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GENETIC STRUCTURE OF BLACK POPLAR (*Populus nigra* L.) POPULATION IN THE AREA OF GREAT WAR ISLAND

Zoran MAKSIMOVIĆ[1], Dijana ČORTAN[2], Vladan IVETIĆ[2], Snežana MLADENOVIĆ DRINIĆ[3], Mirjana ŠIJAČIĆ-NIKOLIĆ[2]

[1] State Enterprise for Forest Management "Srbijašume", Belgrade, Serbia
[2] University of Belgrade, Faculty of Forestry, Belgrade, Serbia
[3] Maize Research Institute, Zemun Polje, Belgrade, Serbia

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The genetic structure of black poplar (*Populus nigra* L.) populations in the area of Great War Island (GWI) was studied at the level of 30 genotypes, based on microsatellite molecular markers (*SSR*). Eleven polymorphic *SSR* loci were used for analysis of intarpopulation genetic variability. Observed and expected heterozygosity in studied population were high (0.70 and 0.82). The fixation index calculated for single locus ranged from -0.055 (PMGC_14) up to 0.424 (PMGC_2607), while the mean value was 0.143. Deviation from Hardy-Weinberg equilibrium (HWE) differed between single loci. Stabile genetic structure and satisfactory level of genetic variability that have been determined at the population level represent a good starting point for conservation and sustainable use of the available gene pool and further breeding of this species.

Key words: black poplar, Great War Island, genetic variability, microsatellites

INTRODUCTION

Black poplar (*Populus nigra* L.) is one of the most important alluvial forest tree species in Europe. According to the REFORGEN data base of forest genetic resources black poplar is classified as endangered species in the whole of Europe. In recent decades, the presence of black poplar in riparian forests of Serbia has been increasingly reduced. In total forest area, autochthonous poplar forests participate with only 0.5-1.0%, and they are considered a rare species (BANKOVIĆ *et al*., 2009).

The protected natural area "Great War Island" belongs to alluvial habitats that are characterized by specific vegetation, which makes this habitat ideal for various plant and animal

Corresponding author: Zoran Maksimović,State Enterprise for Forest Management "Srbijašume", Mihajlo Pupin Boulevard 113, 11070 New Belgrade,Tel: +381 64 313 59 62,Fax: +381 11 711 85 13,E-mail: zoran.maksimovic@srbijasume.rs

species (ŠIJAČIĆ-NIKOLIĆ *et al*., 2014). The presence of black poplar in these habitats is essential for the preservation of sensitive riparian forests. With a share in volume of 5.4 % (2,458.7 m^3) and volume increment of 7.0 % (54.2 m^3), black poplar in this area belongs to the group of rare and endangered species.

From the perspective of conservation and sustainable use of the available gene pool, exploring genetic structure and the level of variability at the population level is a starting point for further activities. Study of neutral variability, which can be measured using DNA profiling techniques, does not include adaptive differences among individuals. The use of molecular markers eliminates numerous misunderstandings on variability, which are a consequence of environmental impacts, especially in the analysis of quantitative traits, the expression of which is much more impacted by interaction between the genetic base and variable environmental conditions (ŠIJAČIĆ-NIKOLIĆ *et al.*, 2009 a, ISAJEV *et al*., 2009). For this reason, last few decades molecular genetics techniques have been increasingly applied in determining the degree of variability in forest species populations from the region (*Querqus petraea* (Matt.) Liebl., ŠIJAČIĆ-NIKOLIĆ *et al.*, 2009 a, 2009 b; *Fagus sylvatica* L., IVETIĆ *et al*., 2010, 2012; *Picea omorica* (Panč.) Purkyně, MILOVANOVIĆ and ŠIJAČIĆ-NIKOLIĆ *et al*., 2010; *Picea abies* Karst., BALLIAN *et al*., 2007; *Pinus nigra* Arnold, LUČIĆ *et al*., 2010, 2013; *Taxus baccata* L., BALLIAN *et al*., 2008).

