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**ORIGINAL ARTICLE**

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# Genetic characterisation of centuries-old oak and linden trees using SSR markers

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## Abstract

The main goal of this study was to identify the unique gene pool of old and historically valuable *Quercus robur* L. and *Tilia cordata* L. to be able to characterise their genetic diversity in order to determine the polymorphism by expressed sequence tag-single sequence repeat (EST-SSR) markers and identify the most valuable specimens.

Morphological description, molecular genetic analysis, and statistical analysis were used in studies. The genetic distances between old-value trees of different *Quercus* L. and *Tilia* L. were determined based on EST-SSR markers and morphological characteristics.

Using polymerase chain reaction (PCR), alleles of the expected size were obtained. It was determined that four to eight alleles were obtained by seven SSR markers in the studied *Q. robur* L. samples. According to the calculated value of the locus polymorphism index (polymorphism information content [PIC]), the most polymorphic was the marker SSRQrZAG 65; the PIC was 0.84. The lowest value of PIC was observed in the marker SSRQrZAG 11; the PIC was 0.69. Intragenetic polymorphism was detected for all studied markers. Among the studied samples of linden, two to five alleles were identified. It was found that the highest value of PIC was obtained for the marker Ts920 – 0.72. The least polymorphic was the marker Ts927 (PIC was 0.28), which is not only due to the small number of alleles, but also their uneven distribution in the sample. Intragenetic polymorphism was detected in four of the six markers analysed for *T. cordata* L.

In this study, polymorphism was detected in all studied samples of *Q. robur* L. and *T. cordata* L., which allows to assess their genetic diversity based on the distribution of alleles.

## Key words

centuries-old trees, DNA analysis, *Quercus* L., *Tilia* L.

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## Introduction

Centuries-old trees are extraordinary organisms. They not only represent a historical landscape and environmental heritage of inestimable value, but also witness a long history (Sliusar and Kushnir 2015) of environmental changes and human interventions and constitute an as yet poorly known reserve of genetic variability, which can be considered a great resource for the management programmes of forest species.

Their size, longevity and strong interactions with global biogeochemistry are some of the traits in which trees reign superlative among extant life forms (Likhanov et al. 2019).

In Ukraine, most of the centuries-old group of trees include representatives of the genera *Quercus* L. and *Tilia* L. European countries with long and strong environmental protection traditions have been long and successfully engaged in the inventory and protection of century-old and other prominent trees (Oleksiichenko and Pidkhovna 2018; Chornobrov et al. 2019). A less-commonly noted, but equally special meaning of old trees is crucial for biodiversity and the mitigation of climate changes. For example, the ecological importance of mature and overmature hardwood forest stands with ancient and virgin trees are relevant in providing carbon (C) stock (Bilous et al. 2019; Matsala et al. 2021).

Studies of genetic diversity by DNA markers of centuries-old populations of *Tilia cordata* Mill. are actively conducted in Denmark and the UK (Logan et al. 2015; Lobo et al. 2018; Erichsen et al. 2019). The genetic structure of *Quercus robur* L. populations is also studied in Poland and Germany (Sandurska et al. 2017; Müller and Gailing 2018). In Ukraine, the problems associated with identifying, researching and conserving such trees are being addressed slowly. Ukrainian scientists are conducting research to identify and describe centuries-old trees *Q. robur* and *T. cordata* in Kyiv and some regions of Ukraine (Masalskyi 2015; Sovakova and Sovakov 2015; Kushnir and Vakulyk 2018).

In order to study the extinction of oaks, the development of methods for their preservation, as well as the practical use and preservation of natural monuments of centuries-old trees, methods of microclonal propagation of *Q. robur* and *T. cordata* are being developed (Galkin et al. 2013; Bilous 2013, 2018). However, due to the influence of abiotic and biotic environmental factors and man-made load, entire populations of some species can be destroyed and the genetic diversity of residual populations can be reduced. Therefore, the development of methods for assessing the genetic diversity of centuries-old *Q. robur* and *T. cordata* trees to determine the optimal ways to preserve them remains relevant. The most common methods for studying tree species polymorphism are morphological assessment of tree populations and the use of protein and DNA markers (Barreneche et al. 1998; Pohjanmies et al. 2016; Chokheli et al. 2016; Mohammad-Panah et al. 2017; Müller and Gailing 2018). Simple sequence repeat (SSR) is one of the most common DNA markers for assessing DNA polymorphism in many plant species. Due to their wide genome distribution, high variability and codominant type of inheritance, SSR markers are effective for determining the genetic diversity of plants, including tree cultures. Therefore, the aim of our study is to evaluate centuries-old *Q. robur* and *T. cordata* trees in Kyiv and some regions in Ukraine by SSR markers and to verify the presence of correlations between genetic distances by SSR markers and the geographical location of the studied samples.

