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Original scientific paper

APPLICATION OF SSR MARKERS FOR ASSESSMENT OF GENETIC DIFFERENTIATION OF SILVER FIR (*Abies alba* Mill.) ORIGINATING FROM JAVOR MOUNTAIN

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Popović V., A. Lučić, Lj. Rakonjac, J. Milovanović, S. Mladenović Drinić, D. Ristić (2019): *Application of SSR markers for assessment of genetic differentiation of silver fir (Abies alba Mill.) originating from Javor mountain*.- Genetika, Vol 51, No.3, 1103-1112.

The process of plant breeding and conservation of gene pool among other things depends on the knowledge of the level of genetic differentiation. The aim of research in this paper was to determine the genetic differentiation of silver fir (*Abies alba* Mill.) populations of regular type and atypical genotypes with pyramidal crown that can be found on Javor mountain, at the site Ogorijevac. The genetic differentiation of silver fir was determined using SSR (Simple Sequence Repeat) markers. Nine SSR pairs of primers gave 29 alleles, while the average number of alleles was 3.2. The primer NFH15 gave the smallest number of alleles (two), while the primer SF78 gave the greatest number of alleles (five). Dice coefficient of the genetic similarity was used to obtain a dendrogram by UPMGA analysis using NTSYSpc statistical program. The genetic similarity recorded among the individuals P1 and P2 was the largest (0.89), while the populations VI and individual P2 showed the lowest similarity (0.61).

Based on the cluster analysis it can be concluded that the studied populations and genotypes of silver fir with different types of crown are clearly differentiated. The basic insight into the level of the genetic diversity of the natural populations of silver fir with

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the various types of crown has been provided using selected SSR markers. The obtained results can be used for creating further strategy for the conservation of the available gene pool and the regeneration of silver fir forests in Serbia.

Keywords: Abies alba Mill., SSR (Simple Sequence Repeat) markers, population, differentiation

INTRODUCTION

Silver fir (*Abies alba* Mill.) belongs the Pinaceae family and it is the most important representative of the genus Abies in Europe. In the nature it can be found in mountainous areas of eastern, western, southern and central Europe where it occurs mainly with beech (*Fagus sylvatica* L.) at lower and middle elevations and with Norway spruce (*Picea abies* L. Karst) at higher elevations (LIEPELT *et al*., 2009). It is a long-standing species which the height of 60 - 65 m and the diameter at breast height of 150 - 200 (380) cm.

Over the past several hundred years, the silver fir forests in Europe have declined significantly (WOLF, 2003). The deterioration of the silver fir forests is primarily due to reduced genetic variability and consequently a reduced ability to adapt to changed environmental conditions (drought, frost, acid rain) as well as the action of biotic pathogens (LARSEN, 1986). In addition to the unprecedented environmental damage, the deterioration of the silver fir forests has a negative economic effect as silver fir is one of the most productive forest species in European forestry (MUSIL and HAMERNÍK, 2007).

In Serbia, the silver fir forests cover an area of 25,600 ha, where natural forests dominate with 95.3% and artificially established forests cover only 4.7% of the total area (BANKOVIĆ *et al*., 2009). During the period from 2011 to 2014 the drought led to drying of the silver fir on large areas. The drying process has slowed down recently, but is still present. Considering the modest share of the silver fir in the growing stock of Serbia, one of the priorities of the forestry profession is to return silver fir to the habitats suitable for that species.

In recent decades, the genetic researches of the silver fir using different markers have been intensified to determine the extent of diversity and to explain the causes of deterioration (KONNERT and BERGMANN, 1995; SAGNARD *et al*., 2002; LONGAUER, 2001; LONGAUER *et al*., 2003; CREMER *et al*., 2006; LIEPELT *et al*., 2009; HALILOVIĆ *et al*., 2009; PIOVANI *et al*., 2010; GÖMÖRY *et al*., 2012; BALLIAN *et al*., 2012; POSTOLACHE *et al*., 2013; BALLIAN, 2003, 2013; SANCHO-KNAPIK *et al*., 2014; CVRČKOVÁ *et al*., 2015; POPOVIC *et al*., 2017).

