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FIRST RESULTS OF RAPD-ANALYSES OF INTRASPECIFIC POLYMORPHISM OF DOUGLAS-FIR CULTIVATED IN BULGARIA AND BELARUS

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Abstract

The aim of the research was to elucidate existing intraspecific polymorphism of Douglas-fir plantations in Bulgaria and Belarus using Random Amplified Polymorphic DNA (RAPD) markers and to estimate genetic similarities and distances. RAPD-primers that reveal high polymorphism were selected. Seven Douglas-fir populations from Bulgaria and one from the Central Botanical Gardens in Belarus were included in the study, and individual RAPD patterns for 8 primers were obtained. The obtained RAPD-patterns were used to calculate the genetic distances among the populations studied.

Key words: *Pseudotsuga menziesii* (Mirb.) Franco, genetic distance.

Introduction

High volume productivity and relative resistance to diseases, cold and drought of Douglas-fir made it one of the most broadly used exotic coniferous species in Europe. The introduction of Douglas-fir in Bulgaria for afforestation started at the beginning of the last century and nowadays the plantations occupy 6,714 hectares. In Belarus, the species was not widely used but also showed good growth and resistance to frost and diseases. The origin of almost all of the established plantations is unknown in both countries, except that of provenance tests in Bulgaria.

The aim of the present study was to examine existing intraspecific polymorphism of Douglas-fir plantations in Bulgaria and Belarus using Random Amplified Polymorphic DNA markers and to estimate genetic similarities and distances.

Materials and Methods

The plantations, from which samples have been collected, can be grouped as follows:

1. Provenance experimental plantations in Bulgaria established in the forest services of Kostenets and Kyustendil with known origin (Table 1 and Figure 1). The plantations are 20 year old and have been established with seedlings produced from seeds supplied by the National tree seed laboratory of the USA.

2. Plantations and groups of trees in Bulgaria and Belarus showing stable growth and health status without information about their origin (Table 1).

Total genomic DNA was extracted from Douglas-fir needles in liquid nitrogen using standard CTAB protocol (Dreiper et al. 1991). DNA concentration and purity was estimated using the Agilent 8453 spectrophotometer.

Polymerase chain reaction (PCR) mixture to amplify RAPD fragments (25 μl) contained 0.2 мМ dNTP (Fermentas); 1× *Taq*-polymerase buffer (Fermentas); 3.5 мМ $MgCl_2$ (Fermentas); 10 pmol primer; 1.5 U Taq- polymerase (Fermentas); 50 ng DNA. PCR was carried out in Mastercycler personal thermocycler (Eppendorf), using an initial "hot start" of 94°C for 4 min, followed by 44 cycles of 1 min at 94°C, 1 min at 38–40°C, and 2 min at 72°C, and terminated with a final extension at 72°C for 8 min.

Amplified fragments were resolved on 2% agarose gel in 1× TAE (tris-acetic acid-EDTA) buffer with ethidium bromide solution (final concentration – 0.5 $\mu g.ml^{-1}$) (Maniatis et al. 1982).

Table 1. The origin of *Pseudotsuga menziesii* (Mirb.) Franco.

Provenance number or name	Seed zone	Origin (region and state in the USA)	Latitude	Longitude	Elevation, m	Location
5	403	Newhalem, Washington	48°5′	121°5′	500	Bulgaria, State forest enterprise Kostenets
6	403	Darrington, Washington	48°0′	121°5′	1167	Bulgaria, State hunting enterprise Kyustendi
19	661	Parkdale, Oregon	45°5′	121°5′	900	Bulgaria, State forest enterprise Kostenets
23 a	482	Bates, Oregon	45°0′	118°5′	1333	Bulgaria, State forest enterprise Kostenets
23 b	482	Bates, Oregon	45°0′	118°5′	1333	Bulgaria, State forest enterprise Kyustendil
Minsk		Unknown	–	–	–	Minsk Belarus, CBG of NAS of Belarus
White water		Unknown	–	–	1400	Bulgaria, State hunting enterprise Alabak
Shipka		Unknown	–	–	800	Bulgaria, State forest enterprise Kazanlak

