Arch. Biol. Sci., Belgrade, 66 (4), 1485-1492, 2014 DOI:10.2298/ABS1404485L

GENETIC DIVERSITY OF SCOTS PINE (*PINUS SYLVESTRIS* L.) POPULATIONS IN SERBIA REVEALED BY SSR MARKERS

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Abstract – This paper presents the results of analysis of the genetic variability of seven Scots pine (*Pinus sylvestris* L.) populations in Serbia using SSR markers. Genomic DNA was isolated from seed tissue of all seven populations. The concentration of DNA samples was within the range of 1-4 μg/μl. Different PCR protocols were used depending on the type of SSR markers. The total number of fragments obtained by SSR analysis with 4 selected primers was 17 (only bands of strong and medium intensity were considered), of which 6 fragments were polymorphic (35.29%). In order to analyze the genetic similarity of the analyzed populations, graphs of correspondence analysis and UPGMA clusters were produced. By comparative analysis of the obtained dendrograms, the dependence of population genetic differentiation and spatial distance was observed, i.e. their isolation by natural barriers. The results indicate that in further research of interpopulation variability it is necessary, when graphically interpreting genetic distances, to use both methods of statistical analysis (UPGMA analysis and correspondence analysis).

Key words: Scots pine; SSR; UPGMA; correspondence analysis.

INTRODUCTION

In Serbia, Scots pine (*Pinus sylvestris* L.) occupies about 26 600 ha and it occurs naturally on 7 527 ha. It is autochthonous primarily in the western and southwestern parts of Serbia, forming a unit with the ranges in eastern Bosnia and Montenegro. Scots pine grows predominantly within the montane belt above 1 000 m, but it also stretches at lower elevations, depending on other site conditions.

For more than a century, Scots pine has been in the focus of forestry science. The interest in this species is first of all justified by its characters: great genetic potential and genetic variability, and the species' taxonomic complexity and plasticity. Its protection and reclamation functions on difficult terrains results from its low environmental requirements. The occurrence of Scots pine on large areas affected by erosion processes, or on degraded, denuded areas where it produces excellent results, classifies it among the most important profitable species used in forestry. All the above qualities, in addition to its wide natural distribution and disjunctive range, have led to an intensive introduction to sites outside its native range, resulting in a great number of subspecies, varieties and forms. Its great natural variability is a considerable genetic potential that presents the

basis and opportunity for a successful improvement of the species.

The basis for the use of SSR markers in researching the genetic structure of Scots pine in seven selected populations in Serbia were the results of the analysis conducted by Karvonen and Savolainen (1993) and Liet et al. (2005). The first authors studied the genetic structure of Scots pine by comparing ribosomal DNA in two Scots pine populations from northern Finland. The coefficient of genetic variation within these populations was 86%, while between these populations it was 14%. In nine northeastern Chinese populations, Liet studied the genetic structure of Scots pine using these markers. Here it was determined that the genetic distance between the populations was 60%.

The research in this paper was based on the analysis of the genetic structure of natural populations of Scots pine by forming a bulk sample for each population. This approach can reduce considerably the financial cost; however, the bulking of DNA samples also results in the potential non-detection of rare alleles and the loss of information about the amount of heterozygosity within samples (Reif et al., 2005). Nevertheless, there are many examples of the application of molecular markers in the detection of DNA polymorphism in the bulk samples of many plant species (Karvonen and Savolainen, 1993; Goto et al., 2001; Reif et al., 2005).

In the analysis of the interpopulation variability of Scots pine in Serbia using genetic markers, studies that used morphometric markers (Lučić et al., 2012), biochemical markers (Lučić et al., 2011) and RAPD molecular markers (Lučić et al., 2011b) have been published. Significant results in multidisciplinary research (genetic and phytocoenological) were obtained in the analysis of five populations of Scots pine in Serbia by combining these research methods (Lučić et al., 2011c).