Individual trees of the silver fir with atypical, narrow-pyramidal crown grow at the Mountain Javor, on the Ogorijevac site. The first scientific observation of these individuals and introduction to the wider professional and scientific community was made by TOŠIĆ (1963). This specific population attracted the attention of scientists and the wider public to explore it, but morphometric characteristics have been usually described (TOŠIĆ, 1963; 1995; 1997; MATOVIĆ *et al*., 1996; TOŠIĆ and VILOTIĆ, 1998). The ecological characteristics of the habitat were also analysed, as well as the possible factors that influence the development of the phenotype (KIŠ *et al*., 2009; RATKNIĆ *et al*., 2013). A basic insight into the genetic variability of the population in Ogorijevac using SSR markers gave a clear picture of the existence of two genetic clusters (TRUDIĆ *et al*., 2016).

The basic characteristic of the phenotype is the growth of lateral branches directed upward, which take a sharp angle in relation to the longitudinal axis of the tree, which influenced the formation of a narrow pyramidal habitus (MATOVIĆ *et al*., 1996).

For the improvement of the genetic structure and the adaptive ability of the species, the appearance of different and rare individuals is of a great importance (ŠIJAČIĆ-NIKOLIĆ *et al.*, 2009). The available gene pool has to be comprehensively studied and guidelines have to be given for its targeted use.

In order to carry out the process of plant breeding and the conservation of the available gene pool to a satisfactory extent, it is necessary to determine the degree of genetic differentiation. The aim of the study was to determine the genetic differentiation using SSR markers of silver fir (*Abies alba* Mill.) populations of the regular type and the atypical genotypes with a pyramidal crown that can be found on Javor mountain, at the site Ogorijevac.

MATERIAL AND METHOD

The research was carried out in the three natural populations of silver fir on the Javor Mountain in southwest Serbia. The silver fir trees with a pyramidal crown were recorded and marked at the Ogorijevac site (Figure 1). The samples were taken from two individuals with the pyramidal crown (P1, P2) and subjected to further analysis. One sampled natural population of silver fir is located at the Ogorijevac site as well as the silver fir trees with the pyramidal crown, while the other two natural populations are several tens of kilometres away. The natural populations were represented by 30 trees per population, and the distance between the trees was more than 50 meters. A branch with needles was taken from each tree. The samples were packaged individually in plastic bags and in a refrigerator taken to the laboratory.

The geographical characteristics of the populations are shown in Table 1.

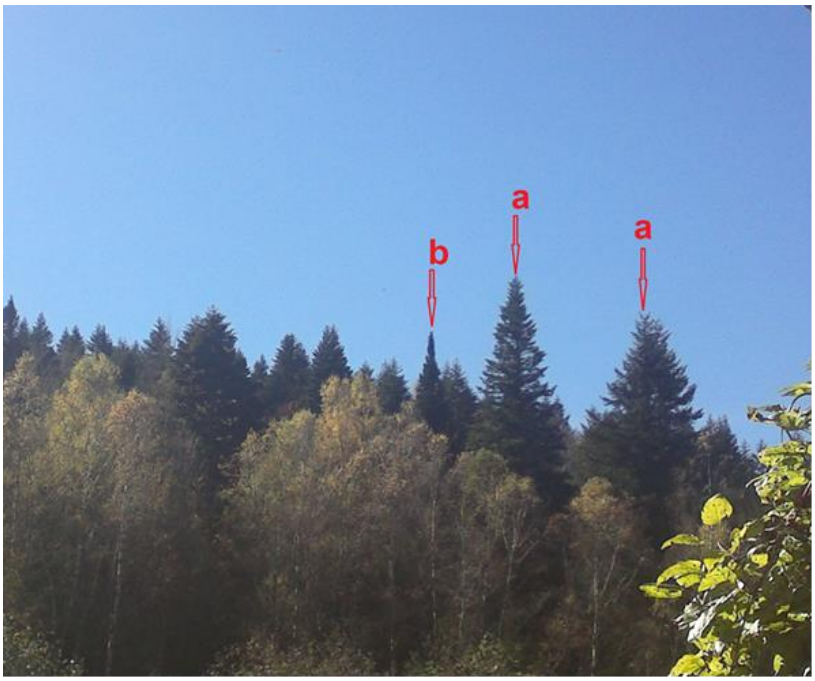

Figure 1. (a) Silver fir with typical crown (b) Silver fir with atypical pyramidal crown on Javor mountain

