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Original scientific paper

ANALYSIS OF INTRA-POPULATION VARIABILITY OF BALD CYPRESS (*Taxodium distichum* L. RICH.) IN SEED STAND NEAR BACKA PALANKA USING RAPD MARKERS

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The analysis of Bald cypress genetic variability at the level of test trees was performed using RAPD (Random Amlified Polymorphic DNA) markers. RAPD analysis was performed on 20 test trees with 13 primers. A total of ten primers gave a clear picture while three primers amplified weakly. 60 is a total number of detected bands obtained by RAPD analysis with 10 selected primers, and the average number of bands is 6. Based on presence/absence of RAPD fragments among all 20 Bald cypress test trees were calculated similarity coefficients by Dice and they range from 0.73 to 1. Based on similarity coefficients was performed the cluster analysis and results were presented as a dendrogram. All 20 test trees were grouped into two sub-clusters. Test trees 1, 4 and 11 were grouped in the first sub-cluster while other test trees were grouped in the second sub-cluster. By analysis of relations within every sub-cluster and sub-sub-cluster the existence of genetic distances between observed test trees can be noticed. The greatest similarity is between test trees 2, 12, 15 and 18. The results of genetic similarity and distance between observed test trees indicate the overwhelming presence of genetic diversity.

Key words: Bald cypress, RAPD, population, variability, test tree

INTRODUCTION

Knowledge on genetic variability of forest tree species is the base of the sustainable forest management. Researches on the variability and adaptive potential of Bald cypress in our conditions which have been published until now refer to trees that grow individually and in smaller or larger groups (DRAŽIĆ and BATOS, 2002; NINIĆ-TODOROVIĆ and OCOKOLJIĆ, 2001, 2002;

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TUCOVIĆ and OCOKOLJIĆ, 2005; ŠIJAČIĆ-NIKOLIĆ *et al.,* 2010; POPOVIĆ *et al*., 2012a, 2012b). Morphological and physiological characteristics of seed and planting material were studied in various environmental conditions (VANN and MEGONIGAL, 2002; MAZHER *et a*l., 2006; POPOVIĆ *et al*., 2012c, 2012d; POPOVIĆ et al., 2013; POPOVIĆ *et al.,* 2014). The possibility of wider application of Bald cypress as a forest species should be based on an assessment of its genetic and adaptive potential as well as quantity and quality of yield, primarily at the level of the existing Bald cypress seed stand in Serbia.

The genetic diversity is more efficiently assessed after introduction of methods that reveal polymorphism directly from DNA. RAPD markers are extensively used in the studies of genetic diversity of a large number of forest tree species (LEE *et al.,* 2002, NKONGOLO *et al.,* 2002, MONTELONE *et al*., 2006; LUČIĆ *et al.,* 2010; LUČIĆ *et al*., 2011; NOWAKOWSKA, 2004; GÓMEZ *et al*., 2000; GOTO *et al.,* 2001). RAPD markers allow us to get a large amount of data on the genetic variability inter and intra populations without detailed prior knowledge on DNA sequence (HOLSINGER *et al.,* 2002).

The aim of the research in this paper is the assessment of the genetic variability of 20 Bald cypress test trees from the seed stand near Bačka Palanka using RAPD markers.

MATERIALS AND METHODS

Material used for the research originated from the Bald cypress seed stand near Bačka Palanka, registration number S 01.10.01.01, which is managed by FE Novi Sad, FMU Bačka Palanka. On the basis of phenotypic characteristics and abundance of yield in 2010, 20 test trees were selected and from each of them were collected needles used for the analysis. The needles were collected in the morning, from the tree height 4 meters on the west side of the canopy. Every sample was marked, separately packed into plastic bags and transported in refrigerator to the laboratory.

Molecular Analysis

DNA isolation

Genomic DNA was isolated from needle of *Taxodium distichum* using the CTAB procedure according to DOYLE and DOYLE (1987). Bulks were prepared by pooling an equal amount of plant material obtained by grinding four needles per test tree.