MATERIALS AND METHODS

The study of Scots pine (*Pinus sylvestris* L.) was conducted with following populations (Table 1, Fig. 1):

The study of Scots pine genetic structure using SSR markers was performed using the seeds of test trees from all seven populations. At each of the sites 20 trees were selected which, based on their phenotypic characteristics, fully represent the population. Cones were collected from the trees and seeds was isolated from the cones by the standard method of processing. Bulk was formed for each population by taking one gram of seeds for each tree. From the seed tissue genomic DNA was isolated. Its concentration was determined on a spectrophotometer and its visualization on agarose gels. The concentration of the samples varied in the range of 1-4 μg/μl.

After DNA isolation, different PCR protocols were used depending on the type of SSR markers.

SPAC primers

SSR markers or microsatellites were selected because of the high degree of information and reliability of results obtained using this method in many population and environmental studies. Microsatellites have proved to be reliable markers in studies of gene fluctuations, the effective size of a population, migration and dispersion processes, parenting and the degree of relatedness (Dow and Ashley, 1996; Streiff et al., 1999).

The sequences of the primers used in SSR analysis are shown in Table 2. The SSR reaction was performed in 25 μl reaction, with 1x reaction buffer, 2.5 U Taq polymerase (Fermentas), 2.5 mM $MgCl_2$, 0.5 μM primer, 100 μM dNTPs, 1xBSA, 10 ng of genomic DNK and 25 μl of mineral oil. The protocol was as follows: initial denaturation at 95°C for 5 min, 15 cycles of denaturation at 95°C for 30 s, annealing at 63.5°C for 1 min (-0.5°C/cycle) and elongation at 72°C for 1 min; the following 22 cycles were carried out at 95°C/30 s, 56°C/1 min and 72°C/1 min.

Upon completion of the reaction, loading buffer was added to the samples (0.25% Bromophenol blue, 30% glycerol in H_2O) and the samples were loaded

Table 1. The studied Scots pine populations (*Pinus sylvestris* L.) and their coordinates

No.	Scots pine populations	Coordinates	
I/1	Dubočica Bare	7410334	4780802
II	Šargan	7380386	4855057
III	Radočelo-Crepuljnik	7456208	4805619
IV	Jablanička Reka	7528069	4808811
V	Bukovik-Aleksinački	7554153	4839527
VI	Kaluđerske Bare	7384720	4862086
VII	Zlatar I	7409044	4805159

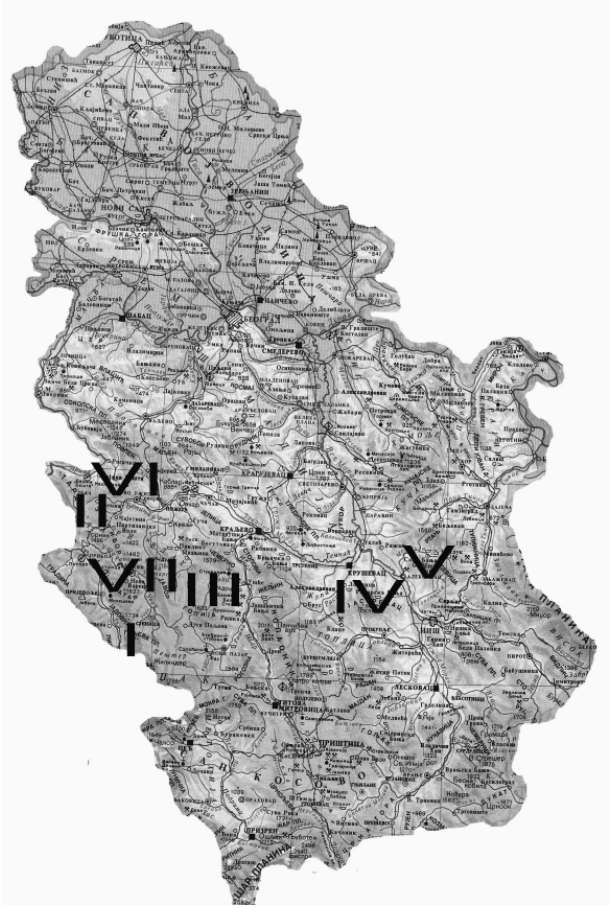

Fig. 1. Map depicting geographical region where *Pinus sylvestris* L. seeds were sampled

