Arch. Biol. Sci., Belgrade, 62 (2), 329-336, 2010 DOI:10.2298/ABS1002329L

GENETIC DIVERSITY OF AUSTRIAN PINE (*PINUS NIGRA* ARNOLD) POPULATIONS IN SERBIA REVEALED BY RAPD

A. LUČIĆ[1], SNEŽANA MLADENOVIĆ-DRINIĆ[2], N. STAVRETOVIĆ[3], V. ISAJEV[3], VERA LAVADINOVIĆ[1], LJUBINKO RAKONJAC[1] and MARIJANA NOVAKOVIĆ[3]

[1]*Institute of Forestry,* 11030 Belgrade, Serbia
[2]*Maize Research Institute,* 10185 Zemun Polje, Serbia
[3]*Faculty of Forestry, University of Belgrade,* 11030 Belgrade, Serbia

Abstract – The ability of random amplified polymorphic DNA (RAPD) to distinguish among populations of Austrian pine from Serbia was evaluated. Ten arbitrary 10mer primers employed in the analysis produced 113 fragments of which 100 were polymorphic (88.5%). All populations had specific RAPD phenotypes. Certain rare and genotype specific bands were identified which could be effectively used to distinguish the populations. Polymorphism in RAPD markers among *P. nigra* population was high and sufficient to distinguish each of the populations. The results obtained suggest that RAPD markers are valuable for the genetic diversity estimation in *Pinus nigra* and for the study of the divergence among populations.

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

UDC 575:582.475(497.11)

INTRODUCTION

Austrian pine (*Pinus nigra* Arnold) is a southern European species extending from Spain in the west to central Turkey in the east. In Serbia its population is most numerous in the west and southwest, where it occupies the area of a large rhomboid, while in the east it occurs very rarely as an autochthonous species.

Many provenance tests in the world show that Austrian pine populations from this region are characterised by their quality and are assessed as the best, the most resistant and with the highest genetic variability. The proof is also given by Wheeler et al., (1976) recommending growers to ask for the seeds with the characteristics of the seeds collected as near as possible to the site of the Forest Estate "Užice", Užice in Serbia.

Knowledge of the genetic variation of a forest tree population is fundamental for sustainable forest management. However, information concerning the genetic diversity of *Pinus nigra* in Serbia is limited. Former studies of the *P. nigra* population were based mainly on morphological and phonological traits (Vidaković, 1974; Liber et al., 2002; Mataruga et al., 2003; Varelides et al., 2001) as well as biochemical markers such as terpenes (Müller-Starck et al., 1992; Bojović, 1995), and isozymes (Nikolić and Tucić, 1983; Fineschi, 1984; Scaltsoyiannes et al., 1994; Aguinagalde et al., 1997; Cengel et al., 2000). Currently, the genetic diversity of plants has been assessed more efficiently after the introduction of methods that reveal the polymorphism directly from DNA levels. As tools in forest tree improvement DNA markers are most useful for estimating genetic diversity, germplasm identification, estimating seed orchard efficiency, QTL analysis and construction of molecular linkage maps. RAPD markers have been intensively used in studies of genetic diversity in natural populations of many plants including pines (Kaya et al., 1993, Gómez et al., 2000, Nkongolo et al., 2002, Monteleone et al., 2006).

Fig. 1. Map depicting geographical region where *P. nigra* seeds were sampled. I – FE Šumarstvo" Raška 27a; II – FE "Šumarstvo" Raška 21a; III – FE "Prijepolje" Prijepolje; IV – FE "Užice" Užice; V – Faculty of Forestry-Beograd-Goč; VI – FE "Stolovi" Kraljevo.

The RAPD markers allow us to obtain a large amount of data on genetic variation within and among populations without detailed prior knowledge of DNA sequences (Holsinger et al., 2002). The purpose of the present study was to analyze RAPD variations in six populations of *Pinus nigra* from Serbia.

MATERIALS AND METHODS

The Register of Seed Stands in Serbia in 2005 defines six seed stands of Austrian pine characterized by different site conditions. Seed stands in the Forest Estate (FE) "Užice" from Užice and FE "Stolovi" from Kraljevo have the highest temperature oscillations. The average amount of precipitation per year during the vegetation period is significantly the highest in the Austrian pine seed stand in the FE "Užice" from Užice. Seed stands in the FE "Prijepolje" from Prijepolje and in the Teaching base "Goč" of the Faculty of Forestry from Belgrade have a considerably more open canopy. The bedrock of all seed stands is serpentine.

