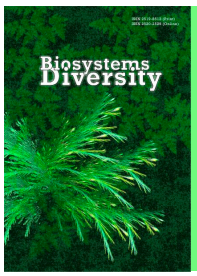

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Studying the genetic structure of *Quercus robur* forest stands on anthropogenically transformed territories using introns of the β-tubulin gene

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Based on the analysis of the intron polymorphism of β-tubulin genes, the genetic variability of old *Quercus robur* L. trees from "Holosiivsky" NPP was investigated. The genotyping of 55 old *Q. robur* trees was carried out; 40 polymorphic and one monomorphic (about 880 bp) TBR fragments were found. High frequency (70–90%) of occurrence of fragments with an approximate molecular weight of 275, 490, 500, and 1110 bp was observed.The genetic polymorphism of old *Q. robur* trees was assessed as quite high: PIC is 0.22 – 0.39, the effective number of alleles per locus was 1.174–1.268. The Shannon information index was in the range of 0.204–0.269.The geographical differentiation of the genetic structure of centuries-old oak trees from "Holosiivsky" NPP was not pronounced. The share of inter-selection genetic variability (AMOVA) accounts for about 6% of genetic variability, and the geographic component – about 1%. Around 93% of genetic variability is concentrated on the individual level. Using the TBP method, we found that *Q. robur* forest stands do not have a stabilized genetic and visible spatial structure, but at the same time they possess a sufficiently large genetic diversity. The absence of a spatial genetic structure may indicate the artificial origin of *Q. robur* trees from different seed materials, and also that a small number of the plants have survived to this time. In this case, the main influence on the structure of oak stands in "Holosiivsky" NPP was from anthropogenic factors, both in the form of cutting down trees and, possibly, the introduction of alien seed material.

Keywords: spatial structure; molecular markers; polymerase chain reaction

Introduction

The common oak (*Quercus robur* L.) is a native species of the Ukrainian flora, one of the main forest-forming species, which covers around 24% of the total forest area of Ukraine (Derzhavne Ahentstvo Lisovykh Resursiv Ukrayiny [State Forest Resources Agency of Ukraine], http://dklg.kmu.gov.ua/forest/control/uk/publish/article?art_id=62921&). It is characterized by high ecological flexibility and has great economic importance. This species can exist in various ecotopes and edaphotopes, distributed from the north of Scandinavia to the south of Europe, from the Iberian Peninsula to the Ural Mountains, and is the key species in many biomes. Historically, over the process of evolution, the oak has divided into separate species, and the process of exchange of genetic material existed and continues to exist (Leroy et al., 2017; McVay et al., 2017).

The common oak is a wind-pollinated (anemogamous) plant, but the majority of acorns spread near the mother plants, and acorns cover significant distances only because of carriers – birds, rodents, humans (Gerber et al., 2014).

This species prefers fertile soil, but is rather resistant to industrial emissions and is actively used in the creation of sanitary zones of industrial enterprises, inner city woodlands and in green building. Oak is used as the main species in field-protection and anti-erosion plantations (Brygadyrenko, 2015). As cities around the world become more and more overloaded and polluted (Blanco et al., 2009), trees can reduce air pollution by absorbing some atmospheric pollutants (Nowak et al., 2006).

Green plantations can also reduce the temperature of the environment by 6 °C (Wenting et al., 2012), which reduces the overheating of urban residents (Nowak et al., 1998; Cummins & Jackson, 2001; Wolch, 2014). Suburban oak forests can efficiently absorb the polluting substances, including reducing concentrations of gaseous NH_3, with an average reduction by 29–38% (García-Gómez et al., 2016). So the powerful structure, hardiness and resistance of *Q. robur* along with its high decorative properties determine its widespread use in green building.

Kyiv has a centuries-long history, during which local forests were actively used, and subsequently artificially renewed. *Q. robur* was one of the main species which were cut down and replanted. So, nowadays, along with young oak species of artificial and natural origin, age-old oak trees are quite common, which may be the remaining part of the former natural forests. A significant proportion of *Q. robur* has protected status and needs more detailed study of origin, belonging to local tree stands, etc.

