UDC 575.630
https://doi.org/10.2298/GENSR1703979P
Original scientific paper

ASSESSMENT OF GENETIC DIVERSITY OF SILVER FIR (*Abies alba* Mill.) IN SERBIA USING SSR MARKERS

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Popović V., A. Lučić, Lj. Rakonjac, B. Cvjetković, S. Mladenović Drinić, D. Ristić (2017): *Assessment of genetic diversity of silver fir (Abies alba mill.) in Serbia using SSR markers* - Genetika, Vol 49, No.3, 979-988.
The paper presents the results of the analysis of genetic variability of eight populations of silver fir (*Abies alba* Mill.) in Serbia obtained using SSR markers. The genomic DNA was isolated from tissue of needles of all eight populations. Due to the costly and lengthy process a small number of the SSR markers for *Abies alba* have been developed, so in this study were used the microsatellite markers of related species. The obtained results indicate a low level of the genetic variability between natural populations of silver fir. The total number of alleles detected with nine SSR markers in eight studied populations of silver fir is 28. The range of alleles varies from two for NFF15 to six for SF78 with an average of 3.1 alleles per locus. The mean value of genetic similarity between populations is 0.92. The smallest genetic similarity between pairs of populations is 0.82 (Dubočica Bare and Stara Planina; Dubočica Bare and Tara) and the greatest genetic similarity is 1 (Zlatar and Stara Planina, Zlatar and Tara, Stara Planina and Tara).
A basic insight into the level of genetic diversity of natural populations of silver fir in Serbia, which are located in a relatively small area, has been given using a set of SSR markers. The obtained results can be used in the future strategy for the management and regeneration of silver fir forests.
Key words: Abies alba Mill., SSR markers, population, variability.

INTRODUCTION

Silver fir (*Abies alba* Mill.) is a conifer from the *Pinaceae* family and it is the highest tree of the genus Abies in Europe. In favorable environmental conditions it can reach the age of 500 - 600 years. Individual trees in their mature age can reach the height of 60-65 m and the

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diameter at breast height of 150 - 200 (380) cm (LIEPELT *et al*., 2009). Natural habitats of silver fir are mountainous regions of eastern, western, southern and central Europe where it occurs mainly with beech (*Fagus sylvatica* L.) at lower and middle elevations and with spruce (*Picea abies* L. Karst) at higher elevations (LIEPELT *et al*., 2009).

Figure 1. Distribution range of silver fir

The main habitats of silver fir are from 52° N in the north (Poland) to 40° N in the south (northern border of Greece) and from 5° E in the west (western Alps) to 27° E in the east (Romania, Bulgaria). It mostly occurs at elevations of 500 to 800 meters above sea level and when going from north to south the elevation increases. Due to the large distribution area this species is not endangered yet, but in the last 200 years the silver fir forests have been significantly reduced in most European countries (WOLF, 2003). The disappearance of forests is a consequence of stress due to environmental factors and silvicultural operations in favor of other conifers, primarily spruce. The decline of silver fir brings not only the environmental but also economic losses, because the silver fir is one of the most productive forest species (MUSIL and HAMERNÍK, 2007).

In Serbia, the silver fir forests occupy a modest area of 25,600 ha where 95.3% are stands of natural origin while the stands of artificial origin occupy 4.7% of the total area. Silver fir as one of the most valuable conifers is not sufficiently represented in the growing stock of Serbia having in mind its potential. A strategically important task of forestry is introducing silver fir into forest areas where this is possible, especially where it is presented by expanding its vertical amplitude (BANKOVIĆ *et al*., 2009).

Decline of the silver fir forests is associated with insufficient genetic variability between populations which leads to the reduction in adaptation ability of this species compared to other forest species (LARSEN, 1986); for the decline of these forests are also responsible the species sensitivity to changes in temperature, lack of water and air pollution (POSTOLACHE *et al*., 2013).