The plant material for this study included the seeds collected from these six populations of Austrian pine (*Pinus nigra* Arnold) (*Fig. 1*).

It is evident that the plant material was collected from a latitudinal range of 43^0 03' N to 43^0 78' N, between 19^0 22' E to 20^0 81' E longitudes within an altitudinal range from 700 m to 1080 m.

Seeds from ten 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 14 RAPD primers (Genosys Biotechnologies) in two rounds of amplification (Williams et al., 1990), of which 10 primers gave clear and reproducible banding patterns. RAPD reactions were done in a volume of 25 μL containing 2.5 mM MgCl2, 100μM dNTPs, 0.2 μM of 10-mer primers (Genosys Biotechnologies), 2.5 U of Taq polymerase (Promega), and 10 ng of template DNA.

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 sec, 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 a 1 x TAE buffer (Tris-acetate 0.04 M and EDTA 0.01 M pH 7.5), 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

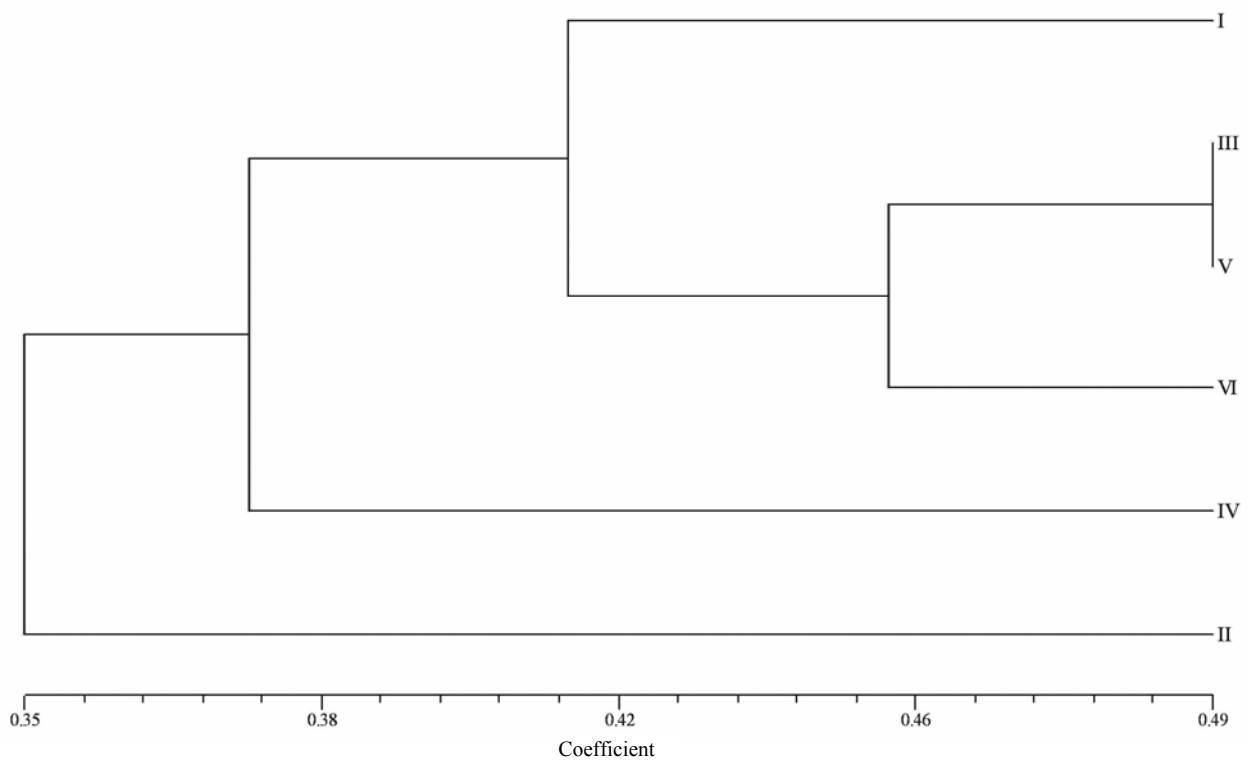

Fig. 2. RAPD-based dendrogram of *Pinus nigra* populations according to Jaccard (1908).