Holosiivsky forest belongs to the territory of Kyiv which is rich in old trees. This forest is a remnant of a native forest, which located on the southwest approaches to Kyiv at the time of Kievan Rus (Vasilyuk et al., 2012). Now the Holosiivsky forest is Kyiv`s largest forest territory, completely surrounded by residential areas. This forest area of more than 1000 hectares is located in the southern part of Kyiv. This is mainly hornbeam-oak forest, formed on gray and light-gray forest soils on loess sediments (Onyshchenko, 2013). The greater part of the forest belongs to the Holosiivsky National Park (HNP) (Pryrodno-zapovidnyy fond Ukrayiny [Nature Reserve Fund of Ukraine], http://pzf.land.kiev.ua/pzf-obl-10.html).

According to the data of surveys of the forests, within the territory, the constantly used area of the NNP is the forest areas with the age of the tree stand of 200 years or older (up to 240), and equals 143 ha (20% of the area of forest vegetation), in all cases the main dominant species, for which the age is determined, is *Q. robur* (Onyshchenko, 2013). Adult oak plants in the area of the "Holosiivsky" park are positioned quite close one to another. Often there is a pattern of growth of old trees along the central paths of the park, in contrast to younger trees. The origin of these tree stands, the source of their seed material, is unknown.

The strong development, the long life of common oak plants make it a model for diverse assessment of the genetic processes, with reforestation in anthropogenically disturbed territories or the preservation of existing tree stands in conditions of intense technogenic load (Petit, 2002; Neophytou, 2010; Frouz, 2015). Due to the popularity of *Q. robur*, depending on the orientations of the study, the following methods are used: morphological (Semerikov, 1976), isoenzyme polymerase chain reaction (PCR) (Kremer, 1993; Aldrich et al., 2003; Grivet et al., 2008; Hampe et al., 2013) and genome sequencing (Plomion et al., 2016).

The variability of *Q. robur* as a subject of research has long been exploited (Semerikov, 1976). To determine the genetic relations, researchers used methods of qualitative phenetics, protein electrophoresis from seeds, isoenzyme analysis, while its genetic diversity with the use of molecular markers, based on polymerase chain reaction (PCR), has been studied within its range in Europe for more than 20 years (Kremer, 1993; Sork et al., 2002; Aldrich et al., 2003; Hampe et al., 2013).

At the same time, introns are becoming increasingly popular as a source of DNA polymorphism. It has been determined that introns are involved in numerous important molecular events as the control of gene expression through the mechanism of alternative splicing and other mechanisms (Rose, 2002; Leet al., 2003; Li et al., 2007; Morello & Breviario, 2008; Braglia, 2010), they are hyper variable regions, comparable in polymorphism terms with microsatellite loci and may have different nucleotide composition and length, even within the limits of one taxonomic unit. In general, the polymorphism of the intron length of the β-tubulin gene is a stable working system of molecular-genetic markers of different species of terrestrial plants, which can be used in genetic analysis of plants (Breviario et al., 2007; Blume et al., 2010; Pirko, 2011; Rabocon et al., 2015).

The objective of the study was to obtain data on the polymorphism of the samples of centuries-old trees of *Q. robur* from Holosiivsky NNP on the basis of genotyping according to the TBR locus, analyzing the spatial component of genetic variability, assessing the anthropogenic impact on the genetic structure of the natural tree species.

Materials and methods

The material from *Q. robur* trees was selected during 2016 in "Holosiivsky" Park, Kyiv (Fig. 1). *Q. robur* trees older than 100 years (we selected trees with the largest trunk diameter) were used, some of the trees have protected status. In total, material from 55 trees was selected.

