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GENETIC DIVERGENCE OF SCOTS PINE (*PINUS SYLVESTRIS* L.) POPULATIONS IN SERBIA REVEALED BY RAPD

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Abstract – The ability of random amplified polymorphic DNA (RAPD) to distinguish among Scots pine populations from Serbia was evaluated. Sixteen arbitrary 10-mer primers employed in the analysis produced 54 fragments of which 21 were polymorphic (38.89%). Certain rare and genotype-specific bands were identified which could be effectively used to distinguish between the populations. Polymorphism in RAPD markers among *P. sylvestris* populations was high and sufficient to distinguish each of the populations. The results obtained suggest that RAPD markers are valuable for the genetic divergence estimation in *Pinus sylvestris* and for the study of divergence among populations.

Key words: *Pinus sylvestris*, population, RAPD, polymorphism

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INTRODUCTION

In Serbia, Scots pine (*Pinus sylvestris* L.) occupies a total of 26,600 ha. It occurs naturally on 7,527 ha. It is autochthonous primarily in the western and southwestern parts of Serbia, making 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 and profession. The interest in this species is first of all justified by its characteristics: a 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 economic species applied in forestry. All the above qualities, in addition to its wide natural distribution and disjunctive range, led to an intensive introduction also into sites outside its native range, resulting in a great number of subspecies, varieties and forms. Its considerable natural variability represents a great genetic potential which provides the base and the opportunity for a successful improvement of the species.

Pinus is considered as one of the most genetically variable plant genera, which is revealed by the assessment of its quantitative genetic variation (Cornelius, 1994), isozyme analysis (Hamrick et al. 1979, Hamrick and Godt, 1990, Lewandowski et al. 2002, Mataruga et al. 2007) and RAPD markers (Lee et al. 2002, Nkongolo et al. 2002, Montelone et al. 2006, Lučić et al. 2010).

In addition to the above authors, significant research was performed by Nowakowska (2004) who analyzed 29 Scots pine populations in Poland, using RAPD and isoenzyme analysis. She reported that the variability of genotypes of pine populations analyzed by the RAPD method was greater than that analyzed by isoenzyme analysis.

MATERIALS AND METHODS

Our study of the natural range and plantations Scots pine (*Pinus sylvestris* L.) in Serbia was continued by focusing the research on the significant natural populations and seed stands formed in older plantations (Table 1 and Fig. 1.)

Seeds from 15 trees of each population were collected. Genomic DNA from bulks of seeds was isolated using the CTAB procedure according to Rogers and Bendich (1985). PCR amplification of genomic DNA was tested on 16 RAPD primers (Genosys Biotechnologies) in two rounds of amplification (Williams et al. 1990), of which all primers gave clear and reproducible banding patterns. RAPD reactions were performed in a volume of 25 μL containing 2.5 mM $MgCl_2$, 100μM dNTPs, 0.2 μM of 10-mer primers (Genosys Biotechnologies), 2.5 U of Taq polymerase (Fermentas), and 10 ng of template DNA.

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

No	Scots pine populations	Coordinates	
I	FMU "Dubočica Bare" 60 a. FE "Golija" Ivanjica	7410334	4780802
II	FMU "Šargan", 25 b. FE "Užice" Užice	7380386	4855057
III	FMU "Radočelo-Crepuljnik" 4b. FE "Stolovi" Kraljevo	7456208	4805619
IV	FMU "Jablanička Reka" 33 d. FE "Rasina" Kruševac	7528069	4808811
V	FMU "Bukovik-Aleksinački", 23b, 4g, FE "Niš", Niš	7554153	4839527
VI	FMU "Kaluđerske Bare", 1a NP "Tara", Bajina Bašta	7384720	4862086
VII	FMU "Zlatar I",22a, FE "Prijepolje", Prijepolje	7409044	4805159

Fig. 1. – Map depicting geographical region where *P. sylvestris* seeds were sampled. I – FE "Golija" Ivanjica, FMU "Dubočica Bare"; II – FE "Užice" Užice, FMU "Šargan"; III – FE "Stolovi" Kraljevo, FMU "Radočelo-Crepuljnik"; IV – FE "Rasina" Kruševac, FMU "Jablanička Reka"; V – FE "Niš", Niš, FMU "Bukovik-Aleksinački"; VI – NP "Tara", Bajina Bašta, FMU "Kaluđerske Bare"; VII – FE "Prijepolje", Prijepolje, FMU "Zlatar I".