Table 2. Primers used in SSR analysis

SPAC 12.5 F SPAC 12.5 R	5' -CTT CTT CAC TAG TTT CCT TTG G- 3' 5' -TTG GTT ATA GGC ATA GAT TGC- 3'
SPAC 11.4 F SPAC 11.4 R	5' -TCA CAA AAC ACG TGA TTC ACA- 3' 5' -GAA AAT AGC CCT GTG TGA GAC A- 3'
SPAC 11.5 F SPAC 11.5 R	5' -TGG AGT GGA AGT TTG AGA AGC- 3' 5' -TTG GGT TAC GAT ACA GAC GAT G- 3'

Table 3. Primers used in analysis of mitochondrial DNA

nad1 F	5' -CTC TCC CTC ACC CAT ATG ATG- 3'
nad1 R	5' -ACA AAG CCC CTT TGA GGG- 3'

on 8% polyacrylamide gel. Electrophoresis was run 1 h/40 mA. A 100 bp PCR marker (Fermentas) was used. Upon completion of electrophoresis, the gels were placed in a water solution of ethidium bromide (0.5 μg/ml) for 30 min at room temperature, illuminated by a *UV*-Transilluminator (302 nm) and photographed with a digital camera. From the photos the SSR profile for each primer as the presence/absence of individual bands in samples was read.

Nad1 primers

Genetic analysis of the interpopulation variability of the analyzed populations of Scots pine was also

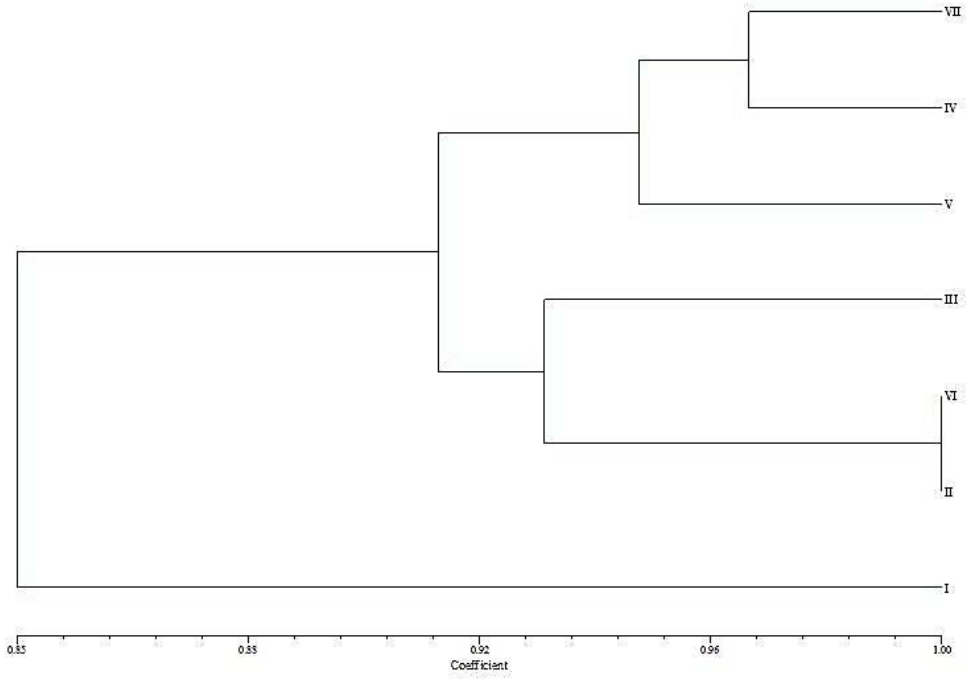

Fig. 2. Cluster analysis dendrogram of populations according to Dice (1945)