Biochemical markers, isozymes and proteins, are widely used in forest genetic studies but they had many limitations (LUČIĆ *et al*., 2011, MATARUGA *et al.*, 2012). The development of DNA markers, including RFLPs (Restriction Fragment Length Polymorphism), RAPDs (Random Amplified Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphism), microsatellites or SSR (Simple Sequence Repeats) and, SNP (Single Nucleotid Polymorphism) has overcome limitations on the number of variable loci and provided the tools to study variation in coding, non-coding, and highly variable regions of both nuclear or organelle genomes (PORTH and EL KASSABY, 2014). Studies of population structure requires sufficient level of intraspecific variability so DNA markers are more appropriate than protein markers. In forest genetics by development of molecular markers, progress has taken place in the study of population genetic structure (WANG and SZMIDT, 2001), genetic diversity (PORTH and EL-KASSABY, 2014; LUČIĆ *et al*., 2014), inter and intra population genetic variability (LUČIĆ *et al*., 2013), and conservation strategy (KONZEN, 2014). In last decade most studies of forest tree have been focused on SSR and SNP markers. The major advantages of using SSR markers over other types of markers, is that they generally have a large number of alleles at a locus, co-dominant inheritance allows discrimination of homo- and hetero- zygotic states in diploid organisms, they display a selectively neutral behavior, can be used among all members of a species, they have a frequent occurrence and an even distribution throughout the nuclear genome, and can also be found in the chloroplast and mitochondrial genomes and they are quickly and efficiently analyzed from very small amounts of plant tissue (LEFORT *et al*., 1999).

Genetic diversity of black poplar has been studied by using morphological markers (ALIMOHAMADI *et al.*, 2012; MAKSIMOVIĆ and ŠIJAČIĆ-NIKOLIĆ, 2013; ČORTAN *et al*., 2013; ČORTAN *et al.*, 2014), molecular *AFLP* markers (GAO *et al.*, 2007; ORLOVIĆ *et al.*, 2009) and microsatellite markers (VAN DER SCHOOT *et al.*, 2000; SMULDERS *et al.*, 2001; POSPIŠKOVA and BARTAKOVA, 2004; SMULDERS *et al.*, 2008; RATHMACHER *et al*., 2010; ALIMOHAMADI *et al*., 2012).

The aim of this study is to determine the intrapopulation genetic variability of black poplar in the area of the Great War Island, as a starting point for the work on conservation and sustainable utilization of the available gene pool and further breeding activities of this species.

MATERIALS AND METHODS

In the area of Great War Island, black poplar occurs in mixed forest stands belonging to the white and black poplar forest type (*Populetim albo- nigrae Slav*.52) on a mosaic of alluvial soils (BANKOVIĆ and MEDAREVIĆ, 2003). In many parts of this island in these populations, the shrub storey is well-stocked with a false indigo bush (*Amorpha fruticosa* L.), which threatens the survival and natural regeneration of these species, leading to a substantial reduction in their natural populations. According to the data from a *Special Forest Management Plan for the management unit GWI 2008-2017*, black poplar is represented with a share in volume of 5.4 % (2,458.7 m^3) and volume increment of 7.0 % (54.2 m^3).

Table 1. Names and sequences of primers, repetitive motifs and the expected length of amplified microsatellite fragments

No	SSR locus	Forward/Reverse primer (5'→3')	Annealing temp	Average bp Length	Motif
1.	PMGC_14	F: TTCAGAATGTGCATGATGG R: GTGATGATCTCACCGTTTG	50°C	210	CTT
2.	PMGC_2020	F: TAAGGCTCTGTTTGTTAGTCAG R: GAGATCTAATAAAGAAGGTCTTC	55°C	150	GA
3.	PMGC_2163	F: CAATCGAAGGTAAGGTTAGTG R: CGTTGGACATAGATCACACG	55°C	220	GA
4.	PMGC_2550	F: AGGTTACAAACTTTGTTGTAGC R: GAACAAACTCTCACTGTGGTC	56°C	118	GA
5.	PMGC_2607	F: TTAAAGGGTGGTCTGCAAGC R: CTTCTTGCACCTCGTTTTGAG	55°C	177	GA
6.	PMGC_2679	F: GGAATCCGTTTAGGGATCTG R: CGTCTGGAGAACGTGATTAG	58°C	118	GA
7.	WPMS_09	F: CTGCTTGCTACCGTGGAACA R: AAGCAATTTGGGTCTGAGTATCTG	60°C	275	GT
8.	WPMS_14	F: CAGCCGCAGCCACTGAGAAATC R: GCCTGCTGAGAAGACTGCCTTGAC	50°C	245	CGT
9.	WPMS_16	F: CTCGTACTATTTCCGATGATGACC R: AGATTATTAGGTGGGCCAAGGACT	65°C	145	GTC
10.	WPMS_17	F: ACATCCGCCAATGCTTCGGTGTTT R: GTGACGGTGGTGGCGGATTTTCTT	55°C	140	CAC
11.	WPMS_18	F: CTTCACATAGGACATAGCAGCATC R: CACCAGAGTCATCACCAGTTATTG	55°C	245	GTG
12.	WPMS_20	F: GTGCGCACATCTATGACTATCG R:	60°C	252	TTCTGG