Amplifications were carried out in a PTC-100 Thermocycler (MJ Research) with the following program: initial denaturation step at 94°C for 2 min, followed by 45 cycles at 94°C for 30 s, 40°C for 1 min, and 72°C for 1 min, and a final cycle at 72°C for 7 min. The amplified products were separated by electrophoresis in 1.4% agarose in 1 x TBE buffer (Tris-borate 89 mM and EDTA 0.5 M pH 8.0), containing 0.15 μg/μL of ethidium bromide. The gels were photographed under UV light.

Only clear and reproducible bands were used for the subsequent statistical analysis. The presence (1) or absence (0) of fragments in each sample was assessed and the data were assembled into a binary matrix. Genetic similarity among populations (GS) was calculated according to Dice (1945) and Sokal and Michener (1958):

Dice $GS_{ij} = 2a/2a+b+c;$
Sokal and Michener (SM) $GS_{ij} = a+d/a+b+c+d$
where:
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)
d – number of fragments absent in both variety *i* and *j* (0.0)
Coefficients of genetic similarity were used to construct a UPGMA.

RESULTS

Sixteen RAPD primers were used to evaluate the level of genetic divergence among different populations of Scots pine (*P. sylvestris* L.) from Serbia. RAPD assays of each population were performed at least two times each, with only reproducible, amplified fragments being scored. A high level of genetic divergence was observed among the seven populations of *Pinus sylvestris* being analyzed. The 16 primers revealed 54 reproducible RAPD loci. Among them, 21 RAPD loci (38.89%) were polymorphic in at least one population. Each primer amplified between 1 and 8 fragments, with an average of 3.38. The total number of polymorphic loci detected varied between primers. Table 2 gives a list of primers and primer sequences for RAPD analysis.

All amplification products with primers GEN 1-70-9/1, GEN 1-70-3, GEN 1-80-9, GEN 1-80-4, GEN 2-80-1 and GEN 2-80-9 were identical in all populations (monomorphic primers). The other 10 primers showed polymorphism between the samples.

Table 2. Primers used in RAPD analysis

Primer	Primer sequence (5'-3')
GEN 2-80-7	GCAGGTCGCG
GEN 4-70-3	CTGTCGGCTC
GEN 2-80-10	CGCGAACGGC
GEN 1-80-5	ACCCCAGCCG
GEN 1-80-4	CGCCCGATCC
GEN 2-80-5	CGAGACGGGC
GEN 1-70-5	GAGATCCGCG
GEN 1-70-3	ACGGTGCCTG
GEN 1-70-9/1	GGACTCCACG
GEN 1-70-9/2	TGCAGCACCG
GEN 2-80-1	GCAGCAGCCG
GEN 2-80-9	GCACGTGAGG
GEN 1-80-9	GCACGGTGGG
GEN 1-70-10	CAGACACGGC
GEN 4-70-2	GGACCGACTG
GEN 4-70-4	GGACCGCTAG

Based on the presence/absence of 54 different RAPD fragments for pairs of all 7 analyzed Scots pine populations, similarity coefficients were calculated after Dice (1945) and Sokal and Michener (1958). The values of similarity coefficient are presented in Table 3.

Dice similarity coefficients ranged from 0.82 (the lowest similarity coefficient between populations I-III) to 0.98 (the highest similarity coefficient between populations III-VII).

The calculated Sokal and Michener similarity coefficients ranged from 0.70 (the lowest similarity coefficient was obtained between populations I-III)

Table 3: Similarity coefficient between Scots pine populations

SM / Dice	Sokal and Michener and Dice similarity coefficients						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
I		0.87	0.70	0.76	0.85	0.76	0.74
II	0.92		0.80	0.81	0.87	0.89	0.83
III	0.82	0.87		0.80	0.78	0.83	0.96
IV	0.84	0.88	0.87		0.83	0.81	0.80
V	0.90	0.92	0.86	0.89		0.80	0.81
VI	0.85	0.93	0.90	0.88	0.87		0.80
VII	0.84	0.90	0.98	0.87	0.88	0.88	

to 0.96 (the highest similarity coefficient between populations III-VII).

The genetic similarity, i.e. distance, between the studied Scots pine populations was analyzed using two methods: correspondence analysis of genetic similarity and UPGMA cluster method (Diagrams 1, 2, 3, 4).