## Material and methods

### Plant material

The test materials were seven samples of centuries-old *Q. robur* trees and six samples of *T. cordata* presented in Table 1. The study was conducted on the basis of laboratory of molecular genetic analysis of the Ukrainian Institute of Plant Variety Examination in the year 2019.

### DNA extraction and polymerase chain reaction procedure

DNA was extracted from 100 mg of green leaves using cetrimonium bromide (CTAB) in duplicate. The resulting total DNA was dissolved in Tris-EDTA (TE buffer) (Prysiazhniuk et al. 2019). The study of molecular genetic polymorphism of *Q. robur* was performed using seven SSR markers and *T. cordata* was evaluated by six SSR markers (Kampfer et al. 1998; Phuekvilai and Wolff 2013). Characteristics of the primers and nucleotide sequences are presented in Tables 2 and 3.

Polymerase chain reaction (PCR) was performed on a T-CY amplifier (Creacon Technologies B.V., Emmen,

**Table 1.** Material characteristics

| Centuries-old trees nomenclature | | Location | Age [years] | Geographical coordinates |
|---|---|---|---|---|
| *Quercus robur* | | | | |
| Q1 | Yuzefinskyy oak | Hlynne, Rivne region | nearly 1000 | 51°55′33″N 27°37′86″E |
| Q2 | Oak T. Shevchenko | Kyiv | more than 600 | 50°49′59″N 30°45′16″E |
| Q3 | M. Rylskyi oak | Kyiv | nearly 600 | 50°38′68″N 30°51′01″E |
| Q4 | Centuries-old oak in the NULES botanical garden | Kyiv | more than 200 | 50°38′16″N 30°50′28″E |
| Q5 | Oak Vitovta | Kyiv | more than 400 | 50°38′42″N 30°49′73″E |
| Q6 | Centuries-old oak 1 on the territory of NULES Ukraine | Kyiv | more than 400 | 50°38′38″N 30°50′53″E |
| Q7 | Centuries-old oak 2 on the territory of NULES Ukraine | Kyiv | more than 400 | 50°38′37″N 30°50′61″E |
| *Tilia cordat*a | | | | |
| L1 | Linden T. Shevchenko | Sedniv, Chernihiv region | more than 600 | 51°63′76″N 31°56′95″E |
| L2 | Linden P. Mohyla | Kyiv | more than 600 | 50°45′75″N 30°51′71″E |
| L3 | Centuries-old linden tree of the Feofaniia Monument Park | Kyiv | more than 400 | 50°43′25″N 50°43′25″E |
| L4 | Linden tree of St. Feodosiya Pechers′koho | Kyiv | more than 700 | 50°34′00″N 30°48′’68″E |
| L5 | Centuries-old linden tree in Holosiivskyi forest | Kyiv | more than 200 | 50°72′18″ N 29°44′92″E |
| L6 | Centuries-old linden tree | v. Irsha, Radomyshl district, Zhytomyr region | more than 200 | 50°38′29″ N 30°50′51″E |

Note: NULES – National University of Life and Environmental Sciences.

**Table 2.** Characteristics of SSR markers for assessment of *Quercus robur* polymorphism

| SSR | Nucleotide sequences of primers, 5′→3′ | Motive | The expected size of the amplicons, bp |
|---|---|---|---|
| ssrQrZAG 7 | F: CAACTTGGTGTTCGGATCAA | $(TC)_{17}$ | 150 |
| | R: GTGCATTTCTTTTATAGCATTCAC | | |
| ssrQrZAG 11 | F: CCTTGAACTCGAAGGTGTCCTT | $(TC)_{22}$ | 273 |
| | R: GTAGGTCAAAACCATTGGTTGACT | | |
| ssrQrZAG 25 | F: GATATGAAAGATTCTTATTCCATCC | $(GA)_{32}$ | 135 |
| | R: GTTAGAACCAATGTACCAAAGTCC | | |
| ssrQrZAG 30 | F: TGCTCCGTCATAATCTTGCTCTGA | $(GA)_{26}$ | 211 |
| | R: GCAATCCTATCATGCACATGCACAT | | |
| ssrQrZAG 31 | F: CTTAGTTTGGTTGGGAAGAT | $(GA)_{31}$ | 190 |
| | R: GCAACCAAACAAATGAAAT | | |
| ssrQrZAG 44 | F: ACCCTTGTAGTCATGTTCGTTG | $(GA)_{29}$ $(TG)_{31}$ | 145 |
| | R: GAAATCTCACCTGCTCCCTATC | | |
| ssrQrZAG 65 | F: CAGTGGTGTCAACTCCTCCCAG | $(TC)_{21}(TA)_{10}$ | 270 |
| | R: GTCAGGTGACCATTCAAACCTAGAA | | |

Note: F – forward primer, R – reverse primer, SSR – simple sequence repeat.