Table 1. Geographic characteristics of analysed populations

Population Code	Population	Latitude	Longitude	Elevation (m)
IV	Javor	43°24'57"	20°03'22"	1222
V	Javor	43°23'58"	20°05'36"	1256
VI	Javor	43°19'09"	20°06'03"	1290
P1	Ogorijevac	43°25'05"	20°03'26"	1139
P2	Ogorijevac	43°25'06"	20°03'27"	1166

Molecular Analysis

The needles were immersing in the liquid nitrogen and the bulk was made by pooling an equal amount of the plant material. The total genomic DNA was isolated from the needles of silver fir using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method for coniferous tree species according to DOYLE and DOYLE (1987). The DNA concentration and quality were measured using a spectrophotometer Eppendorf BioSpectrometer® kinetic.

The polymerase chain reaction (PCR) was optimized for nine SSR (Simple Sequence Repeat) markers which successfully amplified clear and reproducible alleles (Table 2).

The PCR amplification was carried out in a total volume of 25 μL containing: 1xBuffer, 0.8 mM dNTP, 2.5 mM MgCl2, 0.5 μM of each primer pair, 1 U TaqPoly and 50 ng of DNA. Amplifications were completed in thermocycler Biometra TProfessional Standard 96 using the following PCR program: an initial denaturation (95ºC for 15 min), 35 cycles each (denaturation at 94ºC for 30 s, annealing at (table 2) for 1 min and extension at 72ºC for 1 min) and the final elongation was at 72°C for 8 min. The amplified fragments were separated by electrophoresis on 8% polyacrylamide gel with 100 bp molecular weight ladder as a marker. Gels were photographed under UV light on BioDocAnalyse Biometra gel documentation system after staining with 0.5 μg/μL ethidium bromide.

The SSR profiles were assembled into a binary matrix based on the presence (1) or the absence (0) of a specific allele. The genetic similarities (GS) between the population and the atypical genotypes were calculated by DICE (1945).

$GSij = 2a/2a+b+c$

- "a" - number of fragments present in both variety i and j (1, 1)
- "b" - number of fragments present in i and absent in j (1, 0)
- "c" - number of fragments absent in i and present in j (0, 1)

All marker data analyses were performed using statistical NTSYSpc2 program package (ROHLF, 2000) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) was applied for the cluster analysis.

RESULTS AND DISCUSSION

After the last glaciation in Europe, the remaining relict areas of the *Abies alba* and the postglacial recolonization became a very important for studying its dynamics and gene conservation. Due to a maternal inheritance of the mitochondrial DNA, the mitochondrial markers have widely been used for analysing the genetic structure of different conifer species (GUGERLI *et al*., 2001; JOHANSEN and LATTA, 2003; SPERISEN *et al*., 2001). In last two decades,

the DNA markers, such as nuclear microsatellites, had been used to assess genetic variation of *Abies alba* population (HANSEN *et al*., 2005; CREMER *et al*., 2006; CREMER *et al*., 2012; GÖMÖRY *et al*., 2012). POSTOLACHE *et al*. (2013) due to limited number and presence of null alleles among the existing set of the SSRs, developed a new set of microsatellites using transcriptomic and genomic resources.

In this study we analysed the genetic differentiation using SSR markers between regular type of silver fir and atypical genotypes with a pyramidal crown that can be found on Javor Mountain, at the site Ogorijevac. Due to the small number of the available SSR markers for *Abies alba* we used the possibility of transferring microsatellite markers of related species. Therefore we applied four microsatellite markers developed for species *Abies nordmanniana* (HANSEN *et al*., 2005) and developed markers for *Abies alba* (CREMER *et al*., 2006).