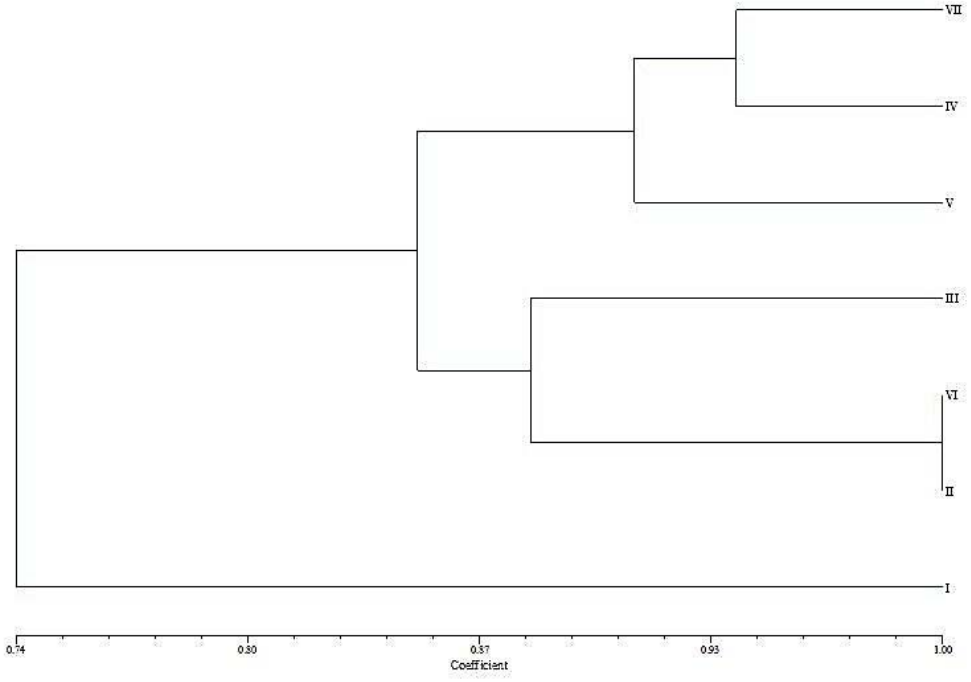

Fig. 3. Cluster analysis dendrogram of populations according to Sokal and Michener (1958)

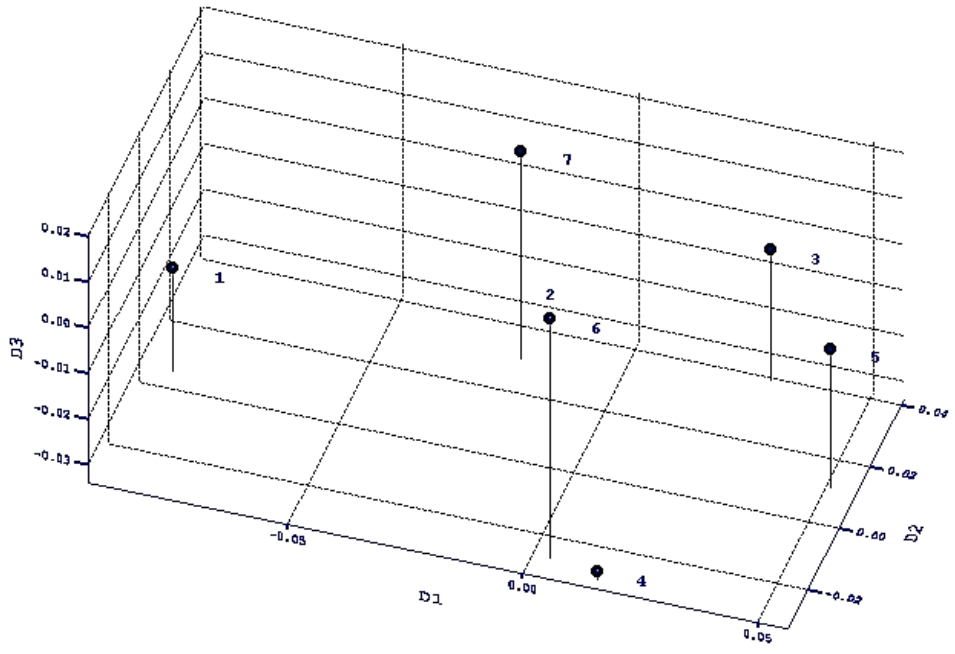

Fig. 4. Correspondence analysis of genetic similarity according to Dice (1945)