**Table 3.** Characteristics of SSR markers for assessment of *Tilia cordata* polymorphism

| SSR | Nucleotide sequences of primers, 5′→3′ | Motive | The expected size of the amplicons, bp |
|---|---|---|---|
| Tc5 | F: TTTTCATACATTTAGAGACTTTTAGCA | $(AG)_{12}$ | 150 |
| | R: TGCATGATTTGTATGTTTAGGG | | |
| Tc915 | F: ACATCGATTGTATTTCCCTTTAAC | $(CT)_{16}$ | 165 |
| | R: GTTGTATTTTGCCCTTAACATTG | | |
| Tc920 | F: AAATGTCTTCAGAGTGACTAGATGG | $(GA)_2(GT)_{15}\ (AG)_4$ | 232 |
| | R: TGCCTCATTATTCTCCTAATTCTC | | |
| Tc927 | F: AGTCCTCCTGTCAAATGCTG | $(AG)_{10}$ | 157 |
| | R: ATCACACTCGTTTATGACATCTTG | | |
| Tc937 | F: AGCCAACCAACTTTTACAATACAG | $(AG)_{13}$ | 162 |
| | R: AGATAAAAGCACATAAATCGATGG | | |
| Tc963 | F: CTAACCCCATTCTCTTTAATTCTG | $(CT)_{11}$ | 238 |
| | R: GCTTTCATTTCAGTTTTCCTCTAC | | |

Note: F – forward primer, R – reverse primer, SSR – simple sequence repeat.

The Netherlands). The reaction mixture with a volume of 10 µl for PCR and the amplification parameters for *Q. robur* and *T. cordata* samples are shown in Tables 4 and 5, respectively.

**Table 4.** Composition of reaction mixture for PCR of *Quercus robur* and *Tilia cordata*

| Components | Final concentration | |
|---|---|---|
| | *Q. robur* | *T. cordata* |
| PCR buffer* | 1× | 1× |
| $MgCl_2$ | 1.5 µM | 2 µM |
| dNTP (dATP, dTTP, dGTP, dCTP) | 100 µM | 200 µM |
| Polymerase Taq | 1 U | 0.5 U |
| Primer (F) | 1 µM | 0.2 µM |
| Primer (R) | 1 µM | 0.2 µM |
| DNA | 50 ng | 50 ng |

Note: * 10 µM Tris-HCl, pH 9.0; 50 µM KCl; 0.01% Triton X-100; F – forward primer, R – reverse primer, PCR – polymerase chain reaction.

The amplification reaction products were visualised by electrophoresis in 2% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer solution according to the conventional method with ethidium bromide (Tkachyk 2015). Electrophoresis was performed for 1.5 h at an electric field strength of 5 V/cm. The size of the fragments was determined using the software TotalLab 12.0.

**Table 5.** PCR protocol for *Quercus robur* and *Tilia cordata*

| Step name | *Q. robur* | | *T. cordata* | |
|---|---|---|---|---|
| | parameters [°C/time] | cycle number | parameters | cycle number |
| Initial denaturation | 95/3 min | 1 | 95/3 min | 1 |
| Denaturation | 94 (89)*/30 s | 33* | 94/30 s | 35 |
| Annealing | 50/30s | | 54**/1 min | |
| Extension | 72/30 s | | 72/30 s | |
| Final extension | 72/3 min | 1 | 72/3 min | 1 |

Note: * first 10 cycles are carried out at 94°C, followed by 23 cycles at 89°C; **60°C for Tc927; PCR – polymerase chain reaction.

### Data analysis

To characterise the genetic structure of the studied genotypes, the frequencies of identified alleles and polymorphism information content (PIC) were calculated (Sivolap et al. 1998). For the purpose of cluster analysis, a matrix was constructed, in which the presence/absence of a specific allele was denoted as 1/0, respectively. The method of hierarchical clustering with Euclidean measure of distance using the computer program STATISTICA 12.0 (trial version) was applied for analysis. Clustering of the studied genotypes was performed using the unweighted pair group average (Ermantraut et al. 2007;

Drozdov 2010). Correlations between the test samples by SSR markers and their geographical location were determined by genetic distances using the Mantel test via a computer program XLSTAT 2018 (trial version) (Lobo et al. 2018; Tommasini et al. 2003; Legendre and Fortin 2010; Diniz-Filho et al. 2013; Klyachenko and Prysiazhniuk 2018).