High levels of the genetic differentiation among populations can be found in some studies. SANCHO-KNAPIK *et al.* (2014) indicate that the genetic differentiation among the analysed populations in the Spanish Pyrenees is mainly established by the longitudinal gradient. A significant genetic differentiation was revealed among populations *Abies ziyuanensis* in South China (TANG *et al*., 2008). In the study of CVRČKOVA *et al*. (2015) was found genetic differences among the studied populations located in different parts of the Czech Republic. In contrast, the population of German silver fir had a lower level of genetic differentiation (CREMER, 2009).

Table 2. List of nine informative primers, repeat motif, number of alleles and unique alleles within populations and genotypes

	Probe	Primer sequence	No. of alleles	Anneal. temp. (°C)	Number of unique alleles and allele size
1.	NFF2	F:GGGGTAGAGAGTTGGCTGCT R:CATAAGGATGAGTGGCTTCCA	3	58	-
2.	NFF3	F:CCAATGGGTTGTCAGAGTGTT R:GGCATTCGAGATTGCTTGAT	3	58	-
3.	NFF7	F:CCCAAACTGGAAGATTGGAC R:ATCGCCATCCATCATCAGA	3	57	-
4.	NFH15	F:CGCCTCCCTCCATTACTTC R:TCGTCTAGAGAGGCGAAATTCT	2	58	-
5.	SF1	F:TTGACGTGATTAACAATCCA R:AAGAACGACACCATTCTCAC	4	47	-
6.	SFb4	F:GCCTTTGCAACATAATTGG R:TCACAATTGTTATGTGTGTGG	3	48	2 (160bp; 190bp)
7.	SFg6	F:TAACAATAAAAGGAAGCTACG R:TGTGACACATTGGACACC	3	49	-
8.	SF78	F:CATTGTTGTCTTTGTTTCACA R:TGCACCGTTTTGTTTTTCC	5	48	2 (170bp; 195bp)
9.	SFb5	F:AAAAAGCATCACTTTTCTCG R:AAGAGGAGGGGAGTTACAAG	3	48	-

The total number of alleles detected by nine SSR markers in observed *Abies alba* populations and atypical genotypes was 29, of which four were unique alleles (Table 2). The

range of allele's richness varied from two to five, with an average of 3.2 alleles per locus. The highest level of polymorphism was detected with SF78, and the lowest with NFF15 (Table 2).

The genotypes P1 and P2 have the unique alleles on same gene loci, as well as the rare alleles on one gene loci. The unique alleles are also found in the population IV on SF78 gene loci and in the population V and the genotype P1 on SFb4 gene loci different alleles. On the other hand, the rare alleles were found in the populations IV and V (SFb4 gene loci), V and VI (SF78 gene loci) as well in P1 and P2 (NFF7 gene loci). The lower level of polymorphism, compared to other populations, was found in the population due to the presence of null alleles in population VI. Due to point mutations or indels in flanking primers primer annealing could failure and cause of microsatellite null alleles, which potentially decreasing population genetic diversity and increasing genetic differentiation among populations.

The genetic similarity coefficients for each pairwise are shown in Table 3. The lowest genetic similarity (0.61) was obtained for VI and P2, while the highest was 0.89 (P1 and P2). The average value of the genetic similarity between analysed genotypes was 0.74. Czech populations showed lower genetic distances ranging from 0.091 to 0.232 (CVRČKOVÁ *et al*., 2015), while WOO *et al*. (2008) get smaller values of Nei's genetic distances (ranged from 0.006 to 0.054) among Korean populations than were distances among the eight Czech populations.

Table 3. Genetic similarity coefficients by Dice

	IV	V	VI	P1	P2
IV	1				
V	0.88	1			
VI	0.81	0.84	1		
P1	0.70	0.68	0.63	1	
P2	0.68	0.67	0.61	0.89	1

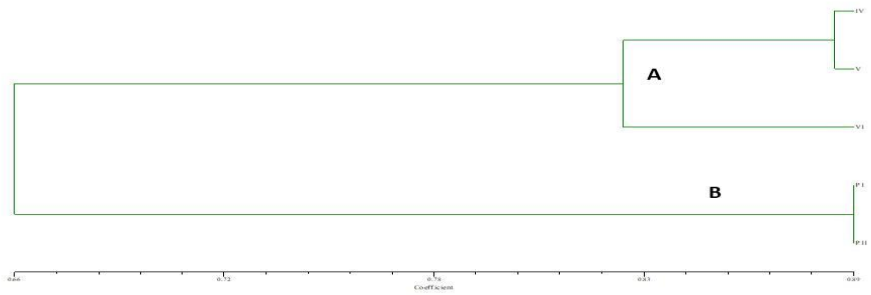

Figure 2. Dendrogram of *Abies alba* genotypes constructed using UPGMA cluster analysis of the genetic similarity values