Fig. 1. A common oak tree in Holosiivsky Forest

The trees selected for analysis were divided into five population samples: 1 – trees growing on the side of the "Holosiivsky Desert" (8 plants), 2 – trees from the upper slope of the ravine (19 plants), 3 – trees from the bottom the slope of the ravine (7 plants), 4 – the trees located between the Main Astronomical Observatory of the National Academy of Sciences of Ukraine and the Museum "Pirogovo" (6 plants), 5 – trees in the area of the Observatory and its surroundings (15 plants) (Fig. 2). As material, the leaf laminae were taken and put into zipper packets with dry silica gel. The dried laminae were maintained at –80 ºC.

The geodata, in relation to the location of the source of the material, were collected at the same time as the material. For this geotags, separated from the photos of the plants from which the material was collected, were processed using scripts in the statistical programming environment "R" (R Core Team, 2011; R Foundation for Statistical Computing, Vienna, Austria; www.R-project.org), packages "googleVis" and "geosphere". The obtained geodata were exported to the GenAlEX package (Peakall & Smouse, 2006).

Isolation of DNA and PCR. Genomic DNA was extracted from the laminae using the CTAB-method (2012). The quality and quantity of DNA were checked using electrophoresis in 1.5% agarose gel and spectrophotometrically on an Eppendorf biophotometer with the determination of the concentration and degree of DNA contamination. DNA samples were maintained at –20 °C.

The TBP analysis was conducted according to Breviario (Bardini et al., 2004). Primer sequences (Breviario et al., 2007):

TBP-F: 5 '-AACTGGGCBAARGGNCAYTAYAC-3';

TBP-R: 5'-ACCATRCAYTCRTCDGCRTTYTC-3 '.

The PCR was performed on the Thermal Cycler 2720 amplifier (Applied Biosystems, USA). The reaction mixture (10 μl) contained a fivefold-diluted PCR buffer with ammonium sulfate, 2.5 mmoles of $MgCl_2$, 50 ng of plant DNA, 1 μM of each primers, 0.2 mm of each dNTPs, 0.5 units of Taq polymerase (Fermentas, Lithuania). The amplification was carried out in accordance with the following protocol: initial denaturation (94 °C during 3 min); 35 cycles of amplification (denaturation 94°C – 30 s, annealing of primers at 55 °C – 40 s, extension 72 °C – 90 s); final extension of 72 °C – 8 minutes, 15 °C – maintenance (Bardini et al., 2004).

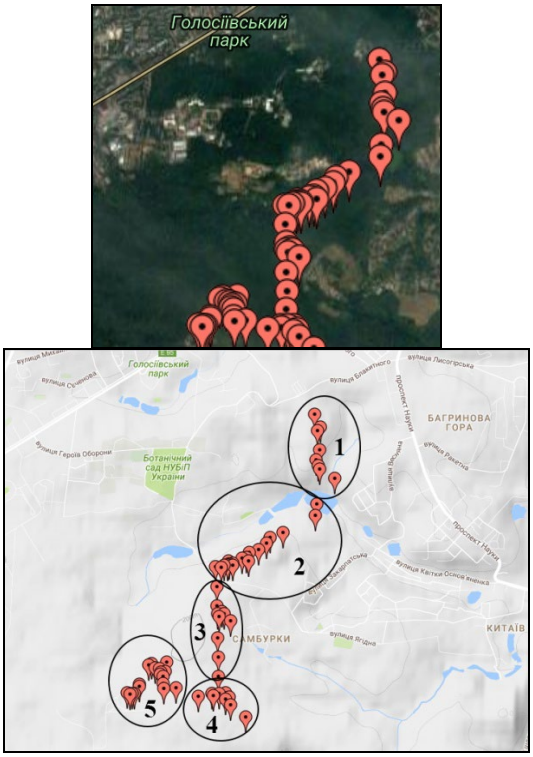

Fig. 2. Locations of oaks in the Holosiivsky park, from which the material was collected

Each PCR reaction was performed at least in triple replication using negative control so during the following electrophoretic analysis, there would be a possibility of detecting nonspecific amplification products differing in the same reactions. Amplification products (0.5 μl) were distributed by electrophoresis in a 6% non-denaturing polyacrylamide gel in 1 × TBE buffer (Green & Sambrook, 2012) at 300 V for 5 hours. Visualization of the fragments was carried out by silver nitrate staining (Rahman et al., 2000; Benbouza et al., 2004). After electrophoresis, the gel was photographed in visible light and the resulting images were subjected to further analysis.

