ggpubr* (Kassambara and Kassambara 2020).

To assess the relationship structure within the detected age classes of the Nagycenk group (old vs young individuals), we employed the ML-Relate software (Kalinowski et al. 2006). This approach was used to determine whether the old individuals were related to the younger trees, similarly to Ikeda et al. (2019). First, we calculated the pairwise relatedness coefficient using a maximum likelihood approach for all individuals (r , the fraction of alleles identical by descent shared by individuals; Blouin 2003). Then, we compared the ratio of the detected pedigree relationships (unrelated (U), half-siblings (HS), full-siblings (FS), and parent-offspring (PO)) and the mean r coefficient between the groups of individuals. Descriptive statistics were calculated in R using the *Dply* package (Wickham 2014). To compare the ratio of related individuals between the groups, a two-sample test for equality of proportions (two-proportions Z-test) was carried out using the *Stats* package v 4.2.0 (R Core Team 2013). Before comparing group means, to test whether distributions of variables met normality requirements, we applied Shapiro–Wilk normality tests implemented in *Stats*. Mean r coefficients were compared using the two-sample (independent) Wilcoxon rank-sum test. Throughout the analysis, 95% confidence intervals (CI) were applied.

Results

Amplification was successful with all nine SSR markers. However, primers for the Tc7 locus amplified four alleles in most samples, suggesting the marker to be multilocus, so we omitted these data from the final evaluation. Micro-Checker test confirmed the lack of null alleles, and all SSR loci conformed to HWE, showing no significant deviation from it.

The number of alleles per locus ranged from 4 (Tc951, Tc31, Tc927) to 18 (Tc963) in the samples of Nagycenk, including the old individuals (Table 1). In the Fertőd samples, the number of alleles ranged from 3 (Tc31) to 12 (Tc915), and in the case of the six cultivars from 2 (Tc31) to 6 (Tc951). The mean number of different alleles (N_a) and effective alleles (N_e) indicated that the largest polymorphism was present at the Tc963, Tc915 and Tc5 loci (Table 2). The eight primer pairs produced a total of 108 alleles. The amplified alleles' sizes were around the expected size

Table 1 The number of the amplified alleles, different alleles and effective alleles at the studied SSR loci by study groups

Locus	Nagycenk old ind. N=20	Nagycenk N=52	Cultivars N=6	Fertőd N=21	N _a	N _e
Tc963	12	18	5	8	10.750	3.227
Tc927	4	10	2	5	5.250	1.618
Tc937	7	10	3	5	6.250	1.948
Tc31	4	9	2	3	4.500	1.495
Tc951	4	5	6	4	4.750	3.053
Tc5	9	11	4	8	8.000	4.492
Tc11	6	10	3	5	6.000	2.921
Tc915	11	16	5	12	11.000	5.459

N_a mean no. of Different Alleles; N_e mean no. of Effective Alleles

Table 2 The observed size of the amplified alleles (in base pairs) in studied SSR loci by study groups compared to Phuekvilai et al. (2013)

Locus	Expected size	Nagycenk old ind	Nagycenk	Cultivars	Fertőd
Tc963	238	231–277	229–275	236–262	236–280
Tc927	157	135–163	135–160	143–149	143–160
Tc937	162	148–168	142–166	148–158	150–194
Tc31	205	192–213	192–211	194–196	194–202
Tc951	160	156–162	156–164	148–162	156–162
Tc5	150	139–176	139–176	139–158	139–164
Tc11	146	116–147	124–149	124–133	130–149
Tc915	165	148–190	148–178	152–172	148–180

(Table 2) based on published data (Phuekvilai et al. 2013).

Diversity parameters are given in Table 3. Samples from Nagycenk, including the old individuals, showed the highest observed and expected heterozygosity (mean $H_o=0.694$, $H_e=0.612$) and the highest number of allelic richness (mean $AR=4.254$). The number of private alleles (group-specific; mean $N_p=18.5$) was over 3-times and 6-times higher compared to Fertőd ($N_p=5$) and the control cultivars ($N_p=3$). The Fertőd group presented slightly lower diversity ($H_o=0.624$, $H_e=0.596$, $AR=3.943$) compared to Nagycenk's. As expected, the investigated control *Tilia* cultivars showed the least diversity

($H_o=0.625$, $H_e=0.515$, $AR=3.339$). Inbreeding coefficients were consistently negative among all groupss (mean $F_{IS}=-0.081$), and none of them was significant at the $p<0.05$ level.