(1) or absence (0) of the fragment in each sample was assessed and the data were assembled into a binary matrix. Genetic (GS) similarity among populations was calculated in accordance with Jaccard (1908) and Sokal and Michener (1958);

Jaccard $GS_{ij} = a/a+b+c;$

Sokal and Michener (SM) $GS_{ij} = a+d/a+b+c+d$

where:

a is the number of fragments present in both variety *i* and *j* (1.1)

b is the number of fragments present in *i* and absent in *j* (1.0)

c is the number of fragments absent in *i* and present in *j* (0.1)

d is number of fragments absent in both variety *i* and *j* (0.0)

Coefficients of genetic similarity are used to construct a UPGMA.

RESULTS

The fourteen RAPD primers were used to evaluate the level of genetic diversity amongst the different populations of Austrian pine (*P. nigra* Arnold) 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 diversity was observed amongst the six populations of *Pinus nigra* being analyzed. Ten of the fourteen primers used in the present study revealed 113 reproducible RAPD loci. Among them 100 RAPD loci (88.5%) were polymorphic in at least one population. Each primer amplified between 3 and 24 fragments, with an average of 11.3. The total number of polymorphic loci detected varied between primers. Table 1 gives

Table 1. List of primers used for the study along with their percent polymorphism

Primer	Sequence	Number of amplification products	Number of Polymorphic bands	Percentage polymorphism
GEN 2-	CGACGG	13	13	100 %
GEN 4-	CTGTCG	11	11	100 %
GEN 2-	CGCGAA	18	17	82.0 %
GEN 1-	ACCCCA	24	22	94.4 %
GEN 1-	CGCCCG	10	10	100 %
GEN 1-	GCACGC	12	11	91.7 %
GEN 1-	GAGATC	11	9	81.8 %
GEN 1-	ACGGTG	3	0	0
GEN 1-	GGACTC	4	0	0
GEN 1-	TGCAGC	7	7	100 %

a list of primers used for the study along with the percent polymorphism. The percent polymorphism of primers ranged from 0% (GEN 1-70-3, GEN 1-70-9) to 100% (GEN 4-70-3, GEN 1-80-4, GEN 2-80-2, GEN 1-70-9/2), the average was 74.9%. With primer GEN 1-80-5 as many as 24 bands were amplified, of which 22 were polymorphic. Certain amplified bands appeared to be common to several populations, whereas others were present in some populations, but absent in others.

Using the dataset with 113 amplification products, similarity matrices were obtained based on Sokal and Michener (1958) and Jaccard (1908) coefficients. Genetic similarity coefficients for all possible pairs of the six populations are presented in Table 2. The average genetic distance was 0.57 (Sokal and Michener) and 0.42 (Jaccard), respectively. The highest genetic similarity based on both coefficients was between population V, Faculty of Forestry-Beograd-Goč - III, FE "Prijepolje" Prijepolje; (0.67, 0.49). According to Jaccard the lowest genetic similarity is noticed between population V, Faculty of Forestry-Beograd-Goč - II, FE "Šumarstvo" Raška 21a; and V, Faculty of Forestry-Beograd-Goč - IV, FE "Užice" Užice (0.31), and according to Sokal and Michener between VI, FE "Stolovi" Kraljevo and II, FE "Šumarstvo" Raška 21a, (0.51). The Mantel test comparing Jaccard's and Sokal and Michener's data gave a value of r=0.916 (P< 0.001).

Table 2. Coefficients of similarity between *Pinus nigra* populations

Sokal and Michener / Jaccard	Coefficient of similarity				
	I	II	III	IV	V
I	1	0.56	0.64	0.56	0.58
II	0.36	1	0.54	0.54	0.52
III	0.47	0.36	1	0.62	0.67
IV	0.36	0.36	0.42	1	0.52
V	0.38	0.31	0.49	0.31	1
VI	0.4	0.35	0.47	0.41	0.44

The dendrograms of *P. nigra* populations, based on UPGMA clustering, were constructed using the genetic similarity coefficients of Jaccard (1908) or Sokal and Michener (1958), and provide a graphic presentation of relationships among the six populations. The dendrogram using the Jaccard's coefficient revealed the presence of one cluster comprising four populations. The populations II, FE "Šumarstvo" Raška 21a and IV, FE "Užice" Užice are loosely linked to cluster (Fig. 2).

The dendrogram using the Sokal and Michener matching coefficient revealed the presence of two distinct clusters (Fig. 3). The cluster A was found to comprise four of the six populations and was thus designated to be a major cluster while cluster B had two populations, II and IV.