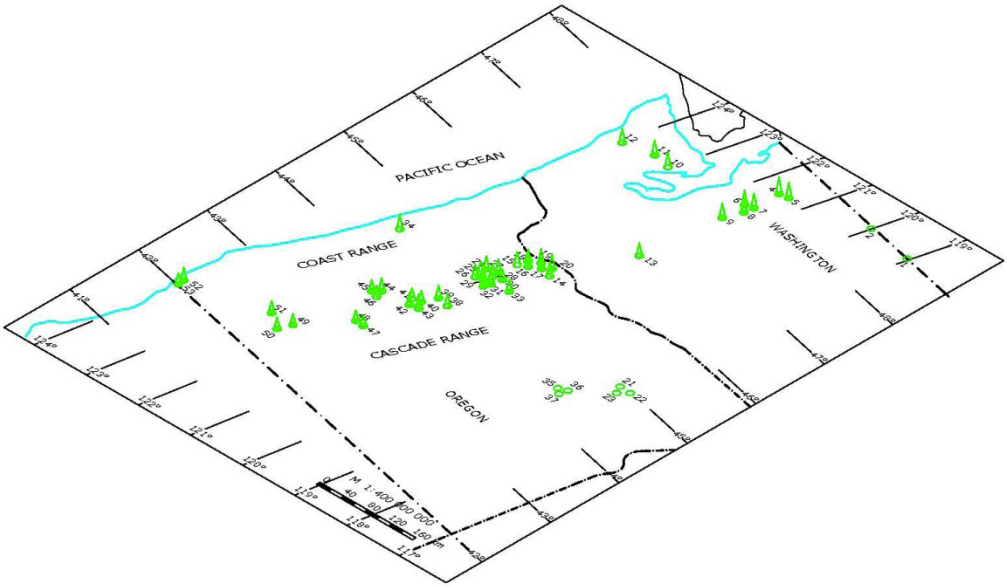

Fig. 1. Geographic location of Douglas-fir seed parents in Oregon and Washington (USA) used for provenance tests in Bulgaria.

Gels were visualized in transilluminator (Hoefer) and photographed. DNA banding patterns sizes were scored using the Phoretics™ 1D program (version 2003.02, Nonlinear Dynamics Ltd). Only distinct, discrete bands were used for analysis. A 100 kb DNA ladder (Fermentas) was used as molecular weight standard.

All analyses were repeated at least three times. Multilocus genetic profiles were composed for each tree using binary scores (1 – presence, and 0 – absence of a RAPD fragment, respectively). The MS Excel program was used to estimate parameters of diversity and differentiation between different samples.

The ability of the most informative primers to distinguish Douglas-fir samples was assessed by calculating their resolving power (**Rp**) according to Gilbert et al. (1999):

$$\mathbf{Rp} = \mathbf{\Sigma I_b},$$

where $\mathbf{I_b} = 1 - (2 \times |0.5 - p|)$, **p** – proportion of trees containing the certain fragment (Gilbert et al. 1999).

The genetic similarity between different samples (S) were estimated using the PHYLIP v. 3.63 program and formula by Nei and Li (1979):

$$\mathbf{S} = \mathbf{2a\,(2a+b+c)^{-1}},$$

where **a** – number of fragments amplified (shared) in both individual trees or samples 1 and 2; **b** – number of fragments amplified in tree or sample 1 but not in 2; **c** - number of fragments amplified in tree or sample 2 but not in 1 (Debnath 2007, Nei and Li 1979). From genetic similarity coefficients (S) the genetic distances matrixes were obtained. The similarity matrix was used as input data for cluster analy-

sis using unweighted pair-group method with arithmetic averages (UPGMA), and the resulted dendrograms were drawn. To evaluate the robustness of the groupings formed, the binary data set was subjected to bootstrap using the TREECON software program.

Results and Discussion

RAPD-primers that reveal higher level polymorphism were selected. We tested 10 different primers developed by University of British Columbia (Canada) and successfully used for Douglas-fir population polymorphism analyses (Aagaard et al. 1998a, 1998b). From 10 primers tested, eight (UBC-234, -328, -336, -460, -570, -504, -330, -428) amplified discrete RAPD products and were used for further work.

The selected primers' characteristics are presented in Table 2. Seven Douglas-fir populations from Bulgaria and one from Belarus were studied, and individual RAPD patterns were obtained for 8 primers.