Nei's pairwise F_{ST} values imply only a slight (0.007–0.06) differentiation among groups. The F_{ST} value of the samples from Nagycenk compared to the old individuals of Nagycenk was almost zero showing shallow genetic differentiation (Fig. 1). The F_{ST} value of the Nagycenk samples compared to the Fertőd samples' value shows very little (0.023–0.027) differentiation. The highest F_{ST} value was found between the cultivars and the Nagycenk group, although it was very low (0.06). Accordingly,

Table 3 Genetic diversity estimation for the studied four populations
N sample size; H_o observed heterozygosity; H_e expected heterozygosity; AR mean allelic richness; N_p number of private alleles; F_{IS} inbreeding coefficient; *ns* non-significant ($p<0.05$)

Population	N	H _o	H _e	AR	N _p	F _{IS}
Nagycenk	52	0.651	0.595	4.311	31	−0.066 ^{ns}
Nagycenk old ind	20	0.737	0.630	4.197	6	−0.144 ^{ns}
Mean		0.694	0.612	4.254	18.5	−0.105
Fertőd	21	0.624	0.596	3.943	5	−0.044 ^{ns}
cultivars	6	0.625	0.515	3.339	3	−0.070 ^{ns}
Mean		0.659	0.584	3.947	11.25	−0.081

Fig. 1 Heatmap of Nei's pairwise F_{ST} calculated between all pairs of populations (Nei 1987). Darker colors indicate stronger differentiation. The UPGMA dendrogram, showing the genetic relatedness between populations, is based on the pairwise F_{ST} values

the UPGMA dendrogram of F_{ST} showed the closest relationship (shortest genetic distance) between the Nagycenk group and the cultivars and Fertőd as separate branches with higher distances.

Bayesian Structure analysis clearly distinguished the samples of Nagycenk from Fertőd (Fig. 2a). The Evanno method indicated that a $K=3$ model fits best the data (Fig. 2b). The Nagycenk individuals (including both the old and younger individuals) form one cluster; another cluster is the Fertőd individuals and the cultivars (with high admixture); the third cluster is formed by four Nagycenk individuals, all identified as *T. plathphyllus*. The neighbour-net network of genetic clusters showed high similarities among the Nagycenk group (0.0358–0.0481), based on their evolutionary distance estimated with a neighbour-joining

Fig. 2 **a** Bayesian estimation of population structure in 99 individuals of *Tilia cordata* at $K=2, 3$ and 4 as determined by STRUCTURE (Pritchard et al. 2000). The most likely number of clusters is defined by Mean $L(K)$ and ΔK values according

ing to the method of Evanno et al. (2005). **b** Neighbor-net network of genetic clusters (Bryant and Moulton 2004). Edge values correspond to the distance of each cluster estimated

method and increasing distances for Fertőd (0.0640) and the control cultivars (0.1614) (Fig. 2c).

The Principal Component Analysis (PCA) of the Nagycenk group, Fertőd and the control cultivars widely scattered all 99 individuals (Fig. 3a). The first two axes (PC1 and PC2) explained only 21.97% of the total variance, while the other two axes (PC3 and PC4) even less, 6.78% and 6.11%. Around the group centroids, confidence ellipses were partly overlapping, but the Nagycenk and Fertőd clearly separated. Similarly to the Structure analysis, few individuals (e.g. 231, 382, 35, and 386) sharply separated from all groups (Fig. 3a). These were the same individuals which formed the third cluster in the Structure analysis, the four *T. platyphyllos* trees. The joint grouping of young and old individuals of Nagycenk was evident when PC3 and PC4 axes were considered (Fig. 3b).

Both age groups presented high number of unrelated individuals (Nagycenk old ind. $U=143$; young ind. $U=1047$), this was about 10-times higher than any types of relationship detected (Nagycenk old ind. $PO=9$, $HS=26$, $FS=12$; young ind. $PO=52$, $HS=146$, $FS=54$). The value of relatedness (r) ranged from 0.00 to 0.82 with a mean of 0.115 in Nagycenk old ind. (sd: 0.163) and 0.081 in the young

ind. group (sd: 0.141). The comparison of estimated relatedness between the age groups using Z-test (p value=0.0817), as well as the comparison of mean r coefficients using Wilcoxon rank-sum test, resulted in non-significant difference (p value=0.0784) at the $p < 0.05$ level (Fig. 4).

Discussion

The assessment of genetic diversity is a critical factor for ecosystem management and conservation of genetic material for population viability. This work aimed to assess the genetic diversity, differentiation and structure of the late-Baroque lime tree allée of Nagycenk.