The reproduction-effective population size of forest trees in the past has been significantly reduced and many genes were lost in the process of genetic drift. Due to improper forest management, the human factor has contributed to the weakening of the genetic structure of the species. In several regions of Central Europe the natural forests have been converted into spruce and pine monocultures (PAULE *et al*., 2001).

In order to ensure the survival and return of silver fir to its natural habitat on a larger scale, it is necessary to acquire a detailed knowledge of the genetic diversity within and between populations (CVRČKOVÁ *et al*., 2015). In the past few decades extensive genetic researches were carried out on silver fir using morphometric markers, terpenes, isozymes, RAPD markers and SSR markers (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). Microsatellite markers are commonly used in population genetic studies for analyses of gene flow, parentage analyses and studies on genetic diversity (PFEIFFER *et al*., 1997). If nuclear microsatellites are highly polymorphic, selectively neutral and codominant markers, they are best suited for the analysis of small-scale genetic diversity (CREMER *et al*., 2006).

The aim of research in this paper was to give an initial overview of the level of genetic diversity and to provide a preliminary picture of differentiation patterns of natural populations of silver fir in Serbia using SSR markers.

MATERIALS AND METHODS

The research was carried out in eight natural populations of silver fir that represent the range of this species in Serbia. The spatial distribution of the studied populations is shown in Figure 2. Each population was represented by 30 trees that are located at a distance greater than 50 meters between each other. Each sample was labeled, packed in a plastic bag and transported to the laboratory in a refrigerator. For each population was formed the bulk by taking 1g of needles from every tree.

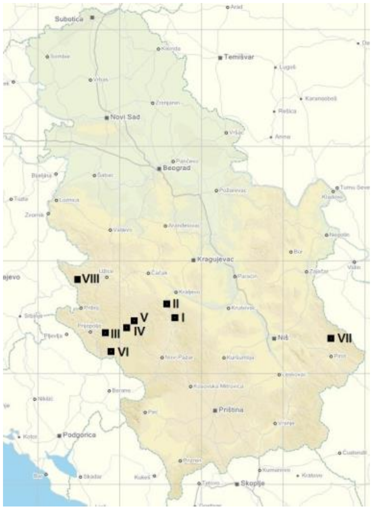

Figure 2. Map of spatial distribution of the studied populations (I-Goč, II-Sokolja, III-Zlatar, IV-Javor 1, V-Javor 2, VI-Dubočica Bare, VII-Stara Planina, VIII-Tara).

Molecular Analysis

DNA isolation

Genomic DNA was isolated from needle of Abies alba using the CTAB procedure according to DOYLE and DOYLE (1987). Bulks were prepared by pooling an equal amount of plant material obtained by grounding four needles per tree. Needels were taken from 30 different plants.

SSR Analysis

Sixty pairs of primers were tested to amplify microsatellite loci in Abies alba. However, seven were removed due to no or very poor PCR amplification and nine of them successfully amplified clear and reproducible products (Table 1).

Polymerase chain reaction was carried out in 25µL reaction volume containing: 1xBuffer. 0.8mM dNTP, 0.5µM of each primer pair, 1U TaqPoly and 50ng of DNA. Amplifications were performd in thermocycler Biometra TProfessional Standard 96 using the following touch-down program: an initial denaturation at 95ºC/5min. by 15 cycles each of denaturation at 95ºC /30 s, annealing at 58ºC/1min (-0.5ºC/cycle) and extension at 72 ºC /1min; another 22 cycles of 95 ºC /30 s. 56ºC/1min and 72ºC/1min were performed. Final elongation was at 72°C for 4min. The amplified fragments were resolved by electrophoresis on 8% polyacrylamide gel. with 100bp ladder as a marker. Gels were run on small format (7.3x10cm) vertical gel system (Mini Protean Tetra-Cell BioRad) at 40mA for 1.5h. After staining with 0.5µg/µL ethidium bromide they were photographed under UV light on BioDocAnalyse Biometra.