The analysis of the electrophoretic gels was carried out in the GelAnalyzer program. The length of the reproducible and distinct bands was determined using a DNA marker (O'Gene Ruler™ 100bp Plus DNA Ladder, ready-to-use; "Fermentas", Lithuania). TBP bands were recorded in the binary code: the presence of the band – one, the absence – zero.

Statistical processing of data. Primary statistical processing of genetic data was carried out in the program GenAlEX (Peakall, 2006). This program assessed the frequency of alleles on locus Ne, and the main parameters of polymorphism (present heterozygosity – HO, expected heterozygosity – HE, Shannon's information index – I), we performed an analysis of the molecular variance (AMOVA). For TBP-markers, we used BinaryDiploid format of data, for SSR CodominantDiploid. The analysis was performed on the basis of the matrices of genetic distances, calculated in GenAlEX, we conducted the analysis in the same program using the method of the main coordinates. The comparison of genetic distance matrices, calculated on the basis of different classes, was performed on the basis of Mantel's test in GenAlEX.

The level of polymorphism of the markers was assessed using the Polymorphism Information Content Index (PIC) using the formula:

$$PIC = \frac{\sum_{i=1}^{n}(1 - f_{ai}^2 - f_{bi}^2)}{n},$$

where, n – the total number of evaluated polymorphic fragments, f_a – the frequency of cases (organisms) in which this fragment was absent, and f_p – the frequency of organisms in which this allele was present (Hongtrakul et al., 1997; Breviario et al., 2007).

Results

Analysis of TBP loci in 55 *Q. robur* trees in total showed 41 bands (Fig. 3). Of these, 40 out of 41 fragments were polymorphic. A fragment with an approximate molecular weight of 880 base pair (bp) was monomorphic in all the samples. The number of polymorphic fragments in the analyzed groups of the oak trees directly correlated with the group size. Accordingly, group 4 (consisting of 6 trees) was characterized by presence of 19 polymorphic fragments, and group 5 (15 trees) – 30 polymorphic fragments. The distribution zone of all fragments was in the approximate range from 250 to 1300 bp.