Both analyses are characterized by high genetic divergence of the study populations expressed in the form of Dendrograms, i.e. Diagrams.

The analyses performed in NTSYS-PC software, based on the coefficients of Dice and Sokal and Michener similarity matrices, produced dendrograms with identical patterns of genotypes, while the

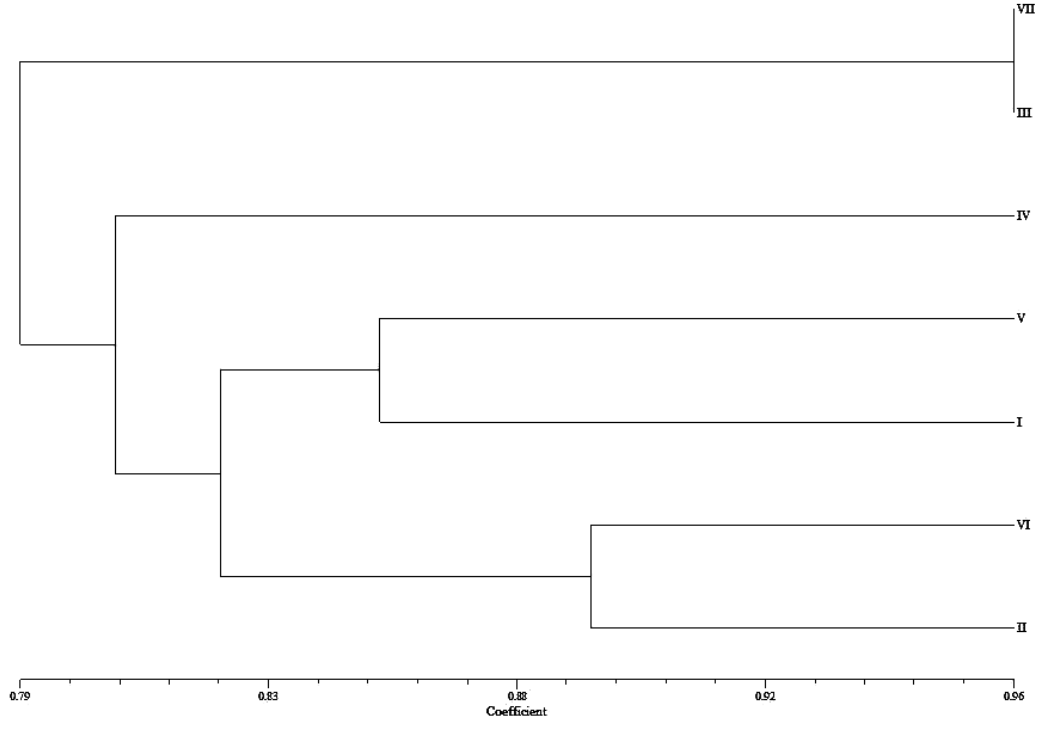

Diagram 1. – RAPD based dendrogram of *Pinus sylvestris* populations according to Sokal and Michener