## Results and discussion

As a result of electrophoretic separation of PCR products, amplicons of the expected size were obtained for samples of *Q. robur* and *T. cordata*. It was determined that the marker ssrQrZAG 11 was characterised by the lowest number of alleles among the studied samples of *Q. robur*. Four alleles were identified by this marker (Fig. 1a, Tab. 6). The marker ssrQrZAG 65 revealed the largest number of alleles – eight alleles (Fig. 1b, Tab. 6).

It was found out that one allele of 300, 332 and 290 bp was identified in the specimens of Yuzefin oak, Shevchenko oak and centuries-old oak in the National University of Life and Environmental Sciences (NULES) botanical garden. Rylskyi oak is polymorphic by this marker and contains two alleles of 268 and 300 bp. It has been found that the allele with a size of 332 bp was unique by the marker ssrQrZAG 11 for the tested samples of common oak and was identified only in Shevchenko oak. As regards the marker ssrQrZAG 65, one allele with a size of 312 bp was identified in Rylskyi oak. Also, two alleles of 312 and 340 bp and 396 and 320 bp, respectively, were revealed in specimens of centuries-old oak in the NULES botanical garden and Vytautas oak. The sizes of alleles, which were obtained for common oak samples, are presented in Table 6.

According to the results of testing of small-leaved linden samples by SSR markers, three alleles were identified in the sample of P. Mohyla linden (154, 174 and 180 bp) among the studied samples of small-leaved linden by one Tc5 marker (Tab. 7).

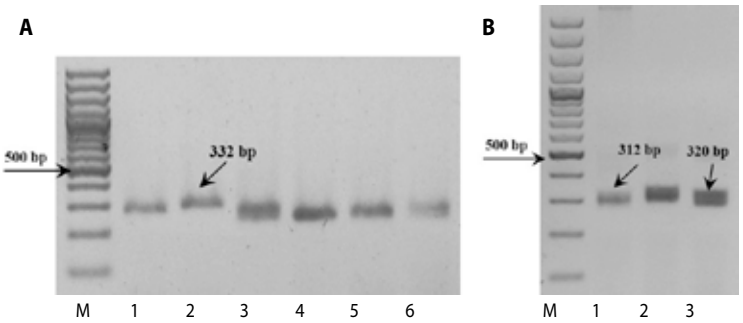


**Figure 1.** Results of PCR testing of samples of common oak with marker: A – ssrQrZAG 11: 1 – Yuzefin oak; 2 – Shevchenko oak; 3 – Rylskyi oak; 4 – centuries-old oak in the botanical garden of the NULES; 5 – Vytautas oak; 6 – centuries-old oak on the territory of NULES Ukraine; B – ssrQrZAG 65: 1 – Rylskyi oak; 2 – centuries-old oak in the NULES botanical garden; 3 – Vytautas oak

Note: M – molecular weight marker 100-bp DNA ladder O'GeneRuler (Thermo Scientific), NULES – National University of Life and Environmental Sciences, PCR – polymerase chain reaction.

**Table 6.** Sizes of obtained alleles for *Quercus robur* with SSR markers

| SSR | Allele size, bp | | | | | | |
|---|---|---|---|---|---|---|---|
| | Q1* | Q2 | Q3 | Q4 | Q5 | Q6 | Q7 |
| ssrQrZAG 7 | 132 | 120, 140 | 132 | 152 | 152 | 140 | 146 |
| ssrQrZAG 11 | 300 | 332 | 268, 300 | 290 | 290 | 268, 300 | 290 |
| ssrQrZAG 25 | 116 | 206 | 206 | 206 | 194 | 158, 206 | 112, 158 |
| ssrQrZAG 30 | 204, 226 | 212, 230 | 182 | 192 | 212 | 192, 212 | 204 |
| ssrQrZAG 31 | 146, 182 | 146, 182 | 140, 158 | 174 | 182 | 158 | 146, 182 |
| ssrQrZAG 44 | 116, 156 | 124, 180 | 144 | 144, 180 | 144 | 156, 216 | 156 |
| ssrQrZAG 65 | 272, 320 | 264, 312 | 312 | 312, 340 | 296, 320 | 396 | 388 |

Note: * Q1 – Yuzefinskyy oak; Q2 – oak T. Shevchenko; Q3 – M. Rylskyi oak; Q4 – centuries-old oak in the NULES botanical garden; Q5 – oak Vitovta; Q6 – centuries-old oak 1 on the territory of NULES Ukraine; Q7 – centuries-old oak 2 on the territory of NULES Ukraine; NULES – National University of Life and Environmental Sciences, SSR – simple sequence repeat.