The amplicon sizes were in the range of 320-2000 bp. Amplicon number varied from 11 to 4 depending on a primer. The maximum number of polymorphic bands (8) was generated by the UBC336 primer, and minimum (3) – by the UBC570 and UBC428 primers, the average number of polymorphic loci per primer was five.

The ability of 8 most informative primers to distinguish between Douglas-fir genotypes was assessed by calculating Resolving power (**Rp**) parameter for each primer according to Gilbert et al. (1999). The **Rp** value was varied from 1.3 (for the UBC428 primer) to 5.0 (for the UBC330 and UBC336 primers) (Table 2).

We revealed 58 amplicons: 40 polymorphic (69%) and 18 monomorphic (31%), identified in all Douglas-fir gen-

Table 2. RAPD-primer names and sequences PCR amplified in Douglas-fir samples, the number of polymorphic, monomorphic and unique fragments per primer, the fragment (amplicon) size in base pairs (bp) and Resolving power (Rp) for all primers.

Primer	Sequence (5′-3′)	Total fragments	Monomorphic fragments	Polymorphic fragments	Unique fragments	Fragment size range (bp)	Resolving power (Rp)
UBC 234	TCCACGGACG	8	3	5	0	400–2000	3.5
UBC 328	ATGGCCTTAC	7	3	4	1	400–1200	2.3
UBC 336	GCCACGGAGA	10	2	8	1	320–900	5.0
UBC 460	ACTGACCGGC	11	6	5	0	350–1200	3.5
UBC 570	GGCCGCTAAT	5	2	3	0	420–900	1.5
UBC 504	ACCGTGCGTC	5	0	5	0	450–1200	3.0
UBC 330	GGTGGTTTCC	8	1	7	1	480–900	5.0
UBC 428	GGCTGCGGTA	4	1	3	0	900–1300	1.3

otype profiles (patterns) that can considered as species specific markers for *Pseudotsuga menziesii*. Some primers amplified unique sample specific fragments: 450 bp fragment in "Shipka" (amplified by the UBC328 primer), 600 bp (UBC336) and 610 bp (UBC330) in "Bates No 23b". This unique amplicons can be used for individual Douglas-fir genotypes identification. Douglas-fir total DNA amplification results with UBC570 and UBC504 primers are presented by Figure 2.

The binary (presence-absence) matrix of was inferred from the RAPD-pattern assuming that amplicons of the same size represent the same RAPD loci. Genetic patterns were compared between individual trees and samples. The genetic similarity and distance coefficients were calculated following the formula suggested by Nei and Li (1979) (Table 3).

The maximum genetic distance of 0.1895 was between Bates No 23a and No 19 samples (Bulgaria). The minimal genetic distance of 0.0310 was between Parkdale No 19 and Newhalem No 5 samples based on 58 RAPD loci. Genetic distances between the Central Botanical Gardens of NAS of Belarus populations and Bulgarian samples were within the 0.0855–0.126 range (Table 3).

Dendrograms representing phylogenetic relationships between Douglas-fir samples were generated using the UPGMA cluster analysis based on the similarity matrices and the Treecon program (Fig. 3). Darrington No 6, White water, Parkdale No 19 and Newhalem No 5 formed one cluster (A) in the dendrogram. Shipka and CBG of NAS of Belarus samples were placed next to this cluster. Bates No 23b and Bates No 23a samples formed a separate group in this dendrogram (cluster B). Bates 23 samples representing provenance from Oregon had shown low indexes of growth capacity and health status in previous assessments (Popov 1996, Georgieva 2009). Big differences were found between Bates 23 and the provenances from Cascade Range and coast range in terms of growth and disease resistance.