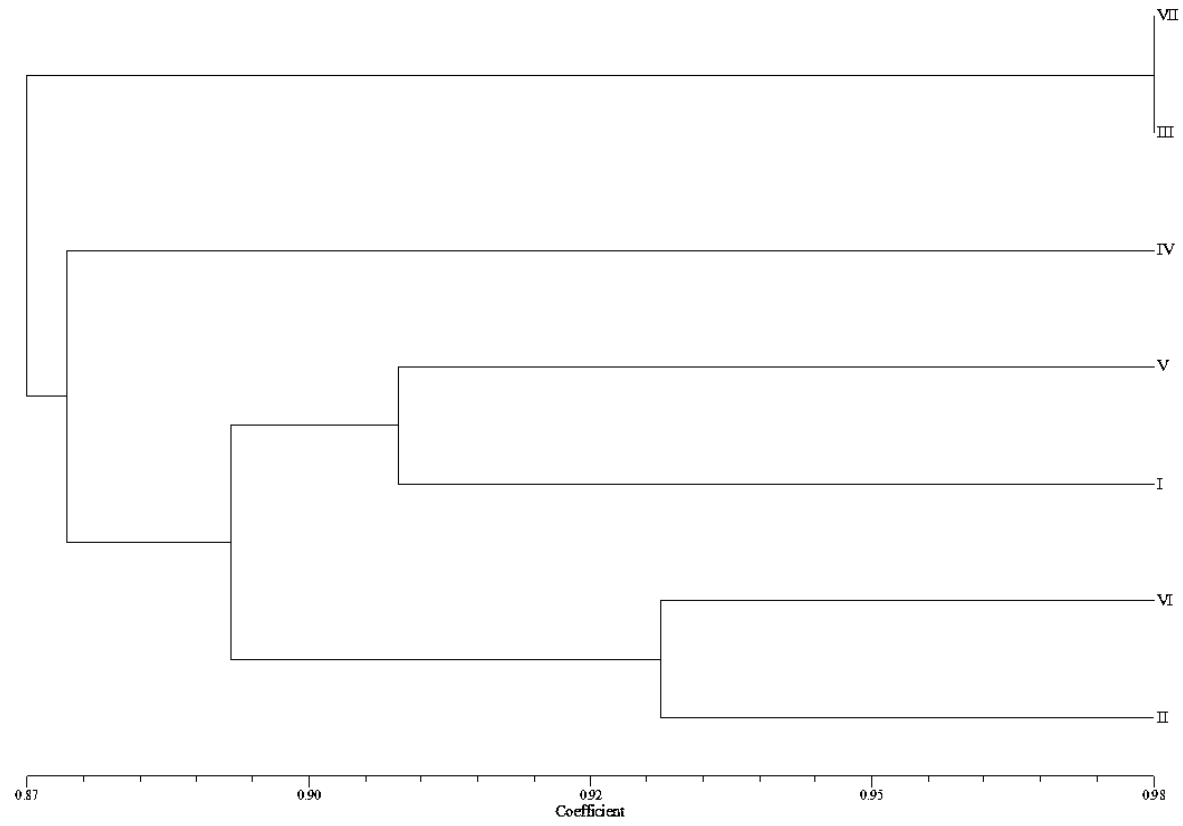

Diagram 2. – RAPD based dendrogram of *Pinus sylvestris* populations according to Dice

values of genetic similarity were different. Comparative analysis of the constructed dendrograms shows that the shortest genetic distances occurred in populations III and VII, which formed one subcluster or group. The other subcluster consisted of populations II, VI, V, I and IV. The analysis of relations within each subcluster showed small genetic distances among the analyzed populations.

Comparative analysis of the constructed diagrams of genetic similarity correspondence analysis of the study populations shows that they were all grouped in two groups. The first group consists of populations III and VII. The second group comprises populations IV, II, VI, I and V. The first three dimensions of the correspondence analysis cover 57, 20 and 16% of the Dice genetic similarity "inertion" (variability), respectively, and 59, 17 and 16% of the SM genetic similarity "inertion", respectively.

The results of the analysis using NTSYS-PC software show a clearer presentation of population clustering, and the results of correspondence analysis give a more detailed presentation of the relations among the study populations, both within groups and between them.

The clustering of population III (FMU "Radočelo-Crepuljnik" FE "Stolovi" Kraljevo) and VII (FMU "Zlatar I", FE "Prijepolje", Prijepolje) in the zone of greater genetic similarity was marked by their geographic closeness. The same also applies to populations II (FMU "Šargan", FE "Užice" Užice) and VI (FMU "Kaluđerske Bare", NP "Tara", Bajina