For small-leaved linden samples, all identified alleles are presented in Table 7.

**Table 7.** Sizes of obtained alleles for *Tilia cordata* with SSR markers

| SSR | Allele size, bp | | | | | |
|---|---|---|---|---|---|---|
| | L1* | L2 | L3 | L4 | L5 | L6 |
| Tc927 | 152, 168 | 152 | 152 | 152, 168 | 152 | 152 |
| Tc5 | 154 | 154, 174, 180 | 154 | 158 | 154 | 154, 180 |
| Tc915 | 154 | 154, 168 | 154 | 154, 174 | 182 | 168 |
| Tc920 | 218, 232 | 224, 240 | 218, 232 | 232 | 232, 252 | 252 |
| Tc937 | 152 | 152 | 152 | 162 | 162 | 162 |
| Tc963 | 246 | 246 | 238 | 238 | 246 | 246 |

Note: * L1 – T. H. Shevchenko *T. cordata*; L2 – P. Mohyla *T. cordata*; L3 – *T. cordata* of St. Theodosius of Pechersk; L4 – a centuries-old *T. cordata* of the Feofaniia Monument Park; L5 – a centuries-old *T. cordata* (the village of Irsha); L6 – a centuries-old *T. cordata* in the Holosiivskyi forest; SSR – simple sequence repeat.

One allele was identified in samples of T.H. Shevchenko linden and century-old linden of the Feofaniia Monument Park; sizes of the identified alleles were 154 and 158 bp, respectively. Two alleles sized 154 and 180 bp were identified in a sample of the linden of St. Theodosius of Pechersk. According to the Tc915 marker, the 154 bp allele has the highest frequency (0.50) among markers with a PIC greater than 0.60. As can be seen from Figure 1b, this allele is characteristic of samples of T. H. Shevchenko linden, P. Mohyla linden, the linden of St. Theodosius of Pechersk and the centuries-old linden of the Feofaniia Monument Park. It was determined that the samples of P. Mohyla linden and the centuries-old linden of the Feofaniia Monument Park turned out to be polymorphic and had another allele of 168 and 174 bp. Characteristics of all obtained alleles by the studied markers for the samples of common oak and small-leaved linden are shown in Table 8. According to the data obtained for the studied common oak samples, the most polymorphic was the marker ssrQrZAG 65, the PIC for which was 0.84.

The lowest PIC value was observed for the marker ssrQrZAG 25 – 0.68. It was determined that for the ssrQrZAG 7 marker, only one sample (Shevchenko oak) is heterozygous and contains two alleles (120 and 140 bp, respectively). Other samples of common oak are homozygous by this marker. Two to four heterozygotes are identified by other markers. Frequencies of the identified alleles range from 0.07 to 0.50. Craciunesc et al. (2011) investigated the genetic diversity of Finnish common oak populations by 15 SSR markers. Scientists have identified from 3 to 15 alleles depending on the marker.

**Table 8.** Characteristics of the obtained alleles by SSR markers for the samples of *Quercus robur* and *Tilia cordata*

| SSR | Number of alleles | Allele size range | Alleles' frequency | PIC |
|---|---|---|---|---|
| *Q. robur* | | | | |
| ssrQrZAG 7 | 5 | 120–152 | 0.07–0.29 | 0.77 |
| ssrQrZAG 11 | 4 | 268–332 | 0.14–0.43 | 0.69 |
| ssrQrZAG 25 | 5 | 112–206 | 0.07–0.50 | 0.68 |
| ssrQrZAG 30 | 6 | 182–230 | 0.07–0.29 | 0.80 |
| ssrQrZAG 31 | 5 | 140–182 | 0.07–0.36 | 0.76 |
| ssrQrZAG 44 | 6 | 116–216 | 0.07–0.36 | 0.76 |
| ssrQrZAG 65 | 8 | 264–396 | 0.07–0.29 | 0.84 |
| *T. cordata* | | | | |
| Tc5 | 4 | 154–180 | 0.08–0.58 | 0.60 |
| Tc915 | 4 | 154–182 | 0.08–0.50 | 0.65 |
| Tc920 | 5 | 218–252 | 0.08–0.25 | 0.72 |
| Tc927 | 2 | 152–168 | 0.17–0.83 | 0.28 |
| Tc937 | 2 | 152–162 | 0.5 | 0.50 |
| Tc963 | 2 | 238–246 | 0.33–067 | 0.44 |

Note: PIC – polymorphism information content, SSR – simple sequence repeat.