Genetic structure of black poplar populations in the area of Great War Island was studied at the level of 30 genotypes using molecular markers (*SSR*). The genotypes were analyzed by 11 different primer pairs, a detailed description of used primers is given in Table 1. Information about the used *SSR* markers was taken from a *SSR* source of International Populus Genome Consortium IPGR (http://www.ornl.gov./sci/ipgc/ssr_resource.htm).

DNA isolation was carried out according to a modified protocol by DUMOLIN *et al.* (1995), while the polymerase chain reaction (PCR) was performed according to PAKULL *et al.* (2009).PCR mixtures in total volume of 25 µl containing 80 ng genomic DNK, (5uM) 1.0 µl primer (5uM), 10 x buffer BD 2.5 µl, $MgCl_2$ (25mM) 1.5 µl, Cy5 dNTPmix 2.0 µl, DMSO 0.5 µl, Taq polymerase 0.2 µl, H_2O 16.3 µl. Annealing temperatures were in the range of 50-70 C^o, depending on used primers (Table 1).

The success of the fragment amplification was checked on a 1% agarose gel that was visualized by Roty-Safe Gelstain (Carl Roth, Karlsruhe, Germany). After determining the success of PCR amplification, the PCR products were separated using an automatic sequencing unit ALFexpress II (GE Healthcare). Fragmentary analysis of the products was carried out using the Fragment Analyser software (version 1.03.01, GE Healthcare).

Data analysis has been performed using statistical program GenAlEx version 6.501 (PAEKALL and SMOUSE, 2005). The following was determined for each microsatellite locus: the number of different alleles (Na), number of effective alleles (Ne), observed (Ho) and expected (He) heterozigosity, and fixation index (Fis). The same program was used to calculate allele frequency, significance test of deviation from Hardy-Weinberg equilibrium (HW) per each locus and genetic distances between test trees within population.

RESULTS

Microsatellite profiles of the analyzed test trees from black poplar populations in the area of Great War Island show that none of the 30 test trees has identical *SSR* profiles. Analyzed loci did not have the same amount of information, while some of genotypes had identical profiles for several loci. The analyzed test trees could not be discerned by using only one *SSR* loci.

The length of base pairs, amplified by polymerase chain reaction and detected by electrophoresis in an automated sequencer, were in the range from 131 up to 282 base pairs depending on the used locus (Table 2). The analysis of 11 microsatellite loci show that studied population had in total 110 different alleles, in average 10 alleles per each locus. Number of effective alleles per locus (Ne) was in the range from 2.499 (WPMS_17) to 11.213 (PMGC_2163). The observed heterozygosity (Ho) per locus was in the range from 0.483 (PMGC_2607) to 0.900 (PMGC_14), while expected heterozygosity (He) per locus values from 0.600 (WPMS_17) to 0.911 (PMGC_2163). The value of fixation index (Fis) was in the range from -0.055 (PMGC_14) to 0.424 (PMGC_2607).

Despite a relatively large number of alleles that have been detected in the tested sample their frequencies in the most loci were not evenly distributed (Table 3). Some alleles make up a large proportion of the total variability of alleles per locus. For instance, at the locus PMGC_2550 alleles with a 135 bp and 145 bp length were noticed in 39 cases out of 60, and together represent 65 % of variability. The minimum number of alleles was detected at the locus WPMS_17, where allele with the size of 134 bp was noticed in 29 cases out of 60, representing allele frequency of 50 %.

Table 2. Levels of genetic diversity within Populus nigra L. population by single locus

Locus	N	Range size (bp)	Na	Ne	Ho	He	Fis
WPMS_16	30	139-160	7	4.972	0.667	0.799	0.166
PMGC_14	30	198-222	9	6.818	0.900	0.853	-0.055
PMGC_2550	30	135-167	9	4.286	0.667	0.767	0.130
WPMS_20	30	221-251	9	6.569	0.833	0.848	0.017
WPMS_14	30	228-282	13	5.769	0.700	0.827	0.153
WPMS_17	29	131-143	3	2.499	0.552	0.600	0.080
PMGC_2163	29	216-246	15	11.213	0.690	0.911	0.243
PMGC_2607	29	151-183	11	6.184	0.483	0.838	0.424
WPMS_18	29	222-246	9	7.509	0.828	0.867	0.045
PMGC_2020	29	135-169	13	6.007	0.828	0.834	0.007
WPMS_9	28	239-281	12	9.739	0.571	0.897	0.363
Total			110				
Mean	29.364		10.000	6.506	0.702	0.822	0.143
SE	0.203		1.000	0.726	0.040	0.025	0.045

