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IDENTIFICATION OF VARIETIES AND GENETIC DIVERSITY OF DOUGLAS-FIR STANDS IN THE REGION OF OSOGOVO, SOUTH WEST BULGARIA

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Abstract

Douglas-fir is one of the most frequently planted non-native coniferous tree species in the recent past in Bulgaria. Based on phenotypic and morphological characteristics, the Douglas-fir plantations in Bulgaria are attributed to the coastal variety (*Pseudotsuga menziesii* var. *menziesii*). Genetic studies on variety identification are missing. The aim of the present study was to assess the variety of four Douglas-fir stands in the region of Osogovo based on genetic markers (isozymes and nuclear microsatellites) and to determine their genetic diversity and differentiation. Both markers types clearly indicated that the investigated stands were of coastal variety. Genetic diversity values were similar or even higher compared to coastal Douglas-fir natural stands, indicating that the genetic variation in the artificial stands in Bulgaria is not significantly reduced. The high level of genetic diversity is a prerequisite for good adaptation and vitality of the studied plantations.

Key words: coastal variety, isozymes, nuclear microsatellites, *Pseudotsuga menziesii*.

Introduction

Douglas-fir is a coniferous tree species native to western North America – from British Columbia in the North, through California and the southern parts of Northern Mexico to the south. The British botanist Murray was the first that recognized in 1869 two geographically distinct groups of Douglas-fir, known as the coastal variety (*Pseudotsuga menziesii* var. *menziesii*) and the interior variety (*Pseudotsuga menziesii* var. *glauca* Beissn. (Franco) (Little 1979). In Bulgaria the coastal Douglas-fir is known as 'green', the interior – as 'blue' Douglas-fir. Literature particularly from Europe (e.g. Göhre 1958, Klumpp 1999, Fitschen 1987, Vidakovic 1991) sometimes mentions another interior variety *Pseudotsuga menziesii* var. *caesia* (Schwerin) Franco situated in the transition zone between coastal and interior variety especially in British Columbia. The coastal variety occurs from central British Columbia southward primarily along the Pacific Coast for about 2200 km, while the continental interior variety – along the Rocky Mountains into the mountains of southern Mexico over a distance of more than 4500 km (Eckenwalder 2009, Farjon 2010, Müller et al. 2012, Lavender and Hermann 2014). Both varieties, coastal

and interior, differ morphologically and ecologically. Var. *menziesii* grows faster and is considerably larger in size than var. *glauca*, which is more shade tolerant, more cold-hardy, and more drought resistant (Eckenwalder 2009). Interior varieties (*glauca* and *caesia*) have high susceptibility to fungal diseases caused by the ascomycete fungi *Phaeocryptopus gaeumannii* (Rohde) Petrak and *Rhabdocline pseudotsugae* (Syd.) (Rohde) Petrak (Hood et al. 1990, Stimm and Dong 2001, Lavender and Hermann 2014).

Douglas-fir is one of the most frequently used non-native coniferous species in the recent past in Bulgaria. It was introduced in Bulgaria about a century ago, but it became widespread only in the late 1950s and during the 1960s (Petkova 2011). The French forester Félix Vogeli established in Bulgaria the first Douglas-fir plantation in 1906 with seeds of unknown origin at 700 m elevation in the Stara Planina mountain range.

At the same time single trees and groups of 'blue' Douglas-fir were introduced near Koprivshtitsa (Central Bulgaria), in the park Borisova Gradina (Sofia) and near Dolni Lozen (Sofia) (Ganchev and Prokopiev 1959, Vakarelov 1970). Vakarelov (1970) studied this variety mainly for the Sofia region (Borisova garden and Dolni Lozen) in relation to its use in parks and gardens. He recommended using it on the northern slopes in the oak altitudinal zone, on slanting seats with fresh and light soil. The same author (Vakarelov 1970) reported high level of phytoncide activity, with values from 12.5 to 93.7 formaldehyde units which prevents the growth of attacking organism or surrounding plant for 'blue' Douglas-fir, and therefore recommended to be used for landscaping on the grounds around hospitals.

Interior variety was introduced in Bulgaria also in provenance tests (Petkova et al. 2014, Popov 2014, Petkova et al. 2015). Damages caused by the ascomycete fungi *Phaeocryptopus gaeumannii* and *Rhabdocline pseudotsugae* were recorded in parts of these test plots (Petkova et al. 2014, Popov 2014).

The main part of the established Douglas-fir plantations in Bulgaria belongs to the coastal variety (*Pseudotsuga menziesii* var. *menziesii*), identified by phenotypic and morphological characters. Genetic similarities and differences between some provenances in provenance test and some of the older Douglas-fir plantations were determined using RAPD analyses of DNA (Chyzhyk et al. 2011), but detailed genetic studies for variety identification have not yet been performed in Bulgaria.

A clear differentiation of the coastal and interior varieties is possible with isozymes, nuclear, mitochondrial, and chloroplast DNA gene markers (e.g. Neal and Adamas 1985, Li and Adams 1989; Aagaard et al. 1995, Aagaard 1997, Aagaard et al. 1998, Klumpp 1999).

Isozymes (Lewontin and Hubby 1966) have been used in forestry to study the genetic variation within and between populations, population structure, phylogeny and mating systems in natural as well as artificial populations of different species (e.g. Mitton 1983, Hamrick and Godt 1989, El-Kassaby and Ritland 1996, Yeh 1979, Namkoong and Koshy 2001). The first range-wide investigation of the genetic structure of Douglas-fir was done by Li and Adams (1989) based on isozymes. Differentiation of coastal (var. *menziesii*) and interior (var. *glauca*) varieties of Douglas-fir was based mainly on the isozyme gene loci 6PGDH-A and PGM-A (Yeh and O'Malley 1980; El-Kassaby et al. 1982a, 1982b; Merkle and Ad-

ams 1987; Merkle et al. 1987; Klumpp 1999; Fussi et al. 2013). In Europe this method was applied to check similarities between Douglas-fir plantations and natural stands in North America (Stauffer and Adams 1993, Fontes et al. 2003), but also for variety identification of planted stands (Fussi et al. 2013). The development of nuclear microsatellite markers for Douglas-fir (Slavov et al. 2004, Krutovsky et al. 2009) offered new possibilities for a more precise classification of varieties (Fussi et al. 2013). Two of the microsatellite markers – PmOSU_3B2, PmOSU_4A7 are most efficient in distinguishing between the two varieties. If the allele 96 at locus PmOSU_3B2 and the allele 242 at locus PmOSU_4A7 have high frequencies, the variety is attributed to interior Douglas-fir, whereas in coastal Douglas-fir the frequencies of these two alleles are very low or equal to zero (allele is absent) (Fussi et al. 2013, van Loo et al. 2015). Thus, the combination of isoenzymes and nuclear microsatellites offers a more precise way for identification of variety.

Beside variety identification, the assessment of the genetic diversity within and among populations is an important issue especially for the artificially established Douglas-fir stands outside of the natural range of the species. Genetic diversity is the basis of adaptation. Thus maintenance of genetic diversity is important in tree breeding and improvement program to develop well-adapted tree populations and strengthen their useful traits and to ensure vitality of forests to resist to diverse biotic and abiotic stressors under changing and unpredictable environmental conditions (Porth and El-Kassaby 2014).

The aim of the present study was to identify the variety of planted Douglas-fir populations from Bulgaria based on