Table 1. Characteristics of selected SSR across the eight analized Abies alba populations

Locus	Primer seqences (5'-3')	Repeat motif	Number of allels
NFF2	F:GGGGTAGAGAGTTGGCTGCT R:CATAAGGATGAGTGGCTTCCA	(GT)13(GA)9	3
NFF3	F:CCAATGGGTTGTCAGAGTGTT R:GGCATTCGAGATTGCTTGAT	-	3
NFF7	F:CCCAAACTGGAAGATTGGAC R:ATCGCCATCCATCATCAGA	(GA)33	2
NFH15	F:CGCCTCCCTCCATTACTTC R:TCGTCTAGAGAGGCGAAATTCT	-	3
SF1	F: TTGACGTGATTAACAATCCA R: AAGAACGACACCATTCTCAC	(CCG)9	3
SFb4	F: GCCTTTGCAACATAATTGG R: TCACAATTGTTATGTGTGTGG	(GT)16	3
SFb5	F: AAAAAGCATCACTTTTCTCG R: AAGAGGAGGGGAGTTACAAG	(CT)15	3
SFg6	F: TAACAATAAAAGGAAGCTACG R: TGTGACACATTGGACACC	(AC)9	6
SF78	F: CATTGTTGTCTTTGTTTCACA R: TGCACCGTTTTGTTTTTCC	(CGCA)8(CA)15G(CA)8	2

Statistical analysis

SSR profiles were scored as presence/absence of fragments in each sample and the data were assembled into a binary matrix. Genetic similarities between populations were evaluated by DICE (1945). Correspondence analysis of genetic similarity was done according to Dice. Unweighted Pair Group Method with Arithmetic mean (UPGMA) was applied for cluster analysis. All marker data analyses to illustrate the genetic relationships among populations, were performed using statistical NTSYSpc2 program package (ROHLF, 2000).

RESULTS AND DISCUSSION

The small number of SSR markers has been developed for *Abies alba* due to expensive and time-consuming process. The possibility of transferring microsatelite markers of related species has been used in this study. Therefore we used markers that had been developed for species *Abies nordmanniana*: NFF2, NFF3, NFH15 and NFF7 (HANSEN *et al*. 2005). CREMER *et al.* (2006) had checked the variability of developted markers (SF b4, SF g6, SF 78, and SF 1) for *Abies alba* among populations in Bulgaria, France, Germany and Switzerland.

Population genetic data based on diferent kind of marker (isozyme, AFLP and SSR) in Abies species indicated narrow genetic variability. Data revealed in our study showed low levels of genetic variation which is expected compared to other studies.

In our work total number of alleles detected with nine SSR markers in observed eight *Abies alba* populations was 28. The range of alleles richness varied from two for NFF15 to six for SF78, with an average of 3.1 alleles per locus.

Genetic similarity coefficients for each pair of populations are shown in Table 2. The lowest genetic similarity between populations determined by SSR analysis was 0.82 (Dubočica Bare and Stara Planina, Dubočica Bare and Tara), while the highest was 1 (Zlatar and Stara Planina, Zlatar and Tara, Stara Planina and Tara). The mean value of genetic similarity between populations was 0.92. Czech populations displayed higher genetic distances ranging from 0.091 to 0.232 (CVRČKOVÁ *et al*., 2015). This might be due to microsatellite markers labelled fluorescently and PCR products were separated by capillary electrophoresis.

Table 2. Similarity coefficients calculated from SSR markers by Dice

	I	II	III	IV	V	VI	VII	VIII
I	1							
II	0.92	1						
II	0.98	0.94	1					
IV	0.85	0.89	0.88	1				
V	0.98	0.94	0.96	0.87	1			
VI	0.84	0.88	0.82	0.92	0.86	1		
VII	0.98	0.94	1	0.88	0.96	0.82	1	
VIII	0.98	0.94	1	0.88	0.96	0.82	1	1

Dendrogram generated on genetic similarity values based on SSR data grouped the populations in one cluster „A“ and loosely tied branche „b“ with population Dubočica Bare (Figure 3). Cluster *A* was devided in subclaster A1 with popluations Goč, Zlatar, Stara Planina and Tara and on the other side populations Javor 2, Sokolja and Javor 1 wich are attached to subclaster A1.