Phuekvilai and Wolff (2013) published 15 SSR primers pairs, of which we have used eight pairs in course of our work, which produced the highest number of alleles. Even though these markers were developed for *T. platyphyllos*, their successful transferability to *T. cordata* has been proven by the cited authors; our study also confirmed the statement. Logan et al. (2015) have used these markers to discriminate between *T. cordata* and *T. platyphyllos* and their hybrid, *T. ×europaea*, to assess the population genetic

Fig. 3 **a** Principal component analysis (PCA) of populations from Nagycenk, Fertőd and the control cultivars genotyped in this study. X-axis denotes the value of PC1, while y-axis denotes the value of PC2, with each dot in the figure representing one individual. The color for individuals corresponds to the population classification. **b** PCA plots of axes PC2-PC3 and PC3-PC4 explaining less variance in the data. Confidence ellipses ($P=0.95$) around population centroids are marked with a solid line

Fig. 4 **a** Maximum Likelihood estimate of individual relatedness (r coefficients) and the determined relationships (unrelated (U), half-siblings (HS), full-siblings (FS), and parent-offspring (PO)) among the individuals of Nagycenk using

ML-relate (Kalinowski et al. 2006). **b** Comparison of age groups by the type of relationship determined (Z-test). **c** Comparison of r coefficients between the age groups (Wilcoxon rank sum test)

diversity of the two species in the UK. They reported high variability and could distinguish between pure species and hybrids using Bayesian clustering and ordination techniques. We identified few *T. platyphyllos* individuals (231, 382, 35 and 386) in the Nagycenk group already at the time of sampling. Identification of *T. cordata* is rather easy based on the leaves, which are hairless on both sides except for the small groups of brown hair in the leaf vein axils. In contrast *T. platyphyllos* and *T. x europaea* both have white hair on the underside of the leaves, but while the leaves of *T. platyphyllos* are covered with downy white hair fully, the leaves of *T. x europaea* has the white hair only in the leaf vein axils. Our morphological observation and the revealed SSR alleles confirm that these individuals belong to the *T. platyphyllos*

species. During allele counting and discrimination, specific alleles were observed for these individuals. Additionally, samples were well separated and positioned at the periphery on the PCA plot (PC1 vs PC2), and the four samples clustered separately (K2 – K4) during the Structure analysis (Figs. 2 and 3).

The Nagycenk allée (and the Fertőd allée) can be regarded as an artificial population. The genetic diversity was in the same magnitude as in the study of Logan et al. (2015) with natural UK populations. However, in that study more SSR loci have been used, of which we only studied the most diverse one. Detected allele numbers by loci are approximately the same; in loci Tc927 and Tc937, even more alleles (Tc927: 4 by Logan and 10 by us, Tc937: 6 by Logan 10 by us) were detected in our study. Similarly, high

levels of genetic diversity, expected and observed heterozygosity, were revealed in our survey for our populations (e.g. Nagycenk mean H_e : 0.694 and H_o : 0.612). However, contrary, Phuekvilai (2014) studied a wild modern-day population from Hungary (HUOL, Oltárc, Hungary; H_e : 0.538, H_o : 0.508), and Logan et al. (2019) four populations from Austria (AUDO, AUSO, AUST, AUTH; H_e : 0.588, H_o : 0.573) and both studies detected much lower genetic diversity. It is possible that our individuals, especially the old trees (H_e : 0.737 and H_o : 0.630), estimated to be approximately 200 years old, could preserve the diversity of the former natural populations of the region. An example is the *T. cordata* remnants of ancient forests in Denmark, where similar diversity was measured (H_e : 0.62 and H_o : 0.62) (Erichsen et al. 2019). Also, in the study of Lobo et al. (2018), comparable diversity was detected (H_e : 0.625 and H_o : 0.632), where trees were sampled from small isolated relict populations preserved in the framework of the “Danish genetic resource conservation program” (Graudal et al. 1995). An alternative hypothesis would be that our populations in Central Europe could be genetically more diverse due to the meeting of several post-glacial migration routes (Phuekvilai 2014; Semerikova et al. 2020). *Tilia* species could retain high genetic diversity for a long time due to their high abundance during warmer periods, outcrossing mating system and increased longevity (Myking 2002; Wolff et al. 2021). However, this would require extensive sampling of the native populations of the geographic region.