Legend: N – sample size, Na – number of alleles per locus , Ne – number of effective alleles per locus, Ho – observed heterozygosity, He – expected heterozygosity, Fis – fixation index

Table 3. Allele frequency of single SSR loci

SSR **locus**																						
WPMS_16		**PMGC_14**		**PMGC_2550**		**WPMS_20**		**WPMS_14**		**WPMS_17**		**PMGC_2163**		**PMGC_2607**		**WPMS_18**		**PMGC_2020**		**WPMS_9**	
N	30	N	30	N	30	N	30	N	30	N	29	N	29	N	29	N	29	N	29	N	28
allele	%	allele	%	Allele	%	allele	%	allele	%	allele	%	allele	%	allele	%	allele	%	allele	%	allele	%
139	23	198	12	135	32	221	20	228	3	131	14	216	9	151	2	222	21	135	5	239	4
142	7	201	15	143	8	227	8	231	30	134	50	218	10	159	14	225	16	137	17	241	4
145	15	204	22	145	33	230	20	240	2	143	36	220	14	161	29	228	3	139	5	245	7
151	25	207	15	147	3	233	20	249	22			222	3	163	12	231	10	143	31	247	9
154	5	210	12	149	7	236	8	252	15			224	7	169	5	234	14	145	7	249	11
157	23	213	2	151	3	239	2	255	5			226	9	171	17	237	9	149	16	251	18
160	2	216	15	153	3	242	10	258	3			228	9	173	5	240	12	151	5	253	9
		219	7	155	8	245	8	261	2			230	2	177	5	243	5	153	2	255	9
		222	2	167	2	251	3	264	7			232	5	179	5	246	10	155	2	261	2
								267	3			234	3	181	3			157	2	263	9
								270	2			236	12	183	2			159	3	279	11
								273	5			238	10					161	3	281	9
								282	2			240	3					169	2		
												242	2								
												246	2								

Table 4. Significance deviation test from Hardy-Weinberg equilibrium (HWE) per each locus

Locus	DF	ChiSq	P	Signif
WPMS_16	21	43.584	0.003	**
PMGC_14	36	38.978	0.337	Ns
PMGC_2550	36	68.240	0.001	***
WPMS_20	36	58.300	0.011	*
WPMS_14	78	78.161	0.474	Ns
WPMS_17	3	4.757	0.190	Ns
PMGC_2163	105	153.631	0.001	**
PMGC_2607	55	74.664	0.040	*
WPMS_18	36	25.067	0.914	Ns
PMGC_2020	78	72.246	0.662	Ns
WPMS_9	66	125.751	0.000	***

Legend: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001

Table 5. The genetic distance between the analyzed black poplar test trees in the area of Great War Island