The clustering of populations in both dendrograms revealed the population III, FE "Prijepolje" Prijepolje and V, Faculty of Forestry-Beograd-Goč,

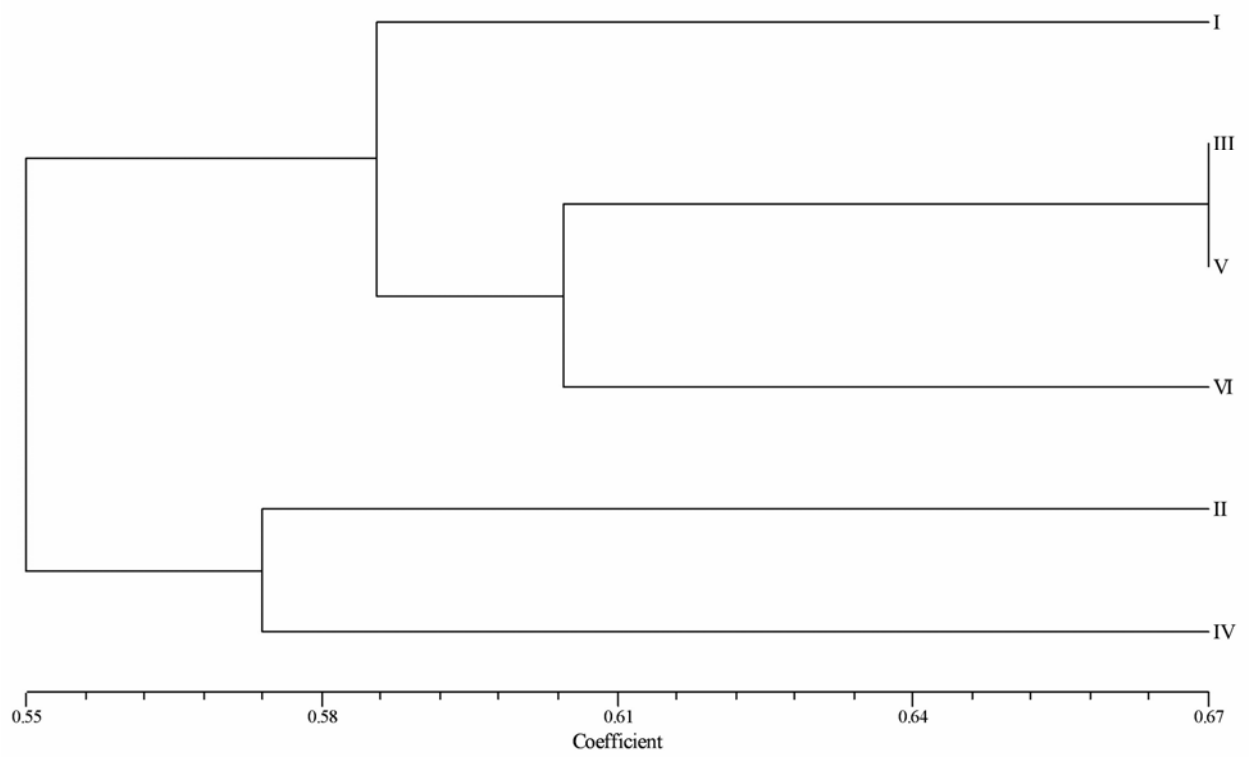

Fig. 3. RAPD-based dendrogram of *Pinus nigra* populations according to Sokal and Michener (1958).

to be the most similar. Population II, FE "Šumarstvo" Raška 21a was found to be the most distant from other members of the major cluster.

DISCUSSION

Genetic diversity is a key factor in the formulation of effective conservation strategies and germplasm management. In general, coniferous trees show very high genetic diversity when compared to other types of organisms. Although pines have a somewhat lower genetic diversity relative to other conifers, *Pinus nigra* was one of the most diverse of the coniferous species (Scaltoyiannes et al., 1994; Lee et al., 2002; Nkongolo et al., 2002; Monteleone et al., 2006). We hereby report the RAPD analysis done to determine the genetic variability in Austrian pine (*Pinus nigra* Arnold) from six 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 the effects of environment and 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 diversity. 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 100 (88.5%) polymorphic and 13 (11.5%) monomorphic banding sites. These

values are comparable to values published for populations of other pine species, including 90.9% for *Pinus silvestris* (Szmidt et al., 1996), 90% for *Pinus halepensis* (Gómez et al., 2000) 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 use in breeding and genetic resource conservation programs.