Thus, 15 alleles were identified by the marker ssrQrZAG 11, which characterises it as the most polymorphic. In our studies, four alleles were revealed by this marker, the frequencies of which were not distributed evenly enough, as indicated by the low PIC value. As to the marker ssrQrZAG 25, which demonstrated the lowest PIC value in our studies, 13 alleles were identified in 38 tested PIC genotypes in studies by Steinkellner et al. (1997). Kampfer et al. (1998) studied the polymorphism of *Q. robur* and *Quercus petraea* species and different populations using 28 SSR markers. The largest number of alleles was identified by sci-

entists with the marker ssrQrZAG 25. Also, 10 alleles were also identified with the markers ssrQrZAG 7 and ssrQrZAG 30. In our studies, these markers also showed a fairly high value of PIC (0.77 and 0.80, respectively). Therefore, a rather uniform distribution of allele frequencies and the obtained PIC values for the studied SSR markers indicate the possibility of their use for the assessment of genetic diversity of centuries-old common oak trees.

For small-leaved linden samples, the most polymorphic was the marker Tc920 with the PIC value of 0.72. The frequencies of alleles by this marker vary from 0.08 to 0.25. The lowest PIC value was found for the marker Tc927, as evidenced by the uneven distribution of allele frequencies (from 0.17 to 0.28). All studied samples of small-leaved linden were homozygous by Tc937 and Tc963 markers. It was noted that low PIC values (0.50 and 0.44, respectively) were also obtained for these markers. Thus, markers Tc5, Tc915 and Tc920 with PIC values from 0.51 to 0.72 proved to be the most effective for determining the polymorphism of centuries-old linden trees. Studies conducted by Phuekvilai and Wolff (2013) showed that 15 SSR markers were effective for determining the polymorphism of *Tilia platyphyllos*. The authors also demonstrated that the markers they developed can also be used to assess the genetic diversity of other species of the *Tilia* genus. The results presented by the authors indicate that the most polymorphic markers in the study of two populations of *T. platyphyllos* were Tc5, Tc915, Tc927, Tc937 and Tc963.

In our studies of *T. cordata* samples, the most polymorphic among these markers were the Tc5 and Tc915 markers. Tc920 marker, by which nine alleles were identified in *T. platyphyllos* (Phuekvilai and Wolff 2013), was the most polymorphic in our studies. Studies by Logan et al. (2015) showed a high level of polymorphism by 13 markers of two linden species *T. platyphyllos* and *T. cordata*. The locus Tc915 was the most polymorphic in *T. platyphyllos*, and the locus Tc963 was the most polymorphic in *T. cordata*. Moreover, 18 and 26 alleles, respectively, were identified by these markers (Logan et al. 2015). Studies for the assessment of genetic diversity of *T. cordata* species were conducted by Cvrčková et al. (2018). In contrast to our results, the most polymorphic in their studies was the Tc963 locus, by which 15 alleles were identified. Furthermore, 7–11 alleles were revealed by markers Tc5, Tc915 and Tc920, which also indicates a high level of polymorphism. Therefore, according to the obtained data, markers Tc5, Tc915 and Tc920 can be recommended for the study of *T. cordata* samples.

To assess the degree of genetic proximity of *Q. robur* and *T. cordata* samples by SSR markers, cluster analysis was performed. The results of clustering in the form of phylogenetic trees are presented in Figures 2 and 3.

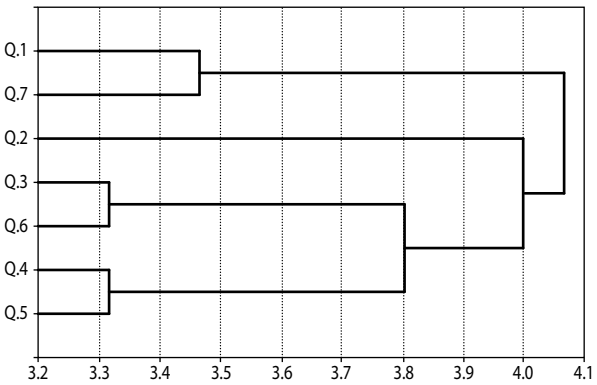


**Figure 2.** Cluster distribution of *Quercus robur* samples by SSR markers

Note: Q1 – Yuzefin oak; Q2 – Shevchenko oak; Q3 – Rylskyi oak; Q4 – centuries-old oak in the NULES botanical garden; Q5 – Vytautas oak; Q 6 and Q7 - centuries-old oak near the territory of NULES Ukraine; NULES – National University of Life and Environmental Sciences, SSR – simple sequence repeat.