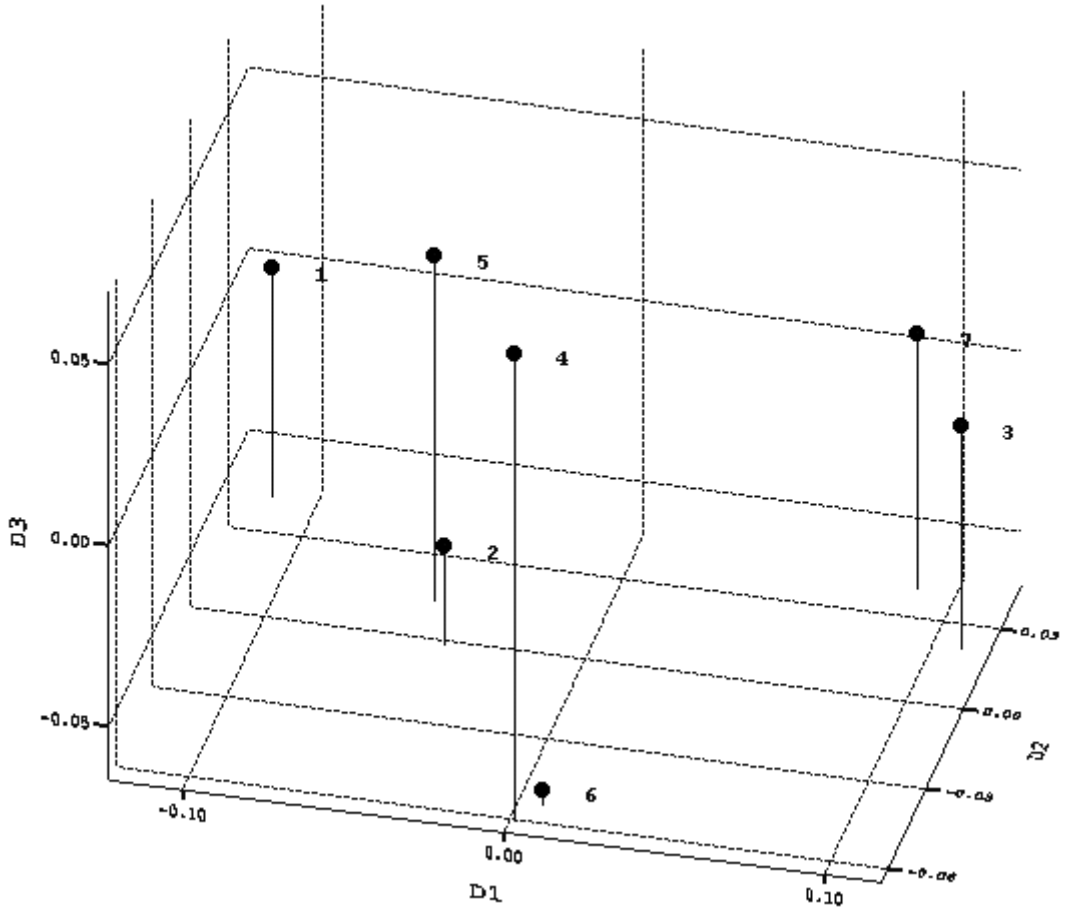

Diagram 4. – Diagram of correspondence analysis of genetic similarity after Sokal and Michener

Bašta). Populations IV (FMU "Jablanička Reka", FE "Rasina" Kruševac) and V (FMU "Bukovik-Aleksinački", FE "Niš", Niš) were genetically very close to populations II and VI, and population I (FMU "Dubočica Bare" FE "Golija" Ivanjica) occurred at somewhat greater genetic distances from populations II and VI. Population I was characterized by its orientation to the forests of Montenegro, as well as by its isolatedness by Pešter Plateau, which resulted in the greatest genetic distance between populations I and III-VII**,** which gravitate from different sides of the Plateau. However, the large genetic distance between populations IV and V was ascribed to their origin, i.e. the fact that the data on their origin were reliable. However, they were genetically closer to populations II and VI in the north-western part of the Scots pine range in Serbia than to populations III and VII**,** which are found in the south-eastern part of the range.

DISCUSSION

Genetic divergence is a key factor in the formulation of effective conservation strategies and germplasm management. In general, coniferous trees show very high genetic divergence when compared to other types of organisms. Although pines have somewhat lower genetic divergence relative to other conifers, *Pinus sylvestris* was one of the most diverse of coniferous species (Szmidt 1996, Nowakowska 2004). We