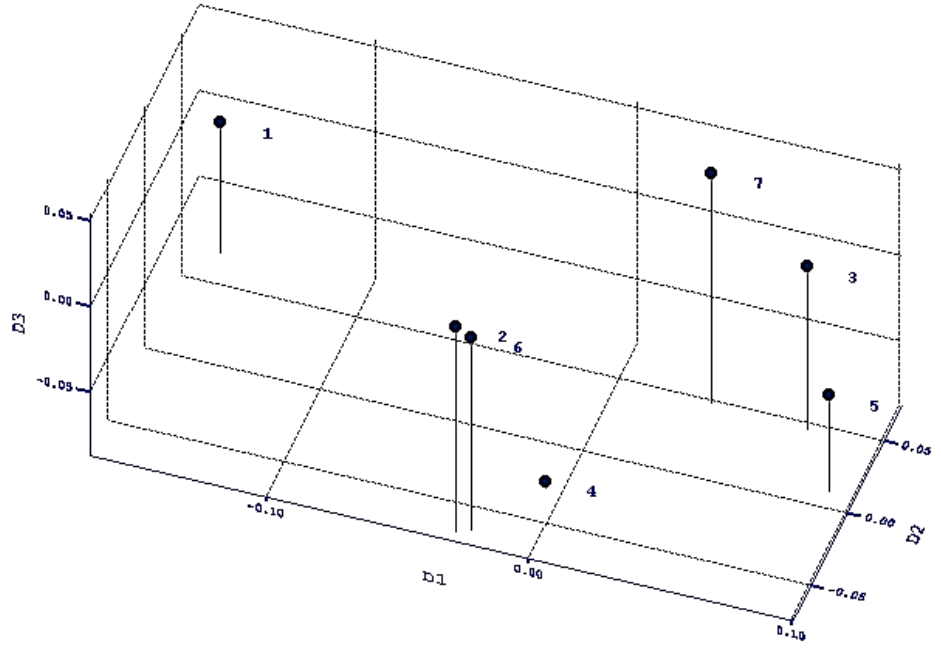

Fig. 5. Correspondence analysis of genetic similarity according to Sokal and Michener (1958)

performed by the analysis of polymorphism of the mitochondrial *nad1* gene. Mitochondrial DNA was selected for analysis because it offers the possibility for monitoring population trends, given that it is only maternally inherited. Population genetic studies of conifers based on mtDNA markers give more precise information on the differentiation among populations than the implementation of chloroplast or nuclear DNA, since it is assumed that the dispersion of seeds takes place within much narrower margins than pollen dispersion.

Nad1 indicates the internal fragment of the second intron of the mitochondrial NADH dehydrogenase subunit 1 of the observed gene. These primers were selected for analysis because of the positive results of its use in conifers in previous research (Sperisen et al., 2001). The primers used for analysis of mitochondrial DNA are shown in Table 3.

PCR was performed according to the modified (Ćorić, 2001) protocol of Williams (1990) in 25 μl reaction mixture containing 2.5 U Taq polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 μM primer, 10 ng genomic DNA, 100 μM dNTPs and 25 μl mineral oil. The protocol was as follows: initial denaturation at 94°C/2 min, 45 cycles at 94°C/30s, 40°C/1 min, 72°C/1 min, and final cycle at 72°C/7 min.

PCR products were digested with EcoRV (E059 Restriction Endonuclease) to improve the resolution. The enzyme mixture (17 μl in ddH_2O, 2 μl buffer and 1 μl enzyme) and 10 μl of the PCR product were incubated in a water bath for 15 min at 37°C. After incubation, the samples were placed on ice to stop the digestion. Upon completion of the reaction, loading buffer was added to the samples (0.25% Bromophenol blue, 30% glycerol in dH_2O) and then they were loaded on 2% agarose gel for electrophoresis. Electrophoresis was run for 1 h/40 mA. 100 bp PCR marker (Fermentas) was used. Upon completion of electrophoresis, gels were photographed with a digital camera. From the photos the profile for each primer as the presence/absence of individual bands in samples was read.

A graphical interpretation of the obtained results was performed using NTSYS-pc software (Rofhl 2000). Correspondence analysis and analysis of the genetic similarity of the analyzed population, according, was performed according to Greenacre (1988) and Greenacre and Blasius (1994). The graphs were made according to Dice (1945) and Sokal and Michener (1958).