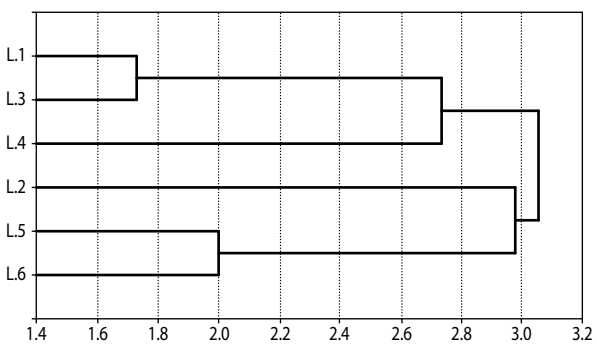


**Figure 3.** Cluster distribution of *Tilia cordata* samples by SSR markers

Note: L.1 T.H. Shevchenko *Tilia cordata*, L.2 – P. Mohyla *Tilia cordata*, L.3 *Tilia cordata* of St. Theodosius of Pechersk, L.4 a centuries-old *Tilia cordata* of the Feofaniia Monument Park, L.5 a centuries-old *Tilia cordata* (the village of Irsha), L.6 a centuries-old *Tilia cordata* in the Holosiivskyi forest.

As a result of cluster analysis of *Q. robur* samples by seven SSR markers, four clusters were obtained, which were formed by samples of Yuzefin oak and the centuries-old oak (NULES educational building 1), Rylskyi oak and the centuries-old oak (on the territory of NULES Ukraine), and the centuries-old oak in the NULES botanical garden and Vytautas oak. One cluster was presented by a sample of Shevchenko oak.

The closest were the samples included in one cluster, and the value of genetic distances between them was 3.32 (samples of Rylskyi oak and the centuries-old oak on the territory of NULES Ukraine, and the centuries-old oak in the NULES botanical garden and Vytautas oak).

The value of genetic distances between the sample of Shevchenko oak and other samples was 3.87–4.36. The most distant by the tested SSR markers were the samples of Yuzefin oak and the centuries-old oak in the NULES botanical garden; the value of genetic distances was 4.47.

Some scientists investigated the genetic diversity of *Q. petraea* and *Q. robur* based on leaf morphology and SSR markers. Multidimensional statistical analysis allowed the authors to classify most of the individual samples as species (Yücedağ and Gailing 2013). Evaluation of the genetic diversity of three *Q. robur* populations by SSR markers was performed by Pohjanmies et al. (2016). It has been shown that differentiation among populations was markedly high. The data obtained from our studies of centuries-old oak trees also showed a sufficient level of polymorphism for determination of the differences between individual trees by SSR markers.

According to the cluster distribution of small-leaved linden samples by six SSR markers, two clusters were obtained. The first cluster was formed by samples of T.H. Shevchenko *T. cordata*, St. Theodosius of Pechersk *T. cordata* and a centuries-old *T. cordata* of the Feofaniia Monument Park; the second cluster included a centuries-old *T. cordata* (the village of Irsha), a centuries-old *T. cordata* in the Holosiivskyi forest and P. Mohyla *T. cordata* (Fig. 3).

According to the obtained data, the closest samples were the samples of T. H. Shevchenko *T. cordata* and St. Theodosius of Pechersk *T. cordata*; the value of genetic distances was 1.73. The highest value of genetic distances (3.74) was observed between the samples of P. Mohyla *T. cordata* and the centuries-old *T. cordata* of the Feofaniia Monument Park, which indicates that these samples are the most distant according to the tested SSR markers. The use of SSR markers for the assessment of genetic diversity of *T. cordata* and *T. platyphyllos* in the UK was shown by Logan et al. (2015). Both species showed a high level of polymorphism. The authors confirmed the intra- and interpopulation genetic structure of the studied species. Based on genetic markers, this structure has been found to have a weak relationship to location (Logan et al. 2015).

In order to check the correlations between pairs of samples of *Q. robur* and *T. cordata* and their geographical location, Mantel test (Pearson linear correlation) was performed (Fig. 4 and 5).

As a result of the analysis, the calculated significance level *p*-value and the correlation factor *r* (AB) for the theoretical significance level $\alpha = 0.05$ were determined, which, according to the interpretation of the test, allows to accept one of the test hypotheses about the presence ($H_a$) or absence ($H_0$) of correlation.