RAPD analysis

Thirteen RAPD primers were tested and ten polymorphic primers were selected, while three primers amplified weakly and they were not used for further work (Table 1). The PCR reaction was carried out in 25 μl reaction mixture containing 2.5 mM MgCl2, 100 μM dNTPs, 0.2 μM of 10-mer primers, 2.5 U of Taq polymerase (Fermentas) and 50 ng of template DNA using a thermocycler Biometra TProfessional Standard 96. Amplification reaction was performed by 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 elongation at 72°C for 7 min. The amplified products were separated by electrophoresis on 1.4% agarose gels in 0.5 x TBE buffer. As a marker 1kb ladder DNA was used. Gels were run on horizontal gel system (DNA Sub-Cell Bio Rad) at 40mA for 2h and photographed under UV light after staining with 0.5 μg/μl ethidium bromide.

Table 1. List of primers used in RAPD analysis of 20 Taxodium distichum test tree

Name	Primer sequence (5'-3')
OPB 15	GGAGGGTGTT
OPB 17	AGGGAACGAG
OPB 01	GTTTCGCTCC
OPB 12	CCTTGACGCA
OPB 10	CCATTCCCCA
GEN 1-70-3	ACGGTGCCTG
OPB 13	TTCCCCCGCT
GEN 1-70-9	GGACTCCACG
OPB 07	GGTGACGCAG
GEN 1-80-9	GCACGGTGGG
GEN 1-70-9	TGCAGCACCG
OPB 16	TTTGCCCGGA
OPB 19	ACCCCCGAAG

Statistical analysis

RAPD profiles were scored as presence/absence of fragments in each sample and the data were assembled into a binary matrix. Genetic similarity between test trees was evaluated by DICE (1945). Unweighted Pair Group Method with Arithmetic mean (UPGMA) method was applied for cluster analysis. All marker data analyses were performed using statistical NTSYSpc2 program package (ROHLF, 2000).

It was also performed the correspondence analysis of genetic similarity by Greenacre (GREENACRE, 1988) of analyzed test trees.

RESULTS AND DISCUSSION

The advantages of RAPD technique are simplicity and speed, but it is sensitive to small changes in experimental conditions, which must be strictly controlled in order to obtain reproducible results. The fact that they are in non-coding parts of the genome allows this technique to detect high levels of polymorphism inter and intra species (LUČIĆ *et al.,* 2011).

RAPD analysis was performed on 20 test trees with 13 primers. A total of 10 primers (OPB 15, OPB 17, OPB 01, OPB 12, OPB 10, GEN 1-70-3, GEN 1-70-9/1, GEN 1-70-9/2, OPB 16, GEN 1-80-9) gave a clear picture, while three primers (OPB 13, OPB 07, OPB 19) amplified weakly and they were not used for further work, so the RAPD analysis for the analyzed test trees was tested in two repetitions. The amplification of these 10 primers gave a clear picture of DNA fragments, so the protocol was used in further analyses without any modification. Sixty bands were scored with ten RAPD markers among the 20 genotypes. The mean number of bands was 6 and it was lower (9.6) than in previous study in which were analyzed 12 genotypes with 17 primers (YUNPENG *et al.,* 2002).

The primer GEN 1-70-3 gave the minimum number of fragments (two), while the highest number of fragments (12) was amplified with primer GEN 1-80-9.

Genetic similarity between test trees determined using Dice's coefficient ranged from 0.73 (11-14) to 1 (2-12, 2-18, 12-18, 12-15, 15-18), while mean value was 0.87 (Table 2).