I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12	I13	I14	I15	I16	I17	I18	I19	I20	I21	I22	I23	I24	I25	I26	I27	I28	I29	I30	
0																														I1
25	0																													I2
19	21	0																												I3
30	21	27	0																											I4
20	20	22	30	0																										I5
15	23	22	32	21	0																									I6
21	20	24	30	15	21	0																								I7
23	23	23	27	18	23	12	0																							I8
27	20	20	27	22	26	21	20	0																						I9
20	19	17	25	19	21	22	19	22	0																					I10
25	28	28	31	26	28	20	22	23	27	0																				I11
17	23	22	30	20	23	13	15	24	20	21	0																			I12
23	26	24	27	24	25	20	19	25	23	20	21	0																		I13
24	20	21	24	22	27	18	13	18	16	21	17	20	0																	I14
24	19	22	27	15	27	16	19	18	21	28	23	27	16	0																I15
26	26	16	29	28	29	26	24	27	17	28	23	23	12	26	0															I16
26	23	15	32	24	27	24	24	23	22	28	22	23	21	23	17	0														I17
27	25	22	32	27	33	27	27	27	25	29	21	27	24	29	24	20	0													I18
21	18	20	25	20	26	18	18	25	14	24	18	21	16	18	18	22	19	0												I19
18	22	23	31	17	20	17	19	26	20	22	18	24	24	23	26	25	28	20	0											I20
22	19	21	30	18	19	15	21	25	20	19	21	23	19	22	22	20	26	19	15	0										I21
20	25	22	27	21	25	18	20	24	22	23	20	22	20	23	22	20	24	20	21	19	0									I22
21	23	22	25	27	29	19	20	26	21	24	20	22	19	23	24	27	22	17	21	26	19	0								I23
25	22	24	29	24	27	21	24	26	20	24	22	17	18	24	20	23	27	16	27	20	20	26	0							I24
22	20	18	28	22	28	22	23	25	20	26	21	26	18	23	18	18	18	15	23	16	20	19	17	0						I25
26	23	19	29	26	26	24	24	24	17	26	23	23	19	24	16	21	21	19	20	22	22	22	19	21	0					I26
22	26	25	32	24	27	19	21	28	22	24	20	24	20	25	20	21	26	14	23	18	18	21	17	13	18	0				I27
23	18	20	25	23	26	22	24	22	18	22	20	23	18	22	18	22	22	17	16	20	23	17	20	19	12	19	0			I28
22	19	19	25	17	23	13	15	18	18	24	18	20	15	17	17	22	26	16	22	18	19	20	18	15	19	21	17	0		I29
23	27	26	28	31	29	22	19	26	23	28	20	26	22	26	26	26	20	19	26	28	20	16	27	23	22	23	22	22	0	I30

Test results of significant deviations from Hardy-Weinberg equilibrium (HWE) per each locus is shown in Table 4. From obtained data it is obvious that the probability of Chi – square values (taking into account the degree of freedom) for loci PMGC_14, WPMS_14, WPMS_17, WPMS_18, PMGC_2020 were higher than 0.05 (P in the range 0.05 to 1.0), thus results for this set of loci were not statistically significant. Considering that the probability of Chi – square values for loci WPMS_16, PMGC_2550, WPMS_20, PMGC_2163, PMGC_2607, WPMS_9 were less than 0.05 (in the range $0<P<0.05$), it can be concluded that the results for this set of loci were statistically significant.

Table 5 shows the genetic distance between the analyzed black poplar test trees in the area of Great War Island. The results show that the smallest genetic distance (12) was found between the test trees E7 and E8, E14 and E16, E26 and E28. The highest genetic distance (32) was found between test trees E4 and E6, E4 and E17, E4 and E18, E4 and E27. Large genetic distances (31, 30) were noted between the test trees E4 and E11, E20 and E4, E5 and E30, E1 and E4, E4 and E5, E4 and E7, E4 and E12, E4 and E21. On the other hand, a relatively small genetic distance (13) was noted between test trees E5 and E7, E5 and E15, E12 and E7, E8 and E14, E7 and E29, E19 and E25, E25 and E27.

DISCUSSION

It is known that microsatellite markers can differentiate closely related species and genotypes and can also be used to investigate genetic diversity in natural populations. In addition, the codominant nature of microsatellites makes them ideally suited for population genetic studies, as it allows assessing loss of heterozygosity, population subdivision and inbreeding (VAN DER SCHOOT *et al*., 2000).

Eleven analyzed primer pairs showed a significant level of intrapopulation polymorphism. Using those primers between 3 and 15 alleles per locus have been detected. A number of effective alleles per locus, which represent estimation of equally frequent alleles in an ideal population, were in the range between 2.499 and 11.213.

Observed heterozygosity in studied population was high (0.552-0.900), aside from the loci PMCG_2607 that had a value of 0.483, where one allele was presented with a high frequency. In research of SMULDERS *et al.* (2001), who developed and used a six trinucleotide repeat microsatellite markers, is stated that observed heterozygosity of 23 genotypes of European black poplar, that represent the diversity across Western and Central Europe, was quite high. Observed heterozygosity for genotypes across Europe was in the range 0.57-0.91, with the exception of WPMS_15 who had a heterozygosity value of only 0.32. Based on obtained results it is evident that the observed heterozygosity (0.70) of 30 black poplar test trees from the area of the Great War Island is in accordance with results of 23 black poplar genotypes from the area of Europe (SMULDERS *et al.* 2001). Number of detected alleles per locus for genotypes across Europe is between 6 (WPMS_15, WPMS_17 and WPMS_20) and 12 (WMPS 14), while in our results is in the range from 3 (WPMS_17) to 15 (PMGC_2163). The obtained results are comparable to the genetic diversity of black poplar populations in other studies across Europe (POSPIŠKOVA and BARTAKOVA, 2004; SMULDERS *et al*., 2008; RATHMACHER *et al*., 2010).