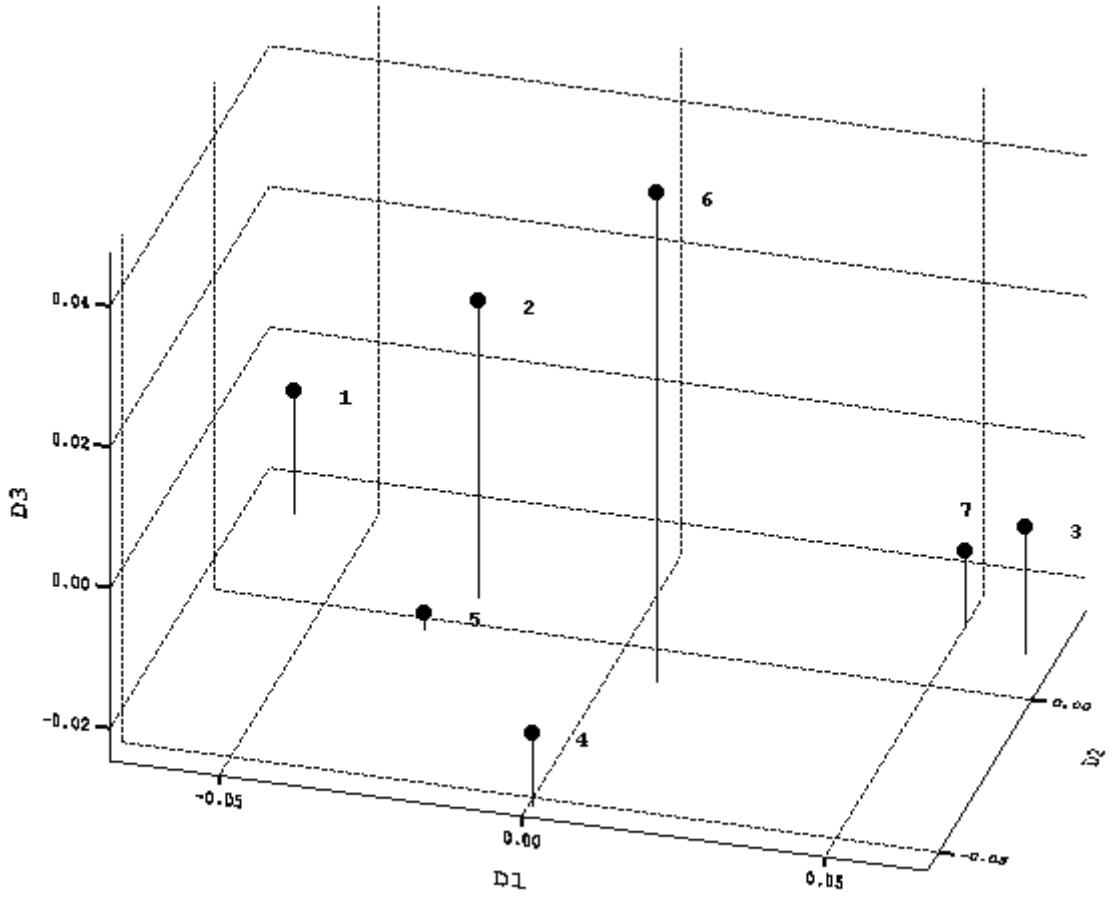

Diagram 3. – Diagram of correspondence analysis of genetic similarity after Dice

hereby report the RAPD analysis done to determine the genetic variability in Scots pine (*Pinus sylvestris* L.) in seven populations from Serbia. The use of RAPD assay to identify genetic variation is preferred over the morphological and biochemical markers since these are completely devoid of environmental effects and of the stage of the experimental material, thus making them highly reliable.

A great number of plants that should be analyzed by molecular markers can be a limiting factor in the study of population divergence. One of the methods to overcome this problem is the analysis of one or several group samples (bulk DNA of several individual plants) per population. This approach had positive results in the study of populations of a great number of plant species. Goto et al. (2001) concluded that RAPD analysis with a bulking procedure would be useful for rapidly checking the genetic identity of ramets in clonal seed orchards.

Ten decamer primers selected for RAPD profiling produced 21 (38.89%) polymorphic and 33 (61.11%) monomorphic banding sites. The percentage of polymorphism was lower than that in other pine species, including 90.9% for *Pinus sylvestris* (Szmidt et al., 1996), 90% for *Pinus halepensis* (Gómez et al., 2000), 88.5% for *Pinus nigra* (Lučić et al., 2010) and 79% for *Pinus mugo* (Monteleone et al., 2006). The high number of polymorphic loci

analyzed allows us to have good relative estimates of the variation between populations to be used in breeding and genetic resource conservation programs The assessed degree of polymorphism allows us to have good relative estimates of the variation between populations to be used in breeding and genetic resource conservation programs. In the RAPD reactions, strong and weak bands were produced. Weak bands resulted from low homology between the primer and the pairing site on the DNA strand (Thormann et al., 1994). Weak bands were, therefore, disregarded to increase analysis precision. Certain rare and genotype-specific bands were identified which could be effectively used to distinguish the populations. RAPD were the dominant markers and null allele present only in heterozygous genotypes could not be detected. These loci appeared as monomorphic and were excluded from data processing. The exclusion of such loci could lead to an underestimation of genetic differentiation among populations in cases where these were characterized by different frequencies of null alleles. On the other hand, RAPDs amplified mostly non-coding DNA sequences subject to weaker selection pressure, thus allowing the scoring of a higher amount of genetic variation (Monteleone et al., 2006).