Table 2. Similarity among test trees using Dice's coefficient

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	1																			
2	0.83	1																		
3	0.80	0.88	1																	
4	0.92	0.92	0.88	1																
5	0.85	0.85	0.89	0.85	1															
6	0.83	0.92	0.80	0.83	0.77	1														
7	0.83	0.92	0.88	0.92	0.85	0.83	1													
8	0.77	0.92	0.89	0.85	0.86	0.92	0.85	1												
9	0.85	0.85	0.89	0.85	0.86	0.92	0.85	0.93	1											
10	0.88	0.88	0.77	0.88	0.81	0.88	0.80	0.89	0.81	1										
11	0.91	0.82	0.78	0.91	0.75	0.82	0.91	0.75	0.83	0.78	1									
12	0.83	1	0.88	0.92	0.85	0.92	0.92	0.92	0.85	0.88	0.82	1								
13	0.92	0.92	0.88	0.92	0.85	0.92	0.92	0.85	0.92	0.80	0.91	0.92	1							
14	0.75	0.83	0.88	0.83	0.77	0.83	0.75	0.92	0.85	0.88	0.73	0.83	0.75	1						
15	0.83	1	0.88	0.92	0.85	0.92	0.92	0.92	0.85	0.88	0.82	1	0.92	0.83	1					
16	0.80	0.88	0.85	0.88	0.81	0.88	0.80	0.96	0.89	0.92	0.78	0.88	0.80	0.96	0.88	1				
17	0.85	0.92	0.89	0.85	0.93	0.85	0.85	0.93	0.86	0.89	0.75	0.92	0.85	0.85	0.92	0.89	1			
18	0.83	1	0.88	0.92	0.85	0.92	0.92	0.92	0.85	0.88	0.82	1	0.92	0.83	1	0.88	0.92	1		
19	0.83	0.92	0.88	0.92	0.92	0.83	0.92	0.85	0.85	0.80	0.82	0.92	0.92	0.75	0.92	0.80	0.85	0.92	1	
20	0.80	0.96	0.85	0.88	0.82	0.96	0.88	0.96	0.89	0.92	0.78	0.96	0.88	0.88	0.96	0.92	0.89	0.96	0.88	1

For easier overview of level of genetic similarity and distance between observed test trees based on RAPD data the cluster analysis was performed and the results are presented as the dendrogram (Figure 1).