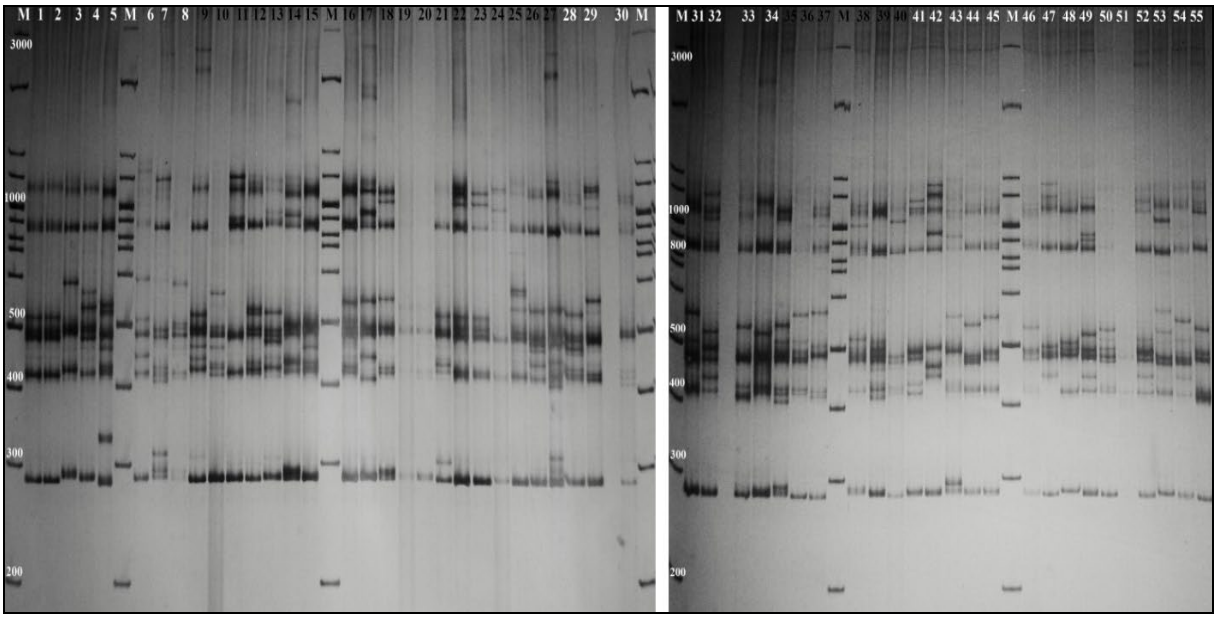

Fig. 3. Electrophoregram with amplicones of β-tubulin genes introns of centuries-old *Q. robur* trees: *1 – 55* (from above) – sample numbers of five groups; m – DNA marker

The allele frequencies obtained by the TBP markers in *Q. robur* plants are presented in Figure 4.

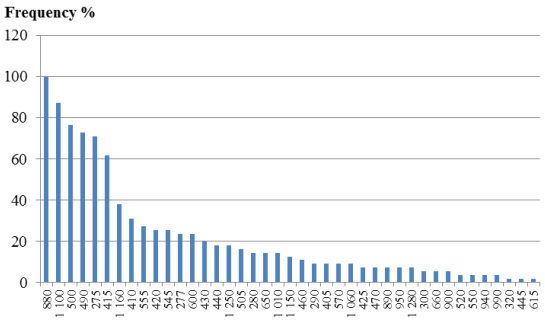

Fig. 4. Frequencies of TBP-markers in leaves of centuries-old *Q. robur* trees from Holosiivsky Park

The frequency of most of the fragments (namely 27) was less than 20%: 19 fragments with a frequency of less than 10% and 8 fragments with a frequency 10–19%. There are 6 fragments in the range 20–39%, and only 3 fragments in the range 30–69%. Most common were fragments with an approximate molecular weight of 275, 490, 500, 1110 bp (frequency ranging 70–90%) and 880 bp (100% frequency). So, as can be seen in the figure, the largest share of the fragments belongs to the rare ones.

The assessment of the allelic polymorphism, performed in each particular selection, demonstrated practically the same effective number of alleles per locus (1.174–1.268), taking into account the errors of the obtained values (Fig. 5). According to the Shannon information index (0.204–0.269), the differences between the studied groups were within the error. That is, there were no significant differences in the genetic polymorphism between all studied groups of old *Q. robur* plants.

Since the TDR method belongs to biallelic markers, it is advisable to use a polymorphic information content index (PIC) to assess the genetic diversity. The lowest value of PIC (0.22) was found in group 2, which included trees from the upper slope of the ravine, and the highest value of PIC (0.39) was in group 4, which included 6 trees from the territory between the Main Astronomical Observatory and Museum

"Pirogovo". In general, the average PIC for all studied groups equals about 0.3. This can be considered a high value, given that for any biallelic marker the maximum PIC equals 0.5.

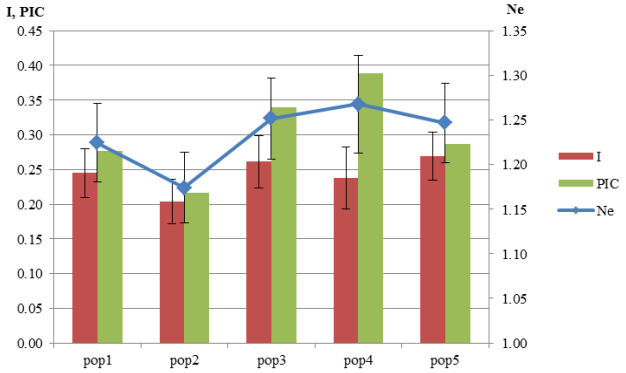

Fig. 5. Main parameters of the allele diversity in the analyzed groups of common oak in relation to TBP-markers