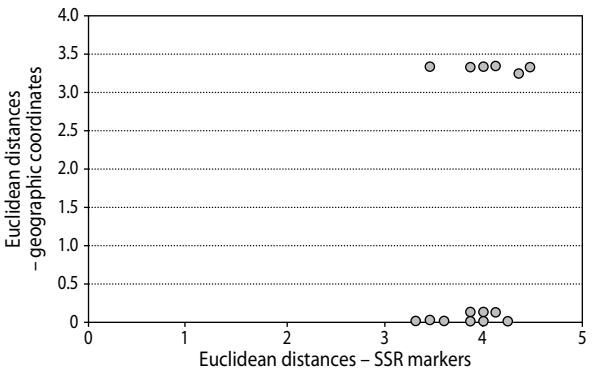


**Figure 4.** Relationship between genetic distances of *Quercus robur* samples according to geographical coordinates and SSR markers (SSR – simple sequence repeat)

Based on the obtained data, it was determined that the calculated *p*-value (0.174) for *Q. robur* samples was higher than the significance level $\alpha = 0.05$. Therefore, we should accept the hypothesis $H_0$ about the lack of correlation, the condition of which is $p > \alpha$ (Diniz-Filho et al. 2013). According to the results of analysis of genetic distances of small-leaved linden samples, the cal-

culated $p$-value (0.350) is higher than the significance level $\alpha = 0.05$, which also indicates the absence of correlations.

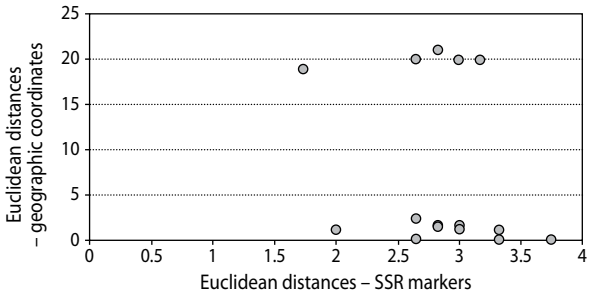


**Figure 5.** Relationship between genetic distances of *Tilia cordata* samples according to geographical coordinates and SSR markers (SSR – simple sequence repeat)

Thus, the obtained data indicate the absence of correlations between the *Q. robur* and *T. cordata* samples and their geographical location.

Studies by Hutchison and Templeton (1999) have shown the use of correlation analysis for assessment of the relative influence of gene flow and drift on the distribution of genetic variability inside and outside a certain region. Studies of *Q. petraea* and *Q. robur* populations based on SSR markers and isoenzymes were conducted by Streiff et al. (1998). The authors found a significant interspecific polymorphism of samples within the population. However, a weaker spatial genetic structure of *Q. robur* was observed them. Studies by Lobo et al. (2018) demonstrate the assessment of *T. cordata* genetic diversity in Denmark based on SSR markers and spring phenology. Also, the authors found significant differences between populations in spring phenology and DNA markers. However, no correlation was found between genetic distances by DNA markers and geographical location. This is explained as a consequence of the fragmentation of *T. cordata* populations in Denmark, which could lead to low gene flow between isolated populations.

Our studies characterise individual trees of *T. cordata* and *Q. robur* of different ages from different regions of Ukraine, but the lack of correlation between genetic distances and geographical location may also be associated with the isolation of populations, as well as significant differences in age (200–1000 years) (Hutchison and Templeton 1999; Mylett 2015).

## Conclusions

According to the results of studies of seven samples of *Q. robur* and six samples of *T. cordata* by molecular SSR markers, molecular genetic polymorphism was determined in all studied samples. It has been found that the most polymorphic among the studied *Q. robur* markers was ssrQrZAG 65 with PIC 0.84. It was determined that the highest level of polymorphism among the studied samples of *T. cordata* was observed by the marker Tc920 (PIC 0.72). According to the results of cluster analysis, it was found that the closest by the studied SSR markers were the samples of Rylskyi oak and centuries-old oak (NULES educational building 1); the value of genetic distances was 3.32. A sample of Shevchenko oak did not enter any cluster; the values of genetic distances were 3.87–4.36. The lowest value of genetic distances (1.73) by SSR markers among the studied samples of *T. cordata* was observed between the T.H. Shevchenko linden and the linden of St. Theodosius of Pechersk. The most distant were the samples of P. Mohyla linden and the centuries-old linden of the Feofaniia Monument Park; the value of genetic distances was 3.74. Therefore, we can recommend the use of SSR markers as an effective system for determining genetic diversity for the assessment of molecular genetic polymorphism of centuries-old oak and linden trees.

It has been determined that there is no correlation between the studied samples of *Q. robur* and *T. cordata* and their geographical location. Due to the small number of samples and the low level of polymorphism of some of the markers used, it is necessary to continue research involving more markers and samples to determine a reliable correlation and obtain a complete characterisation of not only individual samples, but also populations of centuries-old trees.

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