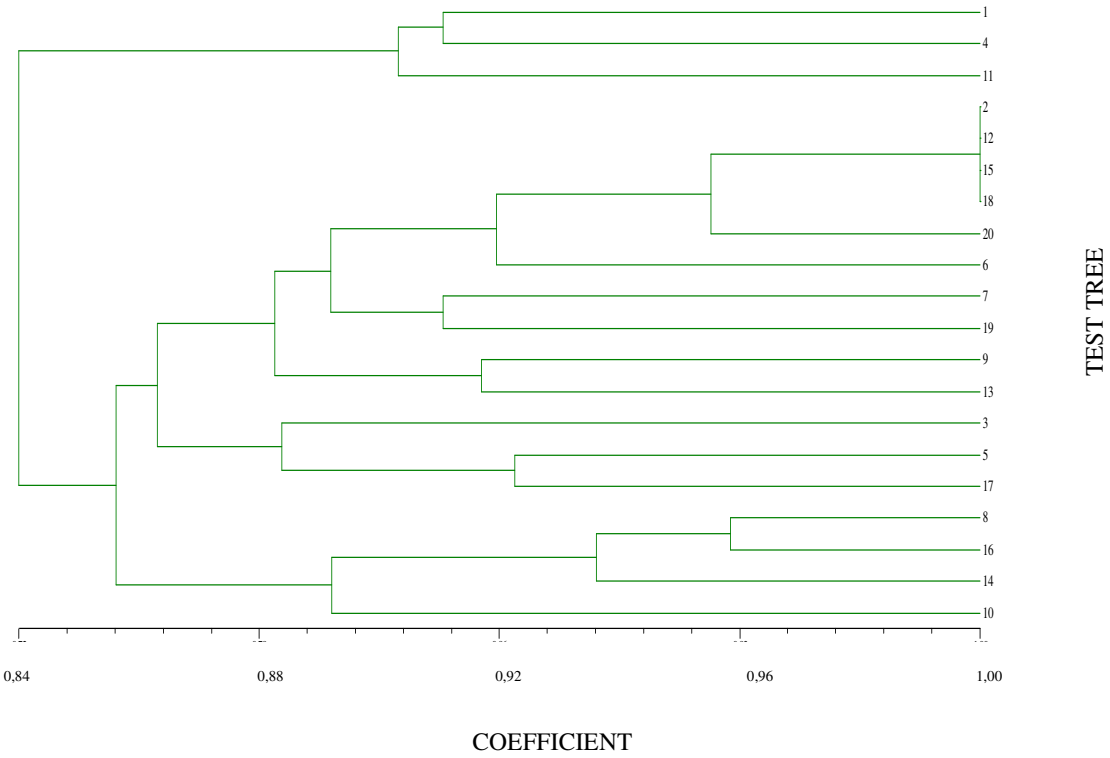

Figure 1. Dendrogram of test trees cluster analysis using Dice's coefficient

All 20 test trees were grouped into two sub-clusters. Test trees 1, 4 and 11 were grouped in the first sub-cluster while other test trees were grouped in the second sub-cluster. The greatest similarity is between test trees 2, 12, 15 and 18. Test trees 1, 4 and 11 are at a greater genetic distance compared to other test trees.

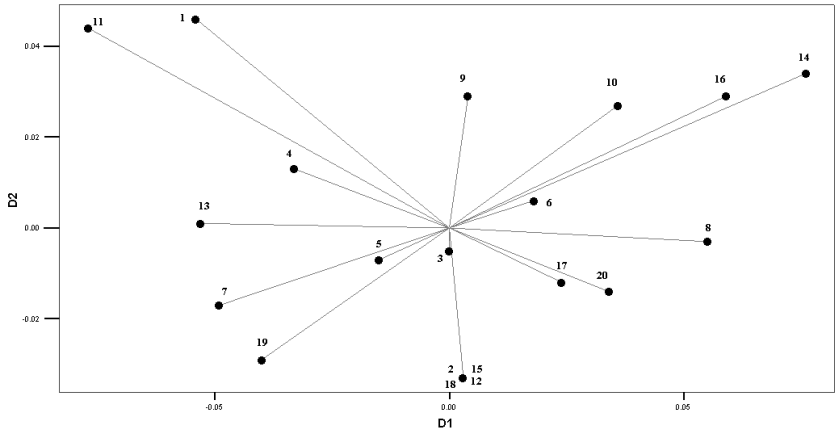

Figure .2. Correspondence analysis of genetic similarities of test trees using Dice's coefficient in two dimensions

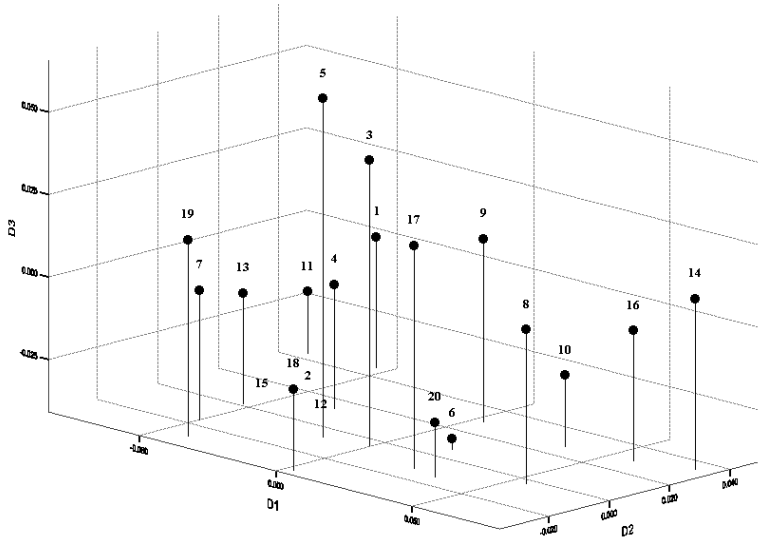

Figure 3. Correspondence analysis of genetic similarities of test trees using Dice's coefficient in three dimensions

The results of the analyses performed in statistical NTSYSpc2 program package can be used to illustrate grouping of test trees depending on the level of genetic similarity and distance

while for more detailed illustration of relation of test trees can be used the results of the correspondence analysis. Based on matrix of genetic similarities obtained using Dice's coefficients the graphs of correspondence analysis in two and three dimensions were made (Figure 2 and 3).