In addition, our diversity estimation supports the hypothesis of Gerzson and Szilágyi (2011), according to which the late Baroque allée’s plantation was done by collecting sprouts or seedlings in the neighbouring forests. Vanden Broeck et al. (2018) compared the genotypic diversity of historical plantings of *T. ×europaea* from the 17th century with the genotypic diversity of currently available planting stock in Belgium and the Netherlands. Despite that, she found mainly two clones (‘Pallida’ and ‘Zwarte linde’) among the investigated trees; the genetic diversity of the historical plantings was about 4-times higher than in the current commercial planting stocks. Wolff et al. (2019) as well as Vanden Broeck et al. (2018) confirmed the theory of Hansen et al. (2014) that the same clones have been produced for decades/centuries by private nurseries in the Netherlands and

Germany. Since neither uniform allele frequencies nor similar allele distributions were observed in our dataset during the statistical analysis, it is unlikely that the studied trees would have been clonally propagated.

None of the studied groups showed signs of inbreeding, and all F_{IS} values were negative (mean F_{IS} : −0.081). Thus, even though small populations are more vulnerable to inbreeding (Ellstrand and Elam 1993), our value indicates that groups experience gene flow (heterozygote excess) and/or did not develop inbreeding in the original population where the propagation material came from. In addition, *Tilia* species are known for being highly outcrossing, with inbreeding coefficients (F_{IS}) close to zero (Wolff et al. 2021).

The lack of substantial genetic differentiation detected between the young and old individuals of Nagycenk is not unexpected. Both the Structure and the Neighbor-Net network analyses tightly linked these groups, with low genetic differentiation between them. Presumably, either the younger individuals originated from the same seed source, or they can be relatives of the old individuals; however, this latter is unlikely since our relatedness analysis and the comparison of r coefficients reject this assumption. Most possibly, both age groups origins from the same seed source but at a different time.

The sharp separation of the Nagycenk samples from the Fertőd group might be the consequence that plant material used during the reestablishment of the park in the early 1900s (Szilágyi and Szabó, 2020) was of native but non-local origin. Allochthonous populations, which could hybridise with local populations, were frequently planted in this period (Pâques 2013). *Tilia* species and cultivars, e.g. *Tilia tomentosa* ‘Petiolaris’, were already planted in the 1700s in Hungary (Pigott 2012). In addition, it should be mentioned, that János Wagner, the prominent Hungarian botanist (1870–1975, Austria-Hungary) obtained his systematical *Tilia* collection from Fertőd. As Vöröss (1984) describes this collection, “Most of the specimens, both as regards numbers and number of forms, came from Eszterháza (=Fertőd), from the famous park of the ESTERHÁZYS. This aristocratic family must have liked lindens, as there are great numbers of varieties in their park”. Thus, we cannot rule out the possibility that the trees of Fertőd were collected or purchased from elsewhere, from a non-local seed

source. According to the land-use system of the Esterházy garden complex, a tree nursery used to be a vital functional part for centuries, where seedling collection or purchase either from local or remote regions created the basic plant stock.

Similarly to Fertőd, the examined six cultivars showed low genetic differentiation and separation from Nagycenk. Neither the Structure nor the PCA analyses were unable to discriminate these individuals. Expectedly, during the diversity estimation, we found minor private alleles and allelic richness. This finding indicates that our markers are not sufficient to detect a genetic pattern specific to the cultivars.

Conclusion

The use of a restricted number of cultivars and clones may pose a risk to global biodiversity, especially in today's rapidly changing world. Our study is the first to explore the genetic composition of small-leaved lime trees growing in a Hungarian historic garden. The trees of Nagycenk preserved substantial amounts of genetic variation, presenting shallow differentiation, lack of inbreeding and genetic erosion due to their seed source origin and high longevity. This collection, despite being artificial, potentially resembles the former genetic diversity of native local lime tree populations. The old trees have shown long-term disease resistance hence could provide valuable genetic material for breeders. Genetic conservation and recurrent selection from such collections, and from where these trees originate, would be an efficient way of maintaining the genetic diversity. For these reasons, assessing the genetic heritage of the late-Baroque historic garden of Nagycenk is essential. As the renewal of the Nagycenk allée is timely, these results can help to decide the strategy to be used. The allée represents a broad genetic base for the species, which is definitely worth preserving.

Author contributions KSZ, EGYT and ZsGy devised the project, EGYT and ZsGy formulated the main conceptual ideas and proof outline. AP and ZsGy collected the plant material and performed the laboratory analyses. EGYT and ZsGy performed the statistical analysis of genetic data. EGYT and ZsGy wrote the manuscript. KSz provided suggestions for data analyses and feedback and editing on the manuscript drafts.

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Data availability The datasets generated and analyzed during the current study are available from the authors.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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