RESULTS AND DISCUSSION

The total number of fragments obtained by SSR analysis with 4 selected primers was 17 (only bands of strong and medium intensity were considered). Of these, 6 fragments were polymorphic (35.29%). The number of obtained SSR fragments with different primers ranged from 3 to 5 and the average number of fragments for each primer was 4.25. Primers 11.4 and 12.5 gave the highest number of fragments (5), primer *Nad1* gave 4 fragments, while primer 11.5 gave only 3 fragments.

A graphical interpretation of the genetic similarity is presented with UPGMA dendrograms (Figs. 2 and 3), and with graphs of correspondence analysis (Figs. 4 and 5).

From the analysis of the graphs of correspondence analysis, it is clear that all analyzed populations are more or less grouped together, except the Dubočica Bare population. Within the grouped populations, the Šargan and Kaluđerske Bare populations are slightly separated, with a genetic distance that is practically negligible.

Analyses performed with NTSYS-pc software, based on the similarity coefficient matrices, gave dendrograms with identical arrangement of genotypes, while the values of genetic similarity were in various ranges. A comparative analysis of the obtained dendrograms clearly shows that the Šargan and Kaluđerske Bare populations are at the smallest genetic distances. Other populations, apart from Dubočica Bare, make a large subcluster. The analysis of relationships within this subcluster shows the existence of small genetic distances.

Population differentiation points to a crucial influence of geographical distance on their grouping. The Šargan and Kaluđerske Bare populations are grouped in an area of larger genetic similarity, which is probably due to their spatial distribution or geographic proximity. On the other hand, the pronounced genetic distance of the Dubočica Bare population compared to the other analyzed populations is probably the result of its spatial isolation from others by the size and position of the Pešter plateau and geographic gravitation toward the forests of Montenegro. As already mentioned, the other populations occupy an area of small genetic distance where the arrangement of groupings differ depending on the method used for the analysis of genetic distance and similarity.

That the results of analysis of interpopulation variability of Scots pine in Serbia depend on the type of markers used confirm the research of Lučić et al. (2011, 2011b, 2012). In the analysis of genetic variability using morphometric markers – cones, seed, seedlings (Lučić et al., 2012), it can be concluded that population differentiation depends on the analyzed character, with a geographic distance that has no significant influence. However, the analysis of biochemical markers – total proteins (Lučić et al., 2011) and molecular markers – RAPD (Lučić et al., 2011b), the results expressed by dendrograms provide a similar arrangement by differentiating populations with the strong influence of geographic distance. In multidisciplinary research (genetic and phytocoenological) (Lučić, et al., 2011c), a very strong relationship was established between site characteristics and genetic differentiation of Scots pine populations.

This paper shows two types of graphic interpretation of Scots pine population variability in Serbia that represents a departure from previous practice where only NTSYS dendrograms are used.

The correspondence analysis (Greenacre, 1988) is a supplementary analysis to the genetic distances and dendrograms that gives a global view of the relationships among populations. It has the advantage of not assuming that the test units belong to certain groups, but more accurately represents the continuous variability. This type of analysis tends to provide results similar to those obtained from cluster analysis dendrograms (Cavalli-Sforza et al., 1994). At the same time, correspondence analysis is more informative and accurate than dendrograms especially when there is considerable genetic exchange between geographically close populations (Cavalli-Sforza et al., 1994), as described in this paper.

The obtained results of analysis performed with the NTSYS-pc software can be used for a more detailed review of the populations' grouping, and the results of correspondence analysis give a more precise review of relationships of analyzed populations both within groups and among them. The results obtained in this research using different statistical methods (NTSYS and correspondence analysis) suggest that both methods are compatible.

A detailed analysis of the obtained results leads to the general conclusion that Scots pine in Serbia is characterized by significant genetic variability. However, to draw any wider conclusions about changes in the genetic structure of Scots pine populations from different locations in Serbia require more detailed studies.

Acknowledgments - The research was financed by the Ministry of Science and Technological Development of the Republic of Serbia, Project TR 31070 "The development of technological procedures in forestry with a view to an optimum forest cover realization" (2011-2014).

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