Numbers at the nodes are bootstrap

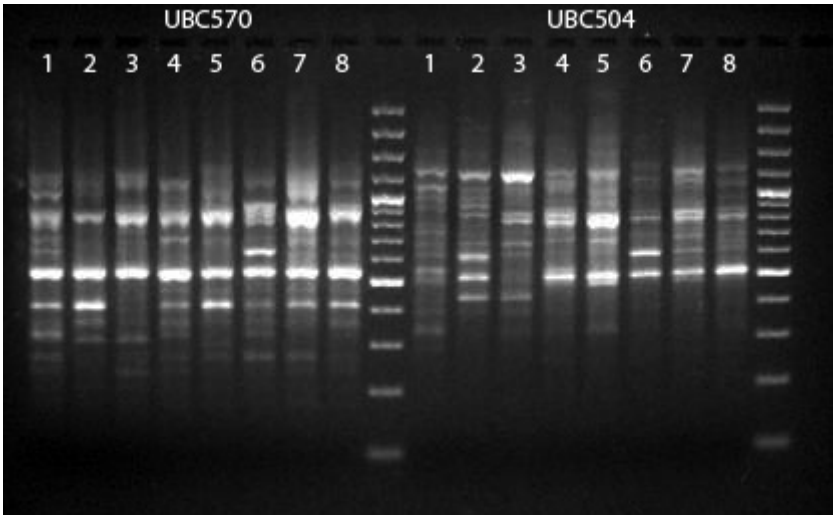

Fig. 2. RAPD pattern of Douglas-fir genotypes amplified using the UBC570 and the UBC504 primers.

Table 3. Matrix of the genetic distance coefficients (Nei and Li 1979) for eight Douglas-fir samples based on the RAPD genotypes.

	1	2	3	4	5	6	7	8
1	**0.0000**							
2	0.1296	**0.0000**						
3	0.1142	0.0685	**0.0000**					
4	0.1194	0.1705	0.1895	**0.0000**				
5	0.0946	0.1446	0.1789	0.0310	**0.0000**			
6	0.1102	0.1444	0.1446	0.0932	0.0833	**0.0000**		
7	0.0855	0.1442	0.1161	0.0598	0.0733	0.0644	**0.0000**	
8	0.1050	0.1395	0.1395	0.0520	0.0537	0.0568	0.0489	**0.0000**

Samples: **1** – Central Botanical Gardens of NAS of Belarus; **2** – Bates No 23b; **3** – Bates No 23a; **4** – Parkdale No 19; **5** – Newhalem No 5; **6** – Shipka (40 years); **7** – Darrington No 6; **8** – White water.

values (%) obtained from 1000 replicate analyses.

In conclusion, RAPD- analysis on 8 primers and 58 loci allowed to differentiate Douglas-fir in Bulgaria from Belarus samples. Primers selected in this study can be used in further studies of Douglas-fir polymorphism.

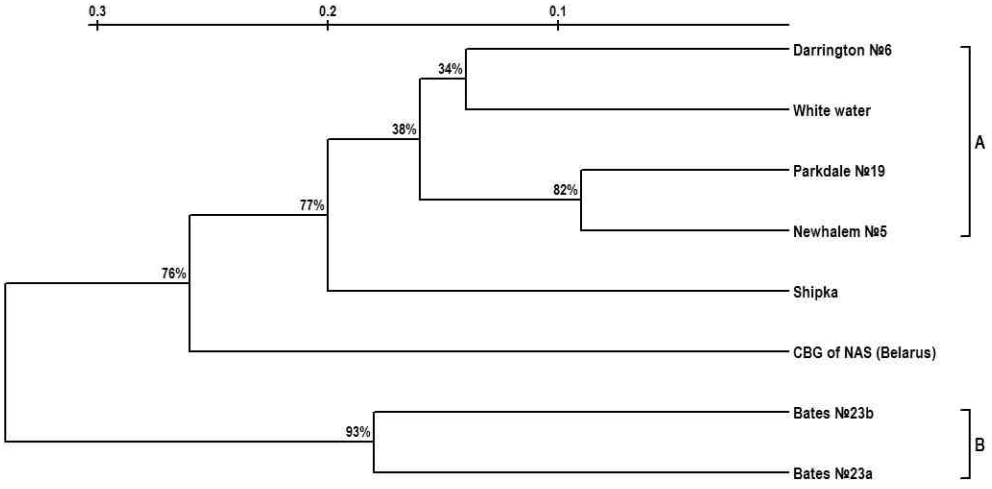

Fig. 3. Unweighted pair-group method with arithmetic averages (UPGMA) dendrogram based on similarity matrix between Douglas-fir samples.

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