The results in this paper showed the practicability of using nuclear microsatellite markers in combination with polyacrylamide gel electrophoresis to study genetic differentiation and intraspecific variation among *Abies alba* populations. More sophisticated methods such as capillary electrophoresis for SSR analysis are now available, but it is still too expensive.

CONCLUSIONS

The results obtained in this study show that there is a significant genetic distance between the silver fir (*Abies alba* Mill.) populations of regular type and atypical genotypes with the pyramidal crown occurring on the Javor Mountain. The used SSR markers gave a basic insight into the level of genetic distance between studied genotypes. In order to answer the question whether the differences between the studied genotypes are the result of a mutation of the genes, the process of adaptability, or the difference in the genome, it is necessary to expand the research by involving a larger number of individuals and perform appropriate genetic analyses.

Based on the genetic similarity matrix it can be concluded that similarity is very high between atypical individual genotypes, as well as between populations IV and V. The cluster analysis based on Dice′s genetic similarities distributed genotypes in two groups. The first group „A“ contained the population IV, which is located near individuals with the pyramidal crown and the population V, which is located at a distance of 10 km from the IV population and individuals with pyramidal type of crown. Also, the population VI located at the distance of 30 km from the population IV was the part of this group. The second group „B“ consists of the genotypes P1 and P2 with a pyramidal crown. The population VI which grows at the greatest geographical distance from the genotypes with the pyramidal type of crown was also the most genetically distant from the P1 and P2.

Having in mind that the preservation of the genetic variability is one of the basic requisites for maintaining the adaptive ability of species, the studied atypical genotypes with the pyramidal crown must be involved in long-lasting processes of conservation and breeding through different types of *in situ* and *ex situ* conservation.

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PROCENA GENETIČKE DIFERENCIJACIJE JELE (*Abies alba* Mill.) SA JAVORA POMOĆU SSR MARKERA

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Izvod

Proces oplemenjivanja biljaka i konzervacije genofonda između ostalog, zavisi i od poznavanja stepena genetičke diferencijacije. Cilj istraživanja u ovom radu bio je da se utvrdi genetička diferencijacija populacija jele (*Abies alba* Mill.) normalanog tipa i atipičnih genotipova piramidalanog tipa krošnje, koje se javljaju na planini Javor, na lokalitetu Ogorijevac. Genetička sličnost odnosno različitost jele određena je upotrebom SSR *(Simple Sequence Repeat)* markera. Devet SSR pari prajmera dalo je ukupno 29 alela, dok je prosečan broj alela bio 3,2. Prajmer NFH15 dao je najmanji broj alela (dva), dok je prajmer SF78 bio sa najvećim brojem alela (pet). Najviša vrednost koeficijenta genetičke sličnosti utvrđena je između genotipova P1 i P2 (0,89), dok je najniža vrednost koeficijenta genetičke sličnosti utvrđena između populacije VI i genotipa P2 (0,61).

Koeficijenti genetičke sličnosti po Dice, upotrebljeni su za dobijanje dendrograma pomoću UPMGA analize, koristeći NTSYSpc statistički program. Na osnovu klaster analize može se zaključiti da su istraživane populacije i genotipovi jele sa različitim tipom krošnje jasno izdiferencirani. Upotrebom izabranih SSR markera dat je osnovni uvid u nivo genetičke raznovrsnosti prirodnih populacija jele različitog tipa krošnje. Dobijeni rezultati mogu poslužiti u budućoj strategiji na konzervaciji raspoloživog genofonda i obnavljanju jelovih šuma u Srbiji.

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