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

The Jaccard similarity coefficient ranged from 0.31 to 0.49, with a mean of 0.42; the Sokal and Michener ranged from 0.51 to 0.67 with a mean of 0.57. The calculation of genetic similarity was based on the two coefficients to estimate if different grouping of genotypes would be obtained, and to determine which type of coefficient is the most adequate for data processing. The two coefficients used to estimate genetic similarity differed by the manner in which the matrix of original data (1, presence of RAPD band or 0, absence) is employed in the similarity estimate. An important aspect to be considered is the inclusion or exclusion of negative co-occurrences in the coefficient. In some cases absence of the trait would indicate similarity but in others this is not necessarily true. The Sokal and Michener coefficient differs from the Jaccard coefficient in that it takes into account the absence of bands in both genotypes (component d). Due to the genetic basis of 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 (Jaccard) are more adequate for use with dominant molecular markers.

The UPGMA dendrograms based on Jaccard and Sokal and Michener coefficients of similarity showed that the population III, GJ "Crni vrh Ljeskovac" 69 c. FE "Prijepolje"-Prijepolje and V, GJ "Goč-Gvozdac" 92b. Faculty of Forestry- Beograd-Goč were similar. On the other hand, the population II, GJ "Divan-Lokva" 21a. FE "Šumarstvo"- Raška has the highest genotype specificity expressed by the highest coefficient of genetic distance compared to other populations.

The obtained results show the existence of inter-population genetic variability, which confirms the high genetic diversity as the base for the differentiation of Austrian pine ecotypes in the part of its range in Serbia. Better understanding of the genetic variability of natural populations of Austrian 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 as to the application in the restoration of degraded populations by the designation of the adequate seed zones.

REFERENCES

Aguinagalde, I., Llorente, F., and *C. Benito* (1997). Relationships among five populations of European black pine (*Pinus nigra* ARN.) using morphometric and isozyme markers. *Silv. Gen.* **46**, 1-5.

Bojović, S. (1995). *Biodiversité du pin noir (*Pinus nigra *Arn.) en région méditerranéenne.* Thèse doctorat, Université d'Aix-Marseille, III. France.

Cengel, B., Velioglu, E., Tolun, A. A., and *Z. Kaya* (2000). Pattern and magnitude of genetic diversity in *Pinus nigra* ARNOLD subspecies pallasiana populations from Kazdagi: Implications for in situ conservation. *Silv. Gen.* **49**, 249-256.

Fineschi, S. (1984). Determination of the origin of an isolated group of trees of *Pinus nigra* trough enzyme gene markers. *Silv. Gen.* **33**, 169-172.

Gómez, A., Alía, R., and *M. A. Bueno* (2001). Genetic diversity of *Pinus halepensis* Mill. populations detected by RAPD loci. *Ann. For. Sci.* **58**, 869-875.

Goto, S., Miyahara, F. and *Y. Ide* (2001). A fast method for checking genetic identity of ramets in a clonal seed orchard by RAPD analysis with a bulking procedure. *Silv. Gen.* **50**, 271-275.

Holsinger, K. E., Lewis, P. O., and *D. K. Dey* (2002). A Bayesian approach to inferring population structure for dominant markers. *Mol. Ecology* **11**, 1157-1164.

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.* **44**, 223 - 270.

Kaya, Z. and *D. B. Neale* (1993). Random amplified polymorphic DNA (RAPD) polymorphisms in *Pinus nigra* subs. *pallasiana* and *Pinus brutia*. *Tr. J. Agr. For.* **17**, 295-306.

Lee, S.-W., Ledig, F. T., and *D. R. Johnson* (2002). Genetic variation at allozyme and RAPD markers in *Pinus longaeva* of the White Mountains, California. *Am. Jour. Bot.* **89**, 566-577.

Liber, Z., Nikolić, T., and *B. Mitić* (2002). Intra- and inter-population relationships and taxonomic status of *Pinus nigra* Arnold in Croatia according to morphology and anatomy of needles. *Acta soc. bot. Pol.* **71**, 141-147.

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Res*. **27**, 209-220.