VAN DER SCHOOT *et al.* (2000) developed 9 dinucleotide repeat microsatellite markers that were tested on 23 black poplar genotypes across Europe and established a high level of microsatellite polymorphism with 10-19 different alleles per locus and level of observed heterozygosity between genotypes in average 0.71 (in range 0.25-1.00).

Expected heterozygosity in the studied population is quite high, in the range of 0.600-0.911, indicating a large genetic diversity within this population. The minimum expected heterozygosity between test trees of studied population was noticed at the locus WPMS_17, while the highest was at the locus PMGC_2163. The levels of heterozygosity were very high, in line with what is generally found for cross-hybridizing species, such as black polar.

Fixation index was calculated for each microsatellite locus and it was in the range of -0.055 (PMGC_14) to 0.424 (PMGC_2607). The mean value of fixation index was 0.143, indicating that within this black poplar population random mating occurs. Considering a mean value that is close to zero, we can say that inbreeding is not considerably represent, therefore variability within population is still high.

Deviations from Hardy-Weinberg equilibrium (HWE) for loci PMGC_14, WPMS_14, WPMS_17, WPMS_18, PMGC_2020 were not statistically significant (P in the range 0.05 to 1.0), and based on that we can accept the null hypothesis that in the studied population random mating is present. Deviation of loci WPMS_16, PMGC_2550, WPMS_20, PMGC_2163, PMGC_2607, WPMS_9 were statistically significant (in the range $0<P<0.05$), based on that we can reject the null hypothesis and conclude that there is no random mating within the studied population. Obtained results from Chi-square test for Hardy-Weinberg equilibrium (HWE) should be checked on a higher number of test trees in the order to ascertain whether random mating is present in population or not.

CONCLUSION

Knowledge of the structure and variability within and between populations is a starting point for conservation and sustainable use of the available gene pool and further breeding of this species.

Conducted research within black poplar population in the area of Great War Island based on the level of genetic variability that was noticed by 11 different primer pairs of microsatellite markers (*SSR*) and variability of the phenotypic characteristics of the test trees (MAKSIMOVIĆ and ŠIJAČIĆ-NIKOLIĆ, 2013) indicate a stabile population structure.

Recorded heterozygosity in the studied population was high (0.483-0.900) and corresponds to the results obtained in the analysis of 23 genotypes of European black poplar which represent the diversity of Western and Central Europe (0.25-1.00).

In order to get familiar with the genetic structure of populations of black poplar in Serbia and the region, as well as assessing the variability level within and among populations, the results of conducted research should be compared and used towards the conservation objectives of the available black poplar gene pool.

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GENETSKA STRUKTURA POPULACIJE CRNE TOPOLE (*Populus nigra* L.) NA PODRUČJU VELIKOG RATNOG OSTRVA

Zoran MAKSIMOVIĆ[1], Dijana ČORTAN[2], Vladan IVETIĆ[2],
Snežana MLADENOVIĆ DRINIĆ[3], Mirjana ŠIJAČIĆ-NIKOLIĆ[2]

[1] Javno predezeće za gazdovanje šumama "Srbijašume", Beograd, Srbija
[2] Univerzitet u Beogradu, Šumarski fakultet, Beograd, Srbija
[3] Institut za kukuruz, Zemun Polje, Beograd, Srbija

Izvod

Genetska struktura populacije crne topole (*Populus nigra* L.) na području Velikog ratnog ostrva proučena je na nivou 30 genotipova primenom mikrosatelitskih molekularnih markera (*SSRs*). Jedanaest heterogenih markera su korišćeni za proučavanje genetske varijabilnosti unutar populacije. Zapažena i očekivana heterozigotnost u istraživanoj populaciji je visoka (0,70 и 0,82). Fiksacioni indeks je izračunat za svaki mikrosatelitski lokus i kreće se u opsegu od -0,055 (PMGC_14) do 0,424 (PMGC_2607). Srednja vrednost fiksacionog indeksa preko lokusa iznosi 0,143. Nivo signifikantnosti odstupanja od Hardy-Weinbergove равнотеже (HWE) se razlikuje između lokusa. Stabilna genetička struktura i zadovoljavajući stepen genetičke varijabilnosti konstatovan na nivou populacije predstavlja dobru polaznu osnovu za konzervaciju i usmereno korišćenje raspoloživog genofonda i dalje oplemenjivanje vrste.

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