The comparative analysis of graphs which present the correspondence analysis of genetic similarities of analyzed test trees shows that test trees have been grouped in a similar manner. Test trees 2, 12, 15 and 18 are, as in the previous analyses, genetically the most similar.

The results of genetic similarities and distances between analyzed test trees using UPGMA cluster method and the correspondence analysis that have been shown on dendrogram and graphs indicate overwhelming presence of genetic diversity among observed test trees.

The analyses of genetic distance between genotypes using results of the correspondence analysis presented in two and three dimensional graphs give important information on relations between studied genotypes. The correspondence analysis is more informative and more accurate than the dendrograms especially where there is a serious genetic exchange between geographically similar genotypes. In his researches conducted on Scots pine LUČIĆ (2012) favors the use of three dimensional graphs in the case of a small number of studied genotypes because they give clearer view of genetic diversity. In the case of multiple genotypes, as it is the case in this paper, the graphs in three dimensions are unclear, so the graphs in two dimensions can be recommended as more informative and easier to understand. Based on previous researches LUČIĆ *et al*. (2011) and this paper it can be recommended the use of three dimensional graphs of correspondence analysis when the number of analyzed genotypes do not exceed 10. In case when the number of genotypes exceeds 10 it is necessary to use two-dimensional graphs.

Researches of authors BABIĆ *et a*l. (2009) and LUČIĆ *et al*. (2011) as well as the results obtained in the analysis of intra-population genetic variability of Bald cypress using RAPD markers indicate the need that in the future researches of inter and intra-line variability have to be used the both methods of statistical analysis, UPGMA cluster and correspondence analysis when graphically present genetic distances.

CONCLUSIONS

The results of the analysis of genetic characterization of test trees using RAPD markers have shown the overwhelming genetic variability which is caused by high degree of heterozygosity. The genetic similarity among test trees calculated using Dice's coefficient ranged from 0.73 (11 - 14) to 1 (2 - 12, 2 - 18, 12 - 18, 12 – 15, 15 - 18), while the mean value was 0.87. The results of genetic similarities and distances using UPGMA cluster method and correspondence analysis indicate overwhelming presence of genetic diversity among observed test trees. The analysis of graphs leads to conclusion that the test trees have been grouped in a similar manner. Test trees 2, 12, 15 and 18 are genetically the most similar.

A satisfactory level of genetic variability among observed test trees is a good starting point for further breeding of species and directed utilization of the available gene pool.

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ANALIZA UNUTARPOPULACIONOG VARIJABILITETA TAKSODIJUMA (*Taxodium distichum* L. Rich.) U SEMENSKOJ SASTOJINI KOD BAČKE PALANKE UPOTREBOM RAPD MARKERA

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Izvod

U radu su prikazani rezultati ispitivanja unutarpopulacione varijabilnosti taksodijuma u semenskoj sastojini kod bačke palanke upotrebom RAPD markera. RAPD analiza je urađena na 20 test stabala sa 13 prajmera. Koeficijenti genetičke sličnosti izračunati po Dice-u, a na osnovu njih su konstruisani grafikoni klaster i korespodencione analize.

Dobijeni rezultati analize genetičke karakterizacije test stabala primenom RAPD markera pokazuju izraženu genetičku varijabilnost, koja je uslovljena visokim stepenom heterozigotnosti. Genetska sličnost između test stabla izračunata po Dice-u kretala se od 0,73 (11 - 14) do 1 (2 - 12, 2 - 18, 12 - 18, 12 – 15, 15 - 18), dok je srednja vrednost bila 0,87. Analizom grafikona klaster i korespodencione analize može se zaključiti da se test stabla grupišu na sličan način. Test stabla broj 2, 12, 15 i 18 su genetički najsličnija.

Zadovoljavajući stepen genetičke varijabilnosti između proučavanih test stabala predstavlja dobru polaznu osnovu za dalji proces oplemenjivanja vrste i usmereno korišćenje raspoloživog genofonda.

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