Dice similarity coefficient ranged from 0.82 to 0.98; the Sokal and Michener ranged from 0.70 to 0.98. The calculation of genetic similarity was based on the two coefficients to estimate if a different grouping of genotypes would be obtained, and to determine which type of coefficient was the most adequate for data processing. The two coefficients used to estimate genetic similarity differed in the manner in which the matrix of original data (1, presence of RAPD band or 0, absence) was employed in the similarity estimate. An important aspect to be considered was the inclusion or exclusion of negative co-occurrences in the coefficient. In some cases, absence of the trait would indicate similarity, but in other cases, this was not necessarily true. The Sokal and Michener coefficient differed from the Dice coefficient by the fact that it takes into account the absence of bands in both genotypes (component d) as well. Due to the genetic basis of the RAPD markers, the absence of amplification of band in two genotypes does not necessarily represent genetic similarity between them. It seems reasonable to consider that the coefficients that exclude negative co-occurrences (Dice) are more adequate for use with dominant molecular markers. The formation of distance matrices was followed by the selection of grouping methods. These methods are sets of rules of grouping based on the measures of similarity/distance. A great number of grouping methods has been proposed, so that cluster analysis is criticized also because the selection of different options is difficult to verify, especially when little is known on the observed problem (Bull and Hogarth, 1990). The most frequently applied UPGMA method for genetic distance clustering starts from the basic hypothesis of an equal rate of evolution along all branches which often is not satisfied. Cluster analysis shows the results in the form of discrete groups – clusters also in cases when there is a continued variability within data. Correspondence analysis is a supplementary analysis of genetic distances and dendrograms, giving a global presentation of the relationships among populations (Greenacre, 1988). Its advantage is in the fact that it does not presuppose that units belong to some groups, but presents continued variability more precisely.

Comparative analysis of the constructed Diagrams of genetic similarity correspondence analysis in two and three dimensions, and the analyses using NTSYS-PC software, based on matrices of similarity coefficients, showed a significant genetic similarity between populations III (FMU "Radočelo-Crepuljnik" FE "Stolovi" Kraljevo) and VII (FMU "Zlatar I", FE "Prijepolje", Prijepolje), as well as between II (FMU "Šargan", FE "Užice" Užice) and VI (FMU "Kaluđerske Bare", NP "Tara", Bajina Bašta), which was marked by their geographic closeness. Greater genetic distances occurred between populations IV (FMU "Jablanička Reka", FE "Rasina" Kruševac) and **V** (FMU "Bukovik-Aleksinački", FE "Niš", Niš), as well as population I (FMU "Dubočica Bare" FE "Golija" Ivanjica). Population I was char-

acterized by its being isolated from the other study populations by Pešter Plateau, while large genetic distances between populations IV and V compared to other populations, were ascribed to their origin, i.e. the fact that the data on the origin of these populations were reliable. There was also a similarity between the patterns of populations I, VI, II, V and IV in the Diagram of correspondence analysis (especially in cases where Dice similarity coefficients were calculated) and their geographic position on the map, with certain skewness. The isolation of populations VII and III can be ascribed to their isolation by altitudinal differences. In their study on human populations, Cavalli-Sforoza et al. (1994) concluded that a two-dimensional correspondence analysis Diagram was often similar to geographic maps of the study populations, with a degree of skewness. In the research of genetic relationships among twelve Chinese indigenous goat populations, Meng-Hua et al. (2002) found perfect coincidence between the results of correspondence analysis and historical-geographical sources. Cavalli-Sforza et al. (1994) concluded that correspondence analysis is more informative and more accurate than dendrograms, especially if significant genetic exchange between geographically close populations occurs. Babić et al. (2009) report that this type of analysis can help to resolve the genetic relationships of the study genotypes. The obtained results show the existence of inter-population genetic variability, which confirms the high genetic divergence, as the base for the differentiation of Scots pine ecotypes in part of its range in Serbia. Better understanding of the genetic variability of natural populations of Scots pine in Serbia can contribute to the development of *in-situ* and *ex-situ* conservation strategies by identifying the genotypes of high interest, as well to the application in the restoration of degraded populations by the designation of adequate seed zones.

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