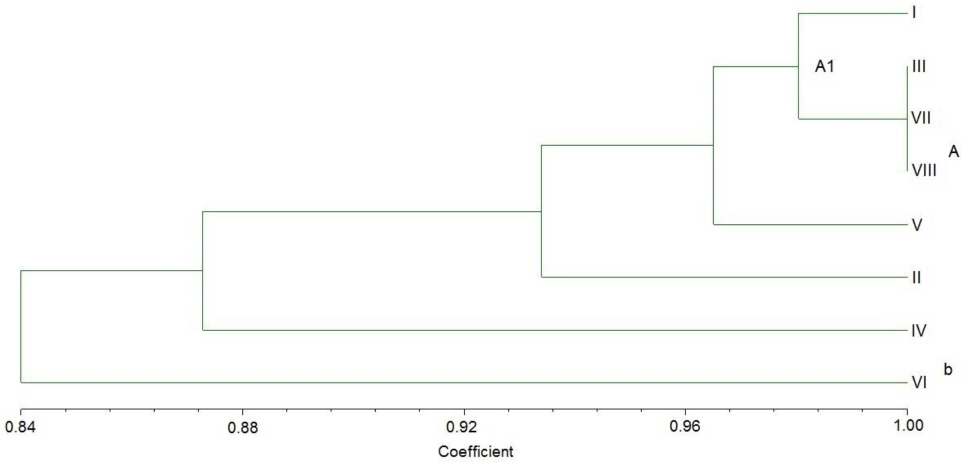

Figure 3. Dendrogram of eight *Abies alba* populations constructed using UPGMA cluster analysis of genetic similarity values (Dice) obtained from SSR data.

Dice's genetic similarity coefficient for each pair of populations was used for creating the three-dimensional chart of the correspondent analysis (Figure 4). By using the comparative analysis of the obtained chart of the correspondent analysis and dendrogram of cluster analysis of studied populations it can be concluded that the populations are grouped in a similar manner. Genetically most similar are silver fir populations on the mountains Zlatar, Stara Planina and Tara and genetically most different is the population Dubočica Bare. The results obtained using the UPGMA cluster method and the correspondent analysis and presented by the dendrogram and the chart, indicate a low level of genetic diversity among the studied populations of silver fir.

The correspondent analysis is more informative and more accurate than dendrograms especially where there is a large genetic exchange between geographically close genotypes (CAVALLI-SFOROZA *et al*., 1994). Using three-dimensional charts when presenting the correspondent analysis gives an important information about the relationship between the studied genotypes, so they should be used when the number of the studied genotypes is less than 10 (LUČIĆ, 2012; POPOVIĆ *et al*., 2015).

In the research of genetic variability of eight natural populations of *Abies nephrolepis* Max. using SSR markers in Korea the small genetic distances (average 0.027) have been determined, indicating that populations are closely related and that there is a free exchange of genes between them (WOO *et al*., 2008).

In silver fir populations from the southwest part of Germany was determined a low level of genetic variability (CREMER *et al*., 2012). Some greater genetic diversity was found in some silver fir populations in the Czech Republic (CVRČKOVÁ *et al*., 2015).

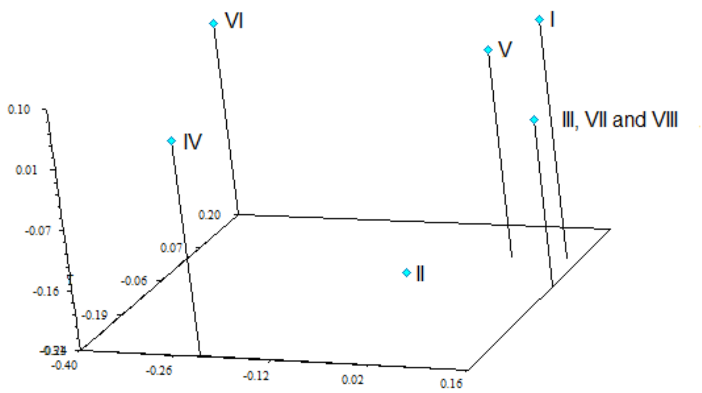

Figure 4. Correspondence analysis of eight *Abies alba* populations of genetic similarity according to DICE (1945)