Mataruga, M., Isajev, V., and *A. Tucović* (2003). Variability of morphometric characteristics of seedlings of 40 Austrian pine (*Pinus nigra* Arn.) half-sib lines, *Book of Abstracts, „Second symposium on the breeding of organisms"*, Vrnjačka Banja, Serbia.

Monteleone, I., Ferrazzini, D., and *P. Belletti* (2006). Effectiveness of neutral RAPD markers to detect genetic divergence between the subspecies *uncinata* and *mugo* of *Pinus mugo* Turra. *Silva Fennica* **40**, 391-406.

Müller-Starck, G., Baradat, P., and *F. Bergmann*, (1992); Genetic variation within European tree species. New Forests **6**, 23-47.

Nkongolo, K. K., Michael, P. and *W. S. Gratton* (2002). Identification and characterization of RAPD markers inferring genetic relationships among Pine species. *Genome* **45**, 51-58.

Nikolić, D., and *N. Tucić* (1983). Isoenzyme variation within and among populations of European black pine (*Pinus nigra* Arnold). *Silv. Gen.* **32**, 80-89.

Rogers, S.O. and *A. J. Bendich* (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* **5**, 69-76.

Rohlf, F. J. (2000). *NTSYS-pc. Numerical taxonomy and multivariate analysis system.* Version 2.0, Exeter Software, Setauket, N.Y.

Scaltsoyiannes, A., Rohr, R., Panetsos, K. P., and *M. Tsaktsira* (1994). Allozyme frequency distributions in five European populations of black pine (*Pinus nigra* Arnold). *Silv. Gen.* **43**, 20-30.

Sokal, R. R. and *C. D. Michener* (1958). A statistical method for evaluating systematic relationships. *Univ. Kans. Sci. Bull.* **38**, 1409-1438.

Szmidt, A. E., Wang, X-R. and *M-Z. Lu* (1996). Empirica. 0l assessment of allozyme and RAPD variation in *Pinus sylvestris* using haploid tissue analysis. *Heredity* **76**, 412-420.

Thormann, C. E., Ferreira, M. E., Camargo, L. E. A., Tivang, J. G., and *T. C. Osborn* (1994). Comparison of RFLP and RAPD markers for estimating genetic relationships within and among cruciferous species. *Theor. Appl. Genet*. **88**, 973-980.

Varelides, C., Brofas, G. and *Y. Varelides* (2001). Provenance variation in *Pinus nigra* at three sites in northern Greece. *Ann. For. Sci.* **58**, 893-900.

Vidaković, M. (1974). Genetics of European black pine (*Pinus nigra* Arn.) *Ann. Forest*. **6/3**, 57-86.

Wheeler, N. C., Kriebel, H. B., Lee, C. H., Read, R. A., and *J. W. Wright* (1976). 15-year performance of European black pine in provenance test in North Central United States. *Silv. Gen*. **25**, 1-6.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and *S. V. Tingey* (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.

ГЕНЕТИЧКА ВАРИЈАБИЛНОСТ ПОПУЛАЦИЈА ЦРНОГ БОРА (*PINUS NIGRA* ARNOLD) У СРБИЈИ ПРИМЕНОМ RAPD МЕТОДЕ

А. ЛУЧИЋ[1], СНЕЖАНА МЛАДЕНОВИЋ-ДРИНИЋ[2], Н. СТАВРЕТОВИЋ[3], В. ИСАЈЕВ[3], ВЕРА ЛАВАДИНОВИЋ[1], Љ. РАКОЊАЦ[1] и МАРИЈАНА НОВАКОВИЋ[3]

[1]*Институт за шумарство,* 11030 Београд, Србија
[2]*Институт за кукуруз,* 10185 Земун Поље, Србија
[3]*Шумарски факултет, Универзитет у Београду,* 11030 Београд, Србија

RAPD маркери су коришћени за процену генетичке различитости популација црног бора (*Pinus nigra* Arnold) у Србији. Десет одабраних 10mer прајмера употребљених за анализу је дало 113 фрагмената од којих је 100 било полиморфно (88.5%). Све популације су имале специфичне RAPD фенотипе. Идентификовани су појединии фрагменти карактеристични за одређену популацију као и генотип специфични фрагменти на основу којих је могуће ефикасно раздвајање анализираних популација. Полиморфизам RAPD маркера међу популацијама *Pinus nigra* је био висок и довољан да се издвоји свака од популација. Добијени резултати показују да су RAPD маркери погодни за проучавање генетичког диверзитета и генетичке сродности популација црног бора.