Further analysis of the molecular variance (AMOVA) revealed almost complete absence of inter-sampling subdivision (Fig. 6). Thus, within the general genetic heterogeneity of the species, 93% of the genetic diversity of *Q. robur* has an internal sampling polymorphism, and only 6% is the fraction of inter-sampling polymorphism (geographical polymorphism can be ignored for its share accounts for only 1%).

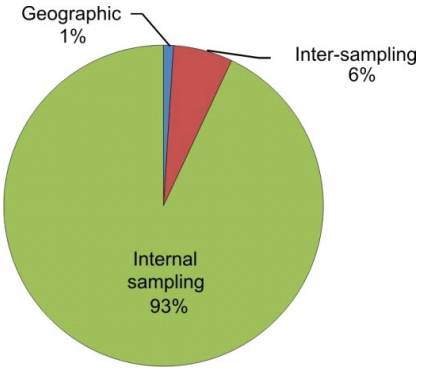

Fig. 6. Distribution of the molecular variations (AMOVA) within the population of old *Q. robur* trees from Holosiivsky Park

Mantel's test between the matrix of geographical and genetic distances in relation to TBP, assesses the significance level of the non-randomness of the distribution of geographic and genetic distances between individual trees at the level ($P \leq 0.034$), at the same time a direct correlation between geographic and genetic distances was practically absent (Fig. 7).

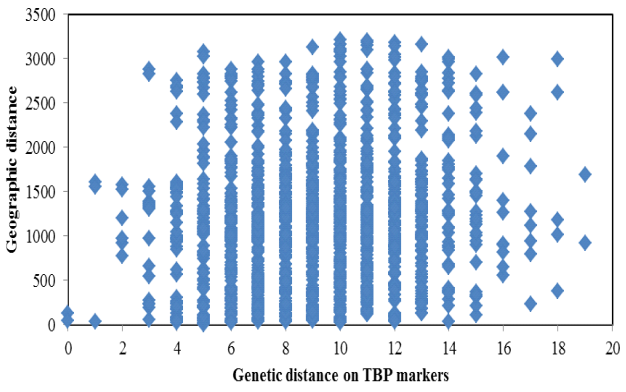

Fig. 7. Mantel's test between the representatives of all studied groups of old *Q. robur* trees on TBR markers and geographic distances

Mantel's test for matrices of distance between oaks, calculated using TBP markers, revealed no significant correlations (Fig. 8). The present correlation coefficient was not distinguished from the selection of distances of correlation coefficients, randomly generated on the basis of these two matrices, with probability of 0.11.

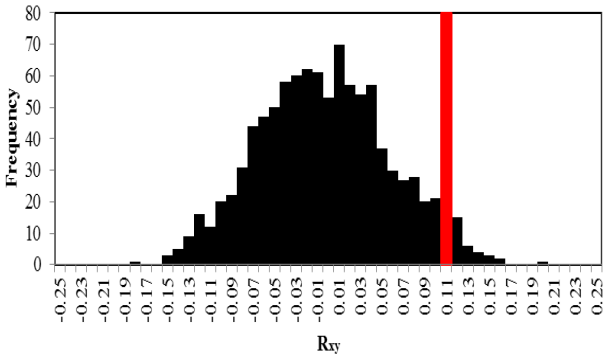

Fig. 8. Distribution of the generated correlation coefficients in Mantel's test and available correlation coefficient (red) for the *Q. robur* studied groups

The analysis of the representatives of the tree stands of common oak using the method of principal components also did not show a high degree of ordination of the principal components of the representatives of the selections in the space (Fig. 9).