The current position related to the history of silver fir is that the sources of recolonization, after the last glacial and post-glacial period, are in the southern Balkans (Greece), north-western Balkans (Croatia, Bosnia) and Apennines (GÖMÖRY *et al*., 2012). Natural distribution of silver fir takes place mainly in the mountainous regions, ranging from the north (Poland) to the south (northern border of Greece) and from the west (Western Alps) to the east (Romania and Bulgaria) (VOLF, 2003).

Anthropomorphic changes in the last 200 years have significantly affected the genetic structure of silver fir in Europe (DUCCI, 1991). The loss of the gene pool which is generated in this manner over time accumulates and becomes bigger and bigger, and that affects the newly created populations in the process of introgression to become very labile and sensitive to changes in the environment. The severe changes can lead to the catastrophic consequences resulting absence of natural regeneration of silver fir (BALLIAN, 2010).

CONCLUSIONS

A basic insight into the level of genetic diversity of natural populations of silver fir in Serbia, which are located in a relatively small area, has been given using selected SSR markers. The obtained results can be used in the future strategy for the management and regeneration of silver fir forests. To come up with the more reliable conclusions about changes in the genetic structure of silver fir populations in Serbia it is necessary to carry out some detailed researches.

In management and regeneration of forests the natural regeneration must always have the priority. However, in order to maintain and increase the level of genetic diversity it is necessary to constantly monitor the genetic structure and to timely take measures for its maintenance. The establishment of provenance and progeny tests is a necessary measure in breeding and acquiring knowledge on gene-environmental potential of silver fir.

Due to all abovementioned the advice can be a creation of optimal conditions for natural regeneration of silver fir whose greater representation in the forests of Serbia will make a positive environmental and economic effect.

ACKNOWLEDGEMENT

The research is financed by the Ministry of Science and Technological Development of the Republic of Serbia, Project TR 31070 "The developments of technological procedures in forestry in order to attain an optimal forest cover percentage" (2011-2014).

Received, February 20th, 2017
Accepted July 15th, 2017

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PROCENA GENETIČKOG DIVERZITETA JELE (*Abies alba* Mill.) U SRBIJI UPOTREBOM SSR MARKERA

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Izvod

U radu su prikazani rezultati analize genetičke varijabilnosti osam prirodnih populacija jele (*Abies alba* Mill.) u Srbiji dobijeni upotrebom SSR markera. Genomska DNA je izolovana iz tkiva četina svih osam populacija. Sa devet izabranih SSR markera u svih osam istraživanih populacija jele ukupno je otkriveno 28 alela. Raspon alela varira od dva za NFF15 do šest za SF78, sa prosekom od 3,1 alela po lokusu. Srednja vrednost genetičke sličnosti između populacija bila je 0,92. Najmanja genetička sličnost između parova populacija iznosi 0,82 (Dubočica Bare i Stara Planina; Dubočica Bare i Tara), a najviša 1 (Zlatar i Stara Planina; Zlatar i Tara; Stara Planina i Tara). Upotrebom izabranih SSR markera dat je osnovni uvid u nivo genetičke raznovrsnosti prirodnih populacija jele u Srbiji, koje se nalaze na relativno malom prostoru. Rezultati dobijeni u ovom radu ukazuju na nizak nivo genetičke varijabilnosti između prirodnih populacija jele. Dobijeni rezultati mogu poslužiti u budućoj strategiji na gazdovanju i obnavljanju jelovih šuma i usmerenom korišćenju raspoloživog genofonda.

Primljeno 20.II.2017.
Odobreno 15. VII. 2017.