UDC 575:630
DOI: 0.2298/GENSR1303641L
Original scientific paper

INTERPOPULATION VARIABILITY OF AUSTRIAN PINE (*Pinus nigra* Arnold) IN SERBIA

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Lučić A., R. Cvetičanin, M. Novaković-Vuković, D. Ristić, V. Popović, Lj.Rakonjac, and S. Mladenović Drinić (2013): *Interpopulation variability of Austrian pine (Pinus nigra Arnold) in Serbia.*-Genetika, Vol 45, No. 3, 641-654.

Multidisciplinary studies (genetic and phytocoenological) of 4 populations of Austrian pine (*Pinus nigra* Arnold) were carried out in western and central Serbia. The obtained results gave the same inter-population arrangement in both methods. Dendograms (NTSYS) differentiate at the greatest genetic distance the population of Austrian pine in Šargan compared to other populations: Crni Vrh, Goč and Studenica. Using phytocoenological analysis it was determined that population of Austrian pine in Šargan forms a community *Erico-Pinetum gocensis*, while the other populations form a community *Seslerio rigidae-Pinetum gocensis.* Multidisciplinary approach that was demonstrated within this paper presents the first studies of Austrian pine that directly link genotype dependence and environmental conditions manifested through the phytocoenological affiliation.

Key words: Austrian pine, RAPD, plant communities.

INTRODUCTION

Austrian pine (*Pinus nigra* Arnold) is a widely distributed species, which in discontinuous range extends from northern Africa across the north Mediterranean and on east it extends to the Black Sea (RUBIO-MORAGA *et al*. 2012). It is also found in Sicily and Corsica.

Austrian pine is one of the economically most important conifers in Serbia. It is the most common tree (CVJETIĆANIN and PEROVIĆ, 2010) in western Serbia (Tara, Povlen, Maljen, Zlatibor, Troglav, Čemerno, Crni Vrh near Priboj), in central Serbia (Goč, Stolovi, Kopaonik),

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and less common tree in eastern Serbia (Zlotska klisura, Beljanica, klisura Sukovske reke, Suva planina and region of Bosilegrad).

The recent classification of the genus *Pinus*, which is based on molecular researches, recognizes six subspecies within this species (BOGUNIĆ *et al*. 2007): *salzmanni, nigra, dalmatica, pallasiana, laricio* and *mauretanica* (North Africa).

Conifers are generally characterized by very high levels of genetic variability (HAMRICK 1979) and the possible factors for this are a) a long life, free pollination with high fertility and b) divergent selection for macro-micro geographic adaptation. Pine is characterized by high total variability due to high intra-population genetic variability which indicates a significant genetic differentiation of local populations and the possibility for the same alleles to be distributed across the whole range of species.

Many provenance tests in the world show that Austrian pine populations from this region are characterized by the quality and they 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 to the growers to ask for the seed with the characteristics of the seeds collected as near as possible to the site FE "Užice" near Užice in Serbia.

Knowledge on genetic variation of forest tree population is fundamental for sustainable forest management. However, information of genetic diversity of *Pinus nigra* in Serbia is limited. The former studies of *P. nigra* population were based mainly on the morphological and phonological traits (LIBER *et al*., 2002; MATARUGA *et al*., 2003) as well as biochemical markers like terpenes (BOJOVIĆ, 1995), and proteins (CENGEL *et al*., 2000; MATARUGA *et al*. 2007; LUČIĆ *et al*. 2008). 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 (GÓMEZ *et al*., 2001, NKONGOLO *et al*., 2002, MONTELEONE *et al*., 2006; LUČIĆ *et al*. 2010). 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).

Scots pine (*Pinus sylvestris* L.) is another species from the genus *Pinus* significant for forestry in Serbia. For this species were carried out extensive researches which are important for this paper because they provide base for better understanding of inter-population variability of pines. In the analysis of inter-population variability of Scots pine in Serbia were used morphometric markers (TOŠIĆ *et al*. 1991; LUČIĆ *et al*. 2012a), biochemical markers (LUČIĆ *et al*. 2011) and molecular markers (LUČIĆ *et al*. 2012b). Significant results in multidisciplinary researches (genetic and phytocoenological) were obtained in the analysis of 5 populations of Scots pine in Serbia combining these research methods (LUČIĆ *et al*. 2012b).

MATERIALS AND METHODS

For this paper, the researches included 4 populations of Austrian pine from 4 different locations in western and central Serbia where the Austrian pine has the largest distribution (Figure 1).

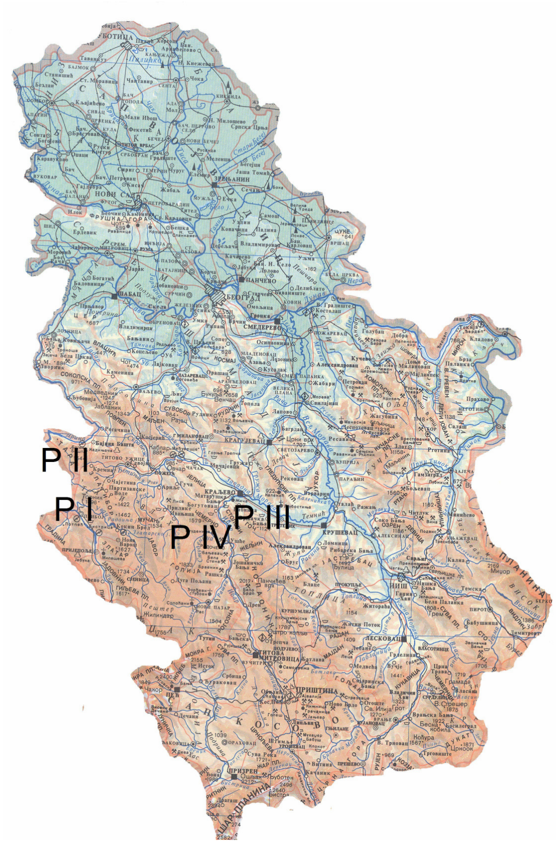

Figure 1. Spatial distribution of the studied Austrians pine (*Pinus nigra* Arnold) populations
Legend:
P I - FMU „Crni vrh-Ljeskovac", FE „Prijepolje" Prijepolje
P II - FMU „Šargan", FE „Užice" Užice
P III - FMU „Goč-Gvozdac" Faculty of Forestry-Beograd-Goč
P IV - FMU „Studenica-Polumir", FE „Stolovi" Kraljevo

Phytocoenological characteristics of the analyzed stands were determined by taking 15 phytocoenological relevés using a method of the Braun-Blanquet Zurich-Montpellier School (BRAUN – BLANQUET, 1931, 1964). Plant species were determined based on Flora of Serbia (JOSIFOVIĆ, 1970-1980) and Ikonographie der flora des südöstlichen Mitteleuropa; (JÁVORKA, CSAPODY, 1979). Names of syntaxa were given after TOMIĆ (2006).

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), and 10 primers gave clear and reproducible banding patterns. RAPD reactions were done 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 (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 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 (1) or absence (0) of the fragment in each sample was assessed and the data were assembled into a binary matrix. Genetic similarity among populations (GS) was calculated in accordance with JACCARD (1908), DICE (1945) and SOKAL and MICHENER (1958):

Jaccard GS_{ij} = a/a+b+c;
Dice GS_{ij} = 2a/2a+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 - the number of fragments present in *i* and absent in *j* (1.0)
c - the 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 are used to construct a UPGMA.

RESULTS

Phytocoenological studies have shown that Austrian pine (*Pinus nigra* Arnold) in locations where the studies were conducted forms two phytocoenoses: *Erico-Pinetum gocensis* and *Seslerio rigidae-Pinetum gocensis*.

Clas. *Erico-Pinetea*
Ord. *Erico-Pinetalia* Oberdorfer 49 *emend*.
All. *Orno-Ericion*
Sub All. *Erico-Pinenion gocensis*
Ass. *Erico-Pinetum gocensis*
Ass. *Seslerio rigidae-Pinetum gocensis*

The community *Erico-Pinetum gocensis* Krause 1957 was studied in **PII - Šargan**, based on 5 phytocoenological relevés. The community is located at an altitude of 939-968 m and on steep slopes (20-35°). It occupies primarily cold aspects (N, NW, E).

In the first layer it was recorded only Austrian pine (*Pinus nigra*). In the second layer, except edificators, there is also single-seeded hawthorn (*Crataegus monogyna*), Mountain ash (*Sorbus aucuparia*), Juniper (*Juniperus communis*) and Manna-ash (*Fraxinus ornus*). The ground flora layer has great degree of coverage, 0.9-1.0, and the dominant species with the greatest degree of coverage are *Erica carnea*, *Pteridium aquilinum, Calamagrostis varia*. In the characteristic set of species are *Erica carnea, Pteridium aquilinum, Rubus idaeus, Dactylis* *glomerata, Tanacetum corymbosum, Potentilla alba, Pinus nigra, Filipendula hexapetalla, Erythronium dens canis, Brachypodium pinnatum, Lilium martagon, Stachys scardica, Thymus pulegioides* and *Vicia cracca.*

Table 1. Phytocoenological Table of community Erico-Pinetum gocensis

Association	*Erico-Pinetum gocensis* Krause 1957					
Management unit	ŠARGAN					Degree of presence
Relevé number	1	2	3	4	5	
Compartment	21	21	21	21	21	
Altitude (m)	950	968	963	952	939	
Aspect	NW	NW	NW	SE	E	
Slope (◦)	20	25	25	35	35	
LAYER I						
Canopy	0.7	0.8	0.7	0.5	0.7	
Mean height (m)	24	25	23	18	22	
Mean distance (m)	3	3	4	4	4	
Pinus nigra	4.4	5.5	4.4	3.3	4.4	V
LAYER II						
Canopy	0.1	0.1	0.1	0.1	0.1	
Mean height (m)	1.5		1	2	3	
Pinus nigra			+.2	+	+	III
Crataegus monogyna	+	+			+	III
Sorbus aucuparia	+	+				II
Juniperus communis	+					I
Fraxinus ornus	+					I
LAYER III						
Degree of coverage	1.0	1.0	1.0	0.9	1.0	
Erica carnea	2.2	2.2	2.2	3.3	4.5	V
Pteridium aquilinum	3.4	+.2	1.2	+		V
Rubus idaeus	1.2	+.2	+	+	+	V
Dactylis glomerata	1.2	+.2	+.2	1.2	+.2	V
Tanacetum corymbosum	+	+	+	+	+	V
Potentilla alba	+.2	1.2	1.2	1.2	+.2	V
Filipendula hexapetalla	+	+	+	+	+	V
Erythronium dens canis	1.1	+	+	1.2	1.1	V
Brachypodium pinnatum	+.2		1.2	1.2	+.2	IV
Lilium martagon	r	r	+		+	IV
Stachys scardica	+.2	+.2		+.2	+.2	IV
Thymus pulegioides	+.2		+.2	2.2	1.2	IV
Vicia cracca	+.2		+	+	+.2	IV
Lathyrus sphaericus	+.2	+	+			III

Fragaria vesca	+			+.2	+	III
Galium vernum	+.2		+.2	+.2		III
Euphorbia amygdaloides	+	+.2	+			III
Daphne blagayana	1.2	1.2	1.2			III
Vaccinium myrtillus	+.2	+.2	1.2			III
Anemone nemorosa	+	+	1.2			III
Galium lucidum	+.2		+.2		+	III
Narcissus radiflorus	+	+.2			+	III
Veronica chamaedrys		+	+		+	III
Pinus nigra			+	+	+	III
Aremonia agrimonioides	+.2			+		II
Silene vulgaris	+			+		II
Stachys officinalis	+.2		+.2			II
Crataegus monogyna	+		+	+	+	II
Rosa pendulina	+	+				II
Sympytum tuberosum	+			+		II
Agrostis capillaris	1.2		+.2			II
Lamium galeobdolon	+.2		+.2			II
Knautia dinarica	+.2			+		II
Calamagrostis varia	4.4	3.4				II
Primula veris	+.2	+				II
Scleropodium purum	+.3	+.3				II
Melittis melysophyllum		+.2	+			II
Galium schultesii		+.2	+.2			II
Sorbus aucuparia		+			+	II
Centaurea stenolepis	+		+			II
Sesleria serbica		+.2			+.2	II
Quercus dalechampii		+			+.2	II
Euphorbia angulata		+	+			II
Dicranum polysetum		1.3	1.3			II
Genista tinctoria				+.2	+.2	II
Euphorbia cyparissias				+.2	+.2	II
Hypericum perforatum				+	+	II
Fraxinus ornus				+	+	II
Campanula cervicaria				+	+	II
Festuca amethystina				2.2	+	II
Polygala amara				+.2	+	II

The community of *Seslerio rigidae-Pinetum gocensis* was recorded in **PI - Crni vrh**, **PIV - Studenica** and **PIII – Goč**, and it is described based on 10 phytocoenological relevés. The community is located at an altitude of about 790 m, on slopes of 7-15° and mostly in sheltered aspects (N, NW), although it was also recorded in warmer aspects (W, SW).

In the tree layer, in all relevés was recorded Austrian pine (*Pinus nigra*), as well as Balkan durmast oak (*Quercus dalechampii*) which also has high degree of presence. In this layer there were also Balkan beech (*Fagus moesiaca*), Wild Service Tree (*Sorbus torminalis*) and Wild cherry (*Prunus avium*). Shrub layer is very rich, there were recorded 17 species. The highest degree of presence has Austrian pine (*Pinus nigra*), then Balkan durmast oak (*Quercus dalechampii*). Other species have lower degree of presence: Balkan beech (*Fagus moesiaca*), Mountain ash (*Sorbus aucuparia*), Wild cherry (*Prunus avium*), Manna Ash (*Fraxinus ornus*) etc. The ground flora layer has great degree of coverage (0.8-1.0), with dominant species *Sesleria serbica, Rubus hirtus* and *Pteridium aquilinum.* In the characteristic set of species are *Pinus nigra, Sesleria serbica, Vaccinium myrtillus, Quercus dalechampii* and *Rubus hirtus.*

Table 2. Phytocoenological Table of community Seslerio rigidae-Pinetum gocensis

Association	*Seslerio rigidae-Pinetum gocensis* Gajić 1954										
Management unit	Crni vrh				Studenica			Goč			
Relevé number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	Degree of presence
Compartment	83b	81c	81a	81a	26c						
Altitude (m)	960	790	820	820							
Aspect	N	N	NW	NW	NNW	SW	NW	W	SW	-	
Slope (◦)	7	15	15	7	15	15	5	10	15	-	
LAYER I											
Canopy	0.6	0.4	0.4	0.5	0.8 0.5	0.6 0.3	0.8 0.2		0.6	0.5	
Mean height (m)	10	20	22	22	24 10	20 4	22 12	20	18		
Mean diameter (cm)	16	24	27	27	38 15	35 15	30 15	40	28		
Mean distance (m)	2	5	4	5	10	5 10	5 9		7		
Pinus nigra	4.4	2.3	3.3	3.3	5.5	4.5	5.5	3.3	3.3	3.3	V
Quercus dalechampii	+					2.1		1.2	1.2	1.1	III
Fagus moesiaca					2.1	2.1	2.1				II
Sorbus torminalis						1.1					I
Prunus avium							1.1				I
LAYER II											
Canopy	0.3	0.1	0.1	0.3	0.5	0.3	0.4	0.3	0.2		
Mean height (m)	2	1	5	5	4	4	4	3	4		
Mean distance (m)	5	7	5	2							
Pinus nigra	+	+	+.2	1.2				2.2	1.2	+	IV

Quercus dalechampii	+				1.1	2.2	1.2		1.2	+	III
Fagus moesiaca					1.2	1.1	+				II
Sorbus aucuparia								+	+	+	II
LAYER III											
Degree of coverage	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Vaccinium myrtillus		1.2	2.3	1.2	1.2	1.3	1.2	1.2	+	1.2	V
Sesleria serbica	3.3	3.2	3.3	2.2				5.5	5.5	5.5	IV
Quercus dalechampii	+.2	+	+	+		+.2		+.2	+		IV
Rubus hirtus	+	+.2			4.4	3.3	3.3	+.2		1.2	IV
Pinus nigra	+	+	+	+.2	+.2	1.3					III
Fragaria vesca	+	+	+	+		+	+				III
Rosa spinosissima		+.2	1.2	+.2				+.2		+	III
Erica carnea		2.3	1.2	2.2				2.2	1.2	+	III
Lotus corniculatus	+	+	+	+							II
Campanula patula		+	+	+							II
Stachys scardica		+	+	+				+			II
Galium cruciata		+	+	+			+				II
Pteridium aquilinum		+.2			3.4	4.4	4.3				II
Prunus avium			+		+		+				II
Dactylis glomerata						1.2	+.2	+.2		+	II
Rosa pendulina								+.2	+	+	II
Epimedium alpinum								+.2	+	+	II
Calamintha officinalis					+	+	+				II
Lembotropis nigricans						+			+	+	II
Hieracium murorum		+			+		+				II

In the part of the research that relates to the determination of inter-population variability using molecular markers – RAPD, there were performed cluster analyses, dendograms 1, 2 and 3.

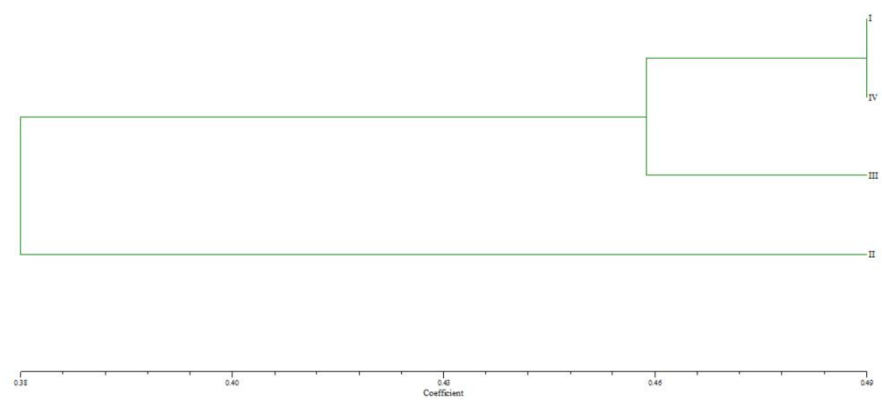

Diagram 1. Cluster analysis dendrogram of Austrian pine populations after Jaccard

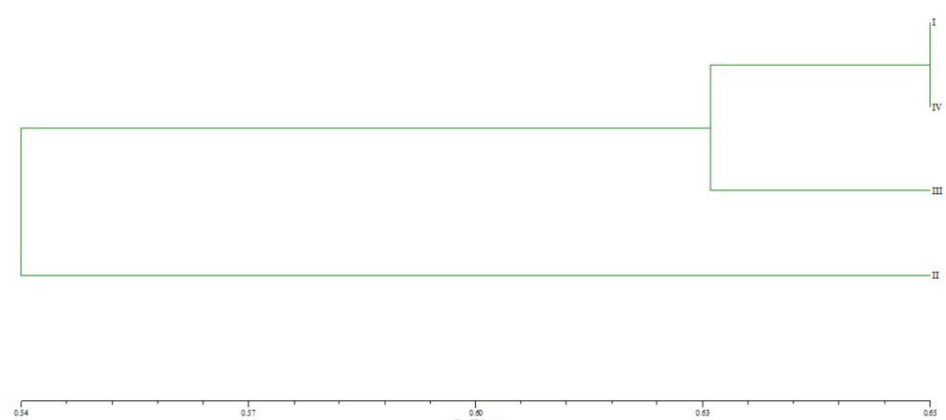

Diagram 2. Cluster analysis dendrogram of Austrian pine populations after Dice

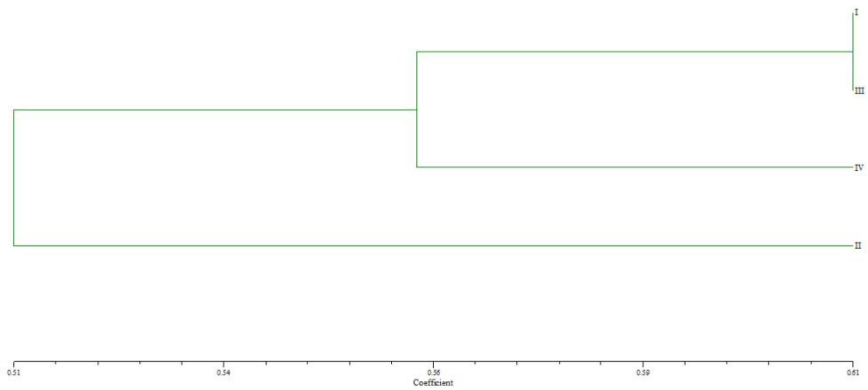

Diagram 3. Cluster analysis dendrogram of Austrian pine populations after Sokal and Michener

Based on cluster analysis dendograms of similarity coefficients of Austrian pine populations it is perceived one sub-cluster which consists of populations **PI - Crni vrh**, **PIV - Studenica** and **PIII – Goč** as well as separated population **PII - Šargan,** which is loosely linked to other populations.

DISCUSSION

The plant community research points out the similarities and differences between the communities with Austrian pine (*Pinus nigra*) as the edificator.

The community *Erico-Pinetum gocensis*, **PII - Šargan** has 75 species, while the community *Seslerio rigidae-Pinetum gocensis* Gajić 1954 **PIV – Studenica, PI - Crni vrh** and **PIII – Goč** has 69 species, although it was described with double number of phytocoenological relevés. This fits character of the community because it has poorer floristic composition than other forests of Austrian pine (TATIĆ and TOMIĆ, 2006). Of 144 species that were recorded in both communities, only 24 are common which represents only 17% of total number of species. Abundance and degree of coverage of common species are very different in two compared phytocoenoses. For example, Bilberry (*Vaccinium myrtillus*) was recorded in almost all relevés in community *Seslerio rigidae-Pinetum gocensis*, while in community *Erico-Pinetum gocensis* it was recorded in 3 phytocoenological relevés, with small abundance and coverage. From other hand, Serbian moor grass (*Sesleria serbica*) in community *Seslerio rigidae-Pinetum gocensis* Gajić 1954 has values of abundance and coverage between 2.2.-5.5., while in the community *Erico-Pinetum gocensis* it was recorded in only 2 phytocoenological relevés, with value +.2.

It is noted that the community *Seslerio rigidae-Pinetum gocensis* has sparser canopy so in Ib and II layer it allows settling of sciophile species: *Fagus moesiaca, Prunus avium, Acer pseudoplatanus, Carpinus betulus* etc. From the other hand, the community *Erico-Pinetum gocensis* Krause 1957 has denser canopy, in I layer it was recorded only *Pinus nigra*, and due to lower light the shrub layer is poorer then in the community *Seslerio rigidae-Pinetum gocensis*. In both communities it was noted the presence of Balkan durmast oak (*Quercus dalechampii*) in offspring, which is a proof of syndinamic relationship of Austrian pine forests with Balkan durmast forests on serpentinites (CVJETIĆANIN, 1999, TATIĆ and TOMIĆ, 2006).

To the great coverage of ground flora layer in community *Erico-Pinetum gocensis* contribute *Erica carnea*, *Pteridium aquilinum,* grasses *Calamagrostis varia* and *Brachypodium pinnatum,* as well as moss *Dicranum polysetum.* In the community *Seslerio rigidae-Pinetum gocensis* Gajić 1954 the highest values of abundance and coverage have *Sesleria serbica, Rubus hirtus* and *Pteridium aquilinum* that are most present in the locality of Studenica. In both communities were recorded species *Brachypodium pinnatum* and *Pteridium aquilinum*, which always indicate a significant degree of degradation of forest phytocenoses. On degradation of habitats also indicate presence of species *Rubus hirtus* in both phytocoenosis, species *Dorycnium germanicum* in community *Erico-Pinetum gocensis, Lembotropis nigricans* in community *Seslerio rigidae-Pinetum gocensis* etc.

Grouping of Austrian pine populations within the cluster analysis of molecular markers coincides with the phytocoenological differentiation of same populations.

In the first group of populations are **PIV – Studenica, PI - Crni vrh** and **PIII – Goč**. On the other side, a quite larger genetic distance of population **PII – Šargan** is specific in comparison with other populations. This is explained not by a large geographical distance but extreme isolation from other populations because of mountains Zlatibor and Zlatar that are located between these populations, and because of gravitation of population **PII – Šargan** towards forests of Bosnia and Herzegovina.

A study of LUČIĆ *et al*. (2011) also indicates genetic distance of population **PII – Šargan;** in that study it was performed an analysis of inter-population variability of Austrian pine in Serbia using seed proteins, where on the greatest genetic distance compared to other populations differentiated the population **PII – Šargan**. The proof is also given by WHEELER *et al*. (1976) recommending to the growers to ask for the seed with the characteristics of the seeds collected as near as possible to the site Šargan in Serbia.

A significant result in multidisciplinary studies (genetic and phytocoenological), but on other species from genus *Pinus*- Scots pine, has been made in the analysis of 5 populations, combining these research methods (LUČIĆ *et al.* 2012b). On that occasion in direct relationship were brought the dependence of the protein complex and environmental conditions manifested through the phytocoenological affiliations.

CONCLUSION

In the paper were analyzed 4 populations of Austrian pine on 4 locations in western and central Serbia. Variability of Austrian pine populations (*Pinus nigra* Arnold) was determined based on phytocoenological and genetic studies. Based on phytocoenological studies two phytocoenoses were determined: *Erico-Pinetum gocensis* and *Seslerio rigidae-Pinetum gocensis*.

Based on cluster analysis dendogram of molecular markers, one group and one separated population were determined. Such differentiation of populations matches with phytocoenoses, so the community *Erico-Pinetum gocensis* determined in Šargan presents special population of Austrian pine which is separated at the greatest distance from all the studied populations in the cluster analysis dendogram. The community *Seslerio rigidae-Pinetum* *gocensis* was recorded in Studenica, Crni vrh and Goč. Based on cluster analysis dendogram these three sites of Austrian pine are grouped at the smallest genetic distance.

Multidisciplinary approach to this issue, demonstrated within this paper, presents the first studies that in direct relationship bring the dependence of genotype and environmental conditions manifested through the phytocoenological affiliations.

ACKNOWLEDGEMENTS

The present work was supported by the Ministry of Science and Technology of the Republic of Serbia (Project TR 31070).

Received February 28th, 2013
Accepted October 05th, 2013

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MEĐUPOPULACIONA VARIJABILNOST
CRNOG BORA (*Pinus nigra* Arnold) U SRBIJI

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Izvod

Multidisciplinarni pristup demostriran u okviru ovog rada predstavlja prva istraživanja na crnom boru koje u direktnu vezu dovodi zavisnost genotipa i uslove sredine ispoljene kroz fitocenološku pripadnost. U radu su izvršena multidisciplinarna istraživanja (genetička i fitocenološka) 4 populacije crnog bora (*Pinus nigra* Arnold) u zapadnoj i centralnoj Srbiji. Izvršena je analiza PCR amplifikacija genomske DNA sa 14 RAPD primera, od čega je 10 polimorfno. Korišćene su tri vrste koeficienata genetičke sličnosti, *Jaccard*, *Dice* and *Socal and Michener*. Dobijeni dendogrami (NTSYS) diferenciraju na najvećoj genetičkoj distanci populaciju crnog bora na Šarganu u odnosu na ostale populacije Crni Vrh, Goč i Studenica. Fitocenološke analize su vršene po metodi *Braun-Blanquet* i tom prilikom je utvrđeno da populacija crnog bora na Šarganu gradi zajednicu crnog bora sa crnjušom (*Erico-Pinetum gocensis*), a ostale populacije formiraju zajednice crnog bora sa uskolisnom šašikom (*Seslerio rigidae-Pinetum gocensis*). Dobijenih rezultata korišćenjem genetičke analize (PCR) i analize fitocenološke pripadnosti dali su isti međupopulacioni raspored.

Primljeno 28. II. 2013.
Odobreno 05. X. 2013.