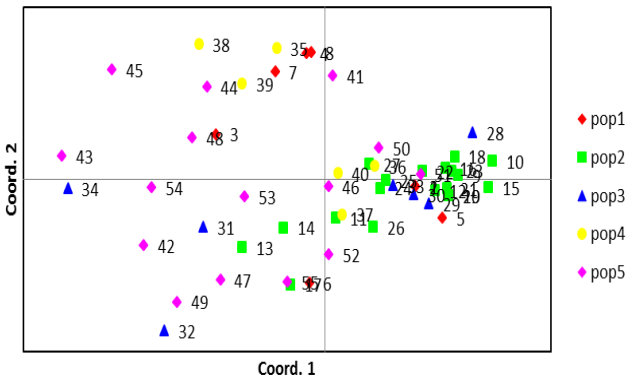

Fig. 9. Ordination of *Q. robur* representatives in the space of the first two principal components

The results of the spatial genetic structure analysis of all specimens determined that neither single selection, nor all samples together have any correlation between genetic distances and the geographical distance between the trees (Fig. 10). That is, there is no spatial genetic structure at the level of separate groups, and in the studied area in general.

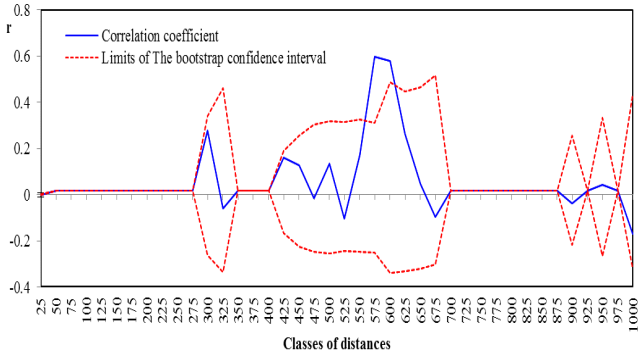

Fig. 10. Results of the analysis of spatial genetic structure of oak tree stands

Discussion

The level of genetic diversity of species is often associated with certain characteristics, such as length of life cycle, pairing and reproduction, size of the geographical range and genetic exchange (Gaudeul et al., 2000;

Hellmanna & Pineda-Kerch, 2007; Li et al., 2008). In our study, most of the fragments found during the analysis of TBR-polymorphisms are rare. Only 3 fragments were found in 30–70% of trees. At the same time, for each of the samples, an effective number of alleles per locus (1,174–1,268) was observed.

As has been demonstrated (Plomion et al., 2018), such large polymorphism may be related to the mechanisms of oak resistance by increasing genetic variability at the individual level. At the same time, according to the Shannon information index (0.204–0.269), the differences between the samples are within the error and no reliable differences in the genetic polymorphism in all studied groups of common species of oak were found. Similar results were observed for 3 natural populations of *Q. petrea* (Pospíšková & Dostálek, 2010).

It should be noted that one of the sources of variability for the oak, as a hardy plant, can be manifestations of somatic variability, which may be due to the mechanisms of plant resistance (Plomion et al., 2018).

The absence of intraspecific subdivision, demonstrated by molecular variation analysis (AMOVA), may be due to a number of causes. Thus, for similar studies of natural populations of sawtoothed oak (*Q. acutissima*), the reduction of inter-sample variability compared to individual variability is associated with a high level of outcrossing in oak woodlands (Chung, 2002).

In Italy a similar study using AFLP in more than 300 trees in three *Q. suber* stands has also demonstrated a very low level of variability at the inter-population level. Thus, among the all studied *Q. suber* H_t = 0.253, only 1.7% were attributed to the differences between the populations. AMOVA analysis, similar to ours, showed that majority of the genetic variability is maintained at the 9% probability level, and among certain populations it equaled 3.6% (F_{st} = 0.036, P < 0.001).

At the same time, differences between populations in geographical boundaries are also proved by a very low level of genetic variability as 2.6% (F_{sc} = 0.026, P < 0.001) of the overall variation and only 1.3% (F_{ct} = 0.013, P = 0.007) by the results of the variation between regions, which characterizes a small differentiation of populations in the range of 700 km (Coelho et al., 2006). In the study of natural populations of oaks (*Q. acutissima*), the variability associated with the population structure was seven times lower than interindividual variability, which is likely associated with prevailing outcrossing in oak woodlands (Chunget al., 2002).

In our study, the Mantel test between the TMR geographic and genetic distances assesses the level of significance of the correlations between genetic and geographical distances between trees at the level (P ≤ 0.034), direct correlations between geographic and genetic distances were absent. In general, the high genetic variability observed in oak populations is associated with biological signs of long-living species such as air pollution, cross-over, large effective population size and a large pollen flow among the population (Sork et al., 2002; Sork & Smouse, 2006; Young & Pickup, 2010). Thus, the assessment of genetic diversity found in our study was close to the assessments for other species of *Quercus* in South and North America and Europe (Fernández & Sork, 2005; Aldrich & Cavender-Bares, 2011; Ashley et al., 2015; Oyamaetal., 2018).

According to the results of the autocorrelation analysis of the spatial genetic structure of all samples, it was determined that some samples as well as all samples have no global correlation between the genetic distances and the geographical distance between the trees (Fig. 10). There was no spatial genetic structure at the level of individual groups, and in the studied area in general. At the same time, two classes of distances (575 and 600 m) were found, for which there is a certain positive coefficient of autocorrelation.

Examination of four closely related oak species (*Q. robur*, *Q. petraea*, *Q. pubescens* and *Q. frainetto*) from Romania (covering a small area), along with a high diversity, demonstrated the presence of spatial genetic auto-correlation at distance of 25 m (Curtu et al., 2015) and its absence at larger distances. Similar studies of red oak (*Q. rubra*) and northern pin oak (*Q. ellipsoidalis*) have demonstrated the presence of a spatial genetic structure at distances up to 83 m in the *Q. rubra* population (Lind-Riehl& Gailing, 2015).

In natural populations of red oak, undergoing minimal anthropogenic impact, American scientists have found a fairly significant degree of spatial autocorrelation (Feng et al., 2008). A certain degree of spatial autocorrelation has been observed for *Q. petraea* in Denmark (Jensen et al., 2003). However, for *Q. robur* seed plantations the spatial correlation was not observed (Katičić et al., 2018). Thus, on the basis of the assessed spatial genetic structure of the tree stands of the "Holosiivsky" NNP we can conclude that there are both primary elements of the spatial structure characteristic for natural populations and populations under anthropogenic influence, which most likely consist of planting of a specific seed material (Katičić et al., 2018) and possibly selective felling.

Conclusion

Expeditionary study and material collection of old *Q. robur* trees has been carried out, the scheme of the location of the samples was developed, the geographical coordinates of the trees on the territory of NNP (Kyiv) were determined.

The genotyping of 55 old trees was carried out. We found 40 polymorphic and one monomorphic (about 880 bp) TBR fragments. The high frequency of occurrence of fragments with an approximate molecular weight of 275, 490, 500, 1110 bp (70–90%) is noted.

The genetic polymorphism of old *Q. robur* trees was quite high: PIC equaled 0.22–0.39, the effective number of alleles per locus was 1.174–1.268, the Shannon information index was 0.204–0.269.

There was no pronounced geographical differentiation of the genetic structure of the samples of old *Q. robur* trees from the Holosiivsky NPP. The share of intraspecific genetic variability (AMOVA) accounts for about 6% of genetic variability, and the geographic component – about 1%. About 93% of genetic variability is concentrated on the individual level.

Using the TBP method, we found that the studied *Q. robur* woodlands had no stabilized genetic and visible spatial structure, but at the same time they had a sufficiently large genetic diversity. The absence of spatial genetic structure in the analyzed old trees may indicate the artificial origin of the trees from different seed materials and, at the same time, a small number of the plants have survived to this time, which destroys the spatial genetic structure of the oak forests. The main factor in this case was anthropogenic impact both in the form of felling, and, possibly, the cultivation of non-adventive material.

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