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Full Length Article

Genetic Variability in Cenopopulations of Pedunculate Oak (*Quercus robur*) in Rostov Region, Russia, with the Use of ISSR-Markers

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Abstract

Using Inter Simple Sequence Repeats (ISSR) markers, we studied genetic diversity in five cenopopulations in pedunculate oak (*Quercus robur* L.), growing on the territory of Rostov-on-Don region in Russia. Four of five cenopopulations were of natural origin. Total 185 ISSR-markers were identified, of which 184 (99.46%) were polymorphic. Five natural cenopopulations indicated different levels of genetic diversity. There was a high level of genetic differentiation in cenopopulations (G_{ST}=1.0350). The results of the study are promising for the development of further programs of forest regeneration and preservation of valuable deciduous woody plant species. © 2018 Friends Science Publishers

Keywords: Population genetics; *Quercus robur*; ISSR-markers; Dendrogram; Genetic diversity

Introduction

Efficient creation of artificial forests should be based on the use of a genetically diversified planting material of high-quality. *Quercus robur* L. is a valuable wood species having long ontogeny and is promising to create long-living artificial plantings of recreational and ameliorative purposes in the steppes zone of Russia and other countries. The area of pedunculate oak has steadily reduced and replaced with fast-growing woody species. *Q. robur* is a widespread species in the natural forest associations of the middle latitudes and mountainous zones of the northern hemisphere and Asia Minor (an area corresponding to the western two-thirds of Turkey), North Africa, the Caucasus, and Europe (Fedorov *et al*., 1980). Actual area occupied by *Q. robur* in Russia is located within the subzone of broad-leaved forests and forest-steppe of the European part of the country (Kazantsev, 2014). The southern boundary of *Q. robur* in Russia is within the forest-steppe of the European part of the country, capturing the territory of the Rostov region (Bulygin and Yarmishko, 2003; Kruzhilin, 2008; Kazantsev, 2014).

Q. robur has a high level of outcrossing in populations, which is 2–5% (Petit *et al*., 2002). Viable pollen of oak can extend on distance upto 80 km, providing mediated gene flow (Buschbom *et al*., 2011). To assess the genetic relationship of different planting material (Novikova *et al*., 2012; Nechaeva *et al*., 2013) are extensively used molecular-genetic methods of analysis, such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Portmanteau (AFLP), Simple Sequence Repeats (SSR) and Inter Simple Sequence Repeats (ISSR). There is information on the use of ISSR and SSR markers for investigation of genetic divergence in cork oak (Lopez-Aljorna *et al*., 2007), evaluation of phylogenetic relationship, and inter simple sequence repeats DNA-markers for genotyping of the family *Fagaceae* (Coutinho *et al*., 2014a, b).

The study of genetic diversity of cenopopulations is possible with the use of modern molecular biology techniques and effective genetic markers, among which in recent years, the most popular is ISSR-marker. The undoubted advantages of microsatellite analysis are: high individual polymorphism, codominant inheritance, and high reproducibility of the method (Sheikina *et al*., 2012). The aim of this work was to study genetic structure of natural and one artificial cenopopulations of pedunculate oak in Rostov region.

Materials and Methods

Plant Material

Populations for the study consisted of five different forest stands of *Q. robur*, selected from the natural distribution of

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pedunculate oak on the territory of Rostov region (Table 1 and Fig. 1). All studied stands were located in different botanically-geographical districts of the Rostov region: a typical example of natural oak forests in the southern district of Dono-Donets hollow (F – cenopopulation is located in the territory of Litvinskogo forestry Belokalitvenskogo forestry enterprise); complex oak-wood on the southern border of distribution with a significant participation of Norway maple, common maple and Tatarian maple, etc. (PL – cenopopulation is located in the territory of Ereminskogo forestry Verhnedonskogo forestry enterprise); planting of Scotch pine and pine Crimean (Pinus nigra subsp. pallasiana) with the participation of pedunculate oak (Cs – cenopopulation is located in the territory of Chernyshevsky forestry Oblivskiy forestry enterprise); high shore of the river Don with the projections ("foreheads") and gully into the slope with islets ravine forests (Rs – cenopopulation is located in the territory between village Razdorskaya and highway, extent from the southern to the northern edge of the village Razdorskaya); ravine forests – simplified oak-wood pluralperlovich (Kr – cenopopulation is located 5 km north-west of the village Man'kovo-Kalitvenskogo). The sampling of selected plant material was carried out in July–August 2017. These samples were placed at -80°C for long term storage.

DNA Extraction

Extraction of DNA was made from leaves, which had previously been disinfected and treated with a weak solution of sodium hypochlorite. DNA extraction was performed with sorbent method using a commercial kit "Sorb-GMO-B" (Syntol, Russia). The concentration was determined by Qubit fluorometer 3.0 (Invitrogen, Russia) according to standard methods. DNA ranged up to concentrations of 5 ng/µL. Isolated DNA was used for population analysis using ISSR-method. ISSR-primers were selected based on published data (Lopez-Aljorna *et al*., 2007; Coutinho *et al*., 2014a; Coutinho *et al*., 2015). Earlier, we highlighted five of the most effective oligonucleotide primers (Chokheli *et al*., 2016) with different annealing temperature (Table 2), which were used in the present study.

Amplification and Electrophoresis

PCR mix was prepared per sample: H_2O (DD): 15.8 µL; 25 m*M* solution of nucleotides 10xdNTP – 2.5 µL; 10x PCR buffer – 2.5 µL; 25 m*M* magnesium chloride ($MgCl_2$) – 2.5 µL; mutant Taq-polymerase – 0.2 µL (5 u/µL), DNA matrix 1 µL and 0.5 µL, primer (10 m*M*). The total volume of the PCR mixture was 25 µL. Amplification was performed in thermal cycler T100 Thermal Cycler (Bio-Rad, USA). The Protocol of amplification was:

1. The first denaturation at 94°C-1 min
2. Denaturation at94°C –30 sec

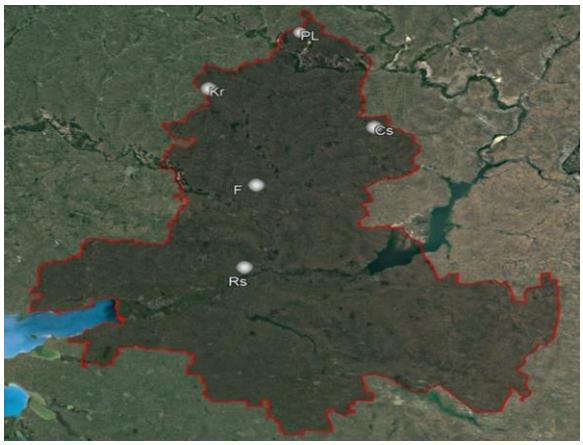

Fig. 1: The location of cenopopulations of the pedunculate oak on the territory of the Rostov region

3. The annealing of the primer at a temperature amplification (T_a°C) –45 sec
4. Elongation at 72°C – 2 min
5. 34 cycles starting with the second paragraph
6. The last elongation at 72°C – 5 min
7. Storage at 4°C

Separation of fragments was performed by electrophoresis in 2% agarose gel using 1xTBE buffer (TRIS, Boric acid, EDTA), at a power of 100 V, 3 h. DNA fragments were stained by dye SYBR Green (x80) in the ratio of 2 µL of dye to 5 µL of DNA. Detection of the fragments was performed in gel documentation system GelDoc XR+ with the software ImageLab 6.0 (Bio-Rad, USA). 100 bpDNA ladder was used as marker (EVROGEN, Russia).

Statistical Analysis

Computer analysis of DNA polymorphism was conducted using the program POPGENE 1.32 (Yeh *et al*., 1997), and GenAlEx6 specialized macro for MS-Excel (Peakall and Smouse, 2006) for the determination of the proportion of polymorphic loci (P) (Williams *et al*., 1990), expected heterozygosity (H_e) (Nei, 1987), absolute number of alleles (n_a), effective number of alleles (n_e) (Kimura and Crow, 1964). To describe the genetic structure of cenopopulations of *Q. robur* following parameters were used. The expected proportion of heterozygous genotypes (H_T) in the whole population as a measure of total gene diversity; the expected proportion of heterozygous genotypes (H_S) in the subpopulation as a measure of its intrapopulation diversity; the proportion of interpopulation genetic diversity in general, diversity or increased differentiation of populations (G_{ST}) (Nei, 1975). Genetic distance (D) between the populations was defined in the program by the formulas of Nei and Li (1979). Based on matrices, binary features were calculated using matrix of genetic differences (Nei, 1972). In the

Table 1: Cenopopulation of pedunculate oak, growing on the territory of Rostov region

Cenopulation name	Geographical localization	Code *	Geographic latitude	Geographic longitude
Hole "Fil'kino"	Rostov region, Belokalitvinskiy district	F	48°26'28,175"	40°48'59,102"
Peskovatsko-Lopatinskiy forest	Rostov region, Verhnedonskoy district	PL	49°58'53,209"	41°18'32,512"
Chernyshevskiesands	Rostov region, Sovetskiy district	Cs	49°01'26,385"	42°12'29,424"
Razdorskie slopes	Rostov region, Ust'-Doneckiy district	Rs	47°37'13,541"	40°41'43,335"
Kriydynniy forest	Rostov region, Chertkovskiyрайон	Kr	49°24'11,360"	40°13'04,400"

*this is conventional sign of cenopopulations, which would be used in text and tables

Table 2: Characteristic of ISSR-primers used for DNA *Q. robur*

Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	The length of the fragments (bp)	The number of fragments for the total sample
UBC 811	$(GA)_8C$	53	250–1100	33
UBC 835	$(AG)_8YC$	52	300–1550	36
UBC 841	$(GA)_8YC$	52	500–1600	29
UBC 857	$(AC)_8YG$	52	250–1550	40
UBC 880	$(GGAG)_4$	53	200–1650	47
Total:				185

Y - isanypyrimidine (Cytosine (C) or Thymine (T))

Table 3: The genetic diversity of cenopopulations of *Q. robur* on the basis of polymorphism of ISSR-PCR markers

Cenopulation name	n_a*	n_e*	H_e*	I*	A*	P* %
Kr	1.9917±0.0913	1.4977±0.2851	0.3078±0.1317	0.4744±0.1621	119	64.32
PL	1.4412±0.5002	1.3120±0.3537	0.1827±0.2072	0.2668±0.3025	30	16.22
Rs	1.9826±0.1313	1.5206±0.2998	0.3158±0.1376	0.4826±0.1698	113	61.08
F	1.9176±0.2765	1.5302±0.3397	0.3123±0.1583	0.4714±0.2056	78	42.16
Cs	1.9402±0.2382	1.5804±0.3214	0.3381±0.1471	0.5050±0.1881	110	59.46
All loci	1.9946±0.0735	1.5549±0.2858	0.3338±0.1280	0.5059±0.1545	184	99.46

* n_a – Observed number of alleles
* n_e – Effective number of alleles
* H_e – gene diversity
* I– Shannon's Information index
* A – The number of polymorphic loci is
* P – The percentage of polymorphic loci is

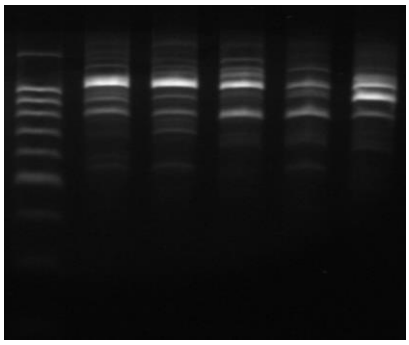

Fig. 2: ISSR-range of the cenopopulations *Q. robur* (Cs) with the primer UBC 841

matrix of genetic differences, unweighted pair-group method (UPGMA) was constructed using dendrograms reflecting the degree of relatedness of the studied populations according to the ISSR-spectra by means of computer programs Treecon 1.3 b and POPGENE 1.32.

Results

In five cenopopulations of *Q. robur*, 185 ISSR-markers of DNA were detected of which 184 were polymorphic (Table 3). The average number of fragments detected with one primer was 37, maximum was 47 (primer UBC 880) and the minimum 29 (primer UBC 841) (Table 2 and Fig. 2). The percentage of polymorphic loci (P), obtained in PCR with all ISSR-primers was 99.46. This index with the lowest values in the cenopopulations PL and F was (P = 16.22, and P = 42.16, respectively). The part of polymorphic loci set with different primers ranged from 30 (the cenopopulation PL) to 119 (the cenopopulation of the Kr).

ISSR-range of the cenopopulations *Q. robur* (Cs) with the primer UBC 841 is given in Fig. 2. The most widespread measure of genetic variation in a population is heterozygosity. The average expected heterozygosity (H_E) for the total sample was 0.3338 (Table 3). The highest level of expected heterozygosity was revealed in the cenopopulation Cs (H_E = 0.3381), and the lowest in cenopopulations PL (H_E = 0.1827). The largest absolute number of alleles detected in the cenopopulations of Kr (n_a = 1.9917) and Rs (n_a = 1.9826). The effective number of alleles was greatest in the cenopopulation Cs (n_e = 1.5804). The lowest indices of effective number of alleles observed in the cenopopulation PL (n_e = 1.3120).

The total gene diversity, heterozygous genotype (H_T)

Q. robur in the total sample was 4.6858, and the average sample gene diversity for all loci (H_S) was 0.1641 (Table 4). The coefficient of cenopopulation differentiation (G_{ST}) indicates that the interpopulation component accounts for 100% of the genetic diversity (G_{ST} = 1.0350).

The dendrogram (Fig. 3) shows that the four natural cenopopulations form between two clusters consisting of 2 cenopopulations each (Kriydynniy forest (Kr) and Razdorskie slopes (Rs); Peskovatsko-Lopatinskiy forest (PL) and the hole "Fil'kino" (F). One artificial cenopopulation placeoutside the clade (Chernyshevskie sands (Cs)) (Fig. 3). However, cenopopulation Cs has been placed near cenopopulations PL and F, which most likely suggests that the genetic material was introduced from Peskovatsko-Lopatinsky forest.

UPGMA dendrogram of genetic similarity of cenopopulations of *Q. robur*, make on the basis of polymorphism of ISSR-markers is given as Fig. 3. Based on the obtained data, a table of the genetic similarities and genetic distances of five cenopopulations of pedunculate oak growing on the territory of Rostov region was constructed (Table 5). From the table we can be concluded that the most genetically similar cenopopulations are F and PL (D = 0.7433) and the far – F and Kr (D = 0.5957).

Table 4: Genetic structure of five cenopopulations of *Q. robur* in the Rostov region

	H_T	H_S	G_{ST}	Nm*
For the total sample	4.6858±49.7533	0.1641±0.0124	1.0350	0.0169

* H_T – the total gene diversity in the total sample
* H_S – average sample gene diversity for all loci
* G_{ST} – the rate of differentiation of populations
* Nm – estimation of gene flow from G_{ST}

Table 5: Table of genetic similarity and genetic distances of cenopopulations of *Q. robur*

	Kr	PL	Rs	F	Cs
Kr	****	0.6010	0.6271	0.5957	0.6093
PL	0.5092	****	0.6168	0.7433	0.6458
Rs	0.4666	0.4833	****	0.6406	0.6194
F	0.5181	0.2967	0.4454	****	0.6176
Cs	0.4954	0.4373	0.4790	0.4820	****

* genetic identity (above diagonal) and genetic distance (below diagonal)

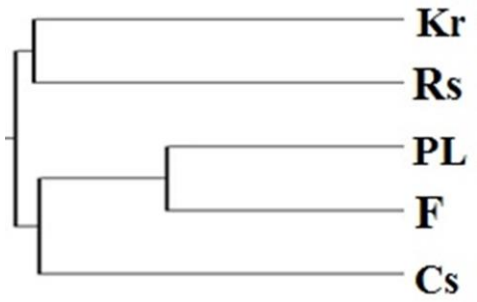

Fig. 3: UPGMA dendrogram of genetic similarity of cenopopulations of *Q. robur*, make on the basis of polymorphism of ISSR-markers

Discussion

Five cenopopulations of *Q. robur* in Rostov region were characterized by high levels of genetic diversity on the basis of polymorphism of ISSR-markers. We found 185ISSR-markers, of which 184 were polymorphic. Each cenopopulation was characterized by its number of polymorphic loci. So for example, in cenopopulation Peskovatsko-Lopatinsky forest (PL) was 30 polymorphic loci, and in cenopopulation Kriydynniy forest (Kr) was 119 polymorphic loci. The average number of fragments detected with one primer was 37, maximum was 47 (primer UBC 880) and the minimum 29 (primer UBC 841). The length of DNA fragments in all markers was approximately the same and ranged from 200–250 bp for UBC 811, UBC 857 and UBC 880 to 1600–1650 bp for UBC 841 and UBC 880 (Table 2). The proportion of polymorphic loci amounted to 99.46%, and average expected heterozygosity was 0.3338. By comparing the level of genetic differentiation, populations of pedunculate oak was similar to other populations of *Q robur*, studied using RAPD-analysis (0.345) in the Republic of Belarus (Kovalevich *et al*., 2010). These factors confirm the hypothesis that widespread species maintain higher level of genetic diversity than rare species (Karron, 1987).

In our study, cenopopulations of pedunculate oak in the Rostov region showed a significant level of genetic differentiation. A large proportion of diversity accounted for between-population component (G_{ST} = 1.0350). Genetic distance (D) between the studied cenopopulations of *Q. robur* ranged from 0.2967 (between PL and F) to 0.5181 (between Kr and F). The most genetically similar populations are cenopopulations hole "Fil'kino"(F) and cenopopulations Peskovatsko-Lopatinskiy forest (PL), and the level of genetic similary is equal to 0.7433. From the resulting dendrogram, it can be conclude that the four natural cenopopulations form between two clusters consisting of two cenopopulations each (Kriydynniy forestthe forest (Kr) and Razdorskie slopes (Rs) (the level of genetic similary is equal to 0.6271); Peskovatsko-Lopatinskiy forest (PL) and the hole "Fil'kino"(F) (the level of genetic similary is equal to 0.7433)). One artificial cenopopulation stood outside the clade (Chernyshevskie sands (Cs)) (Fig. 3). However, cenopopulation Cs takes place near cenopopulations PL and F (the level of genetic similary between Cs and PL is equal to 0.6458, and between Cs and F is equal to 0.6176), which most likely suggests that the genetic material was introduced from Peskovatsko-Lopatinsky forest. Formed clusters of cenopopulations are characterized by their geographical distribution. Thus, the studied cenopopulations *Q. robur* location in similar botanically-geographical districts of the Rostov region, are characterized by similar genetic structure.

Conclusion

Cenopopulations of pedunculate oak in the Rostov region showed a significant level of genetic differentiation. A large proportion of diversity accounted for between-population component (G_{ST} = 1.0350). Genetic distance (D) between the studied cenopopulations of *Q. robur* ranged from 0.2967 (between PL and F) to 0.5181 (between Kr and F). From the resulting dendrogram, it can be conclude that the four natural cenopopulations form between two clusters consisting of 2 cenopopulations each (Kriydynniy forest the forest (Kr) and Razdorskie slopes (Rs); Peskovatsko-Lopatinskiy forest (PL) and the hole “Fil’kino” (F)). One artificial cenopopulation stands outside the clade (Chernyshevskie sands(Cs)). Thus, the studied cenopopulations *Q. robur* location in similar botanically-geographical districts of the Rostov region, are characterized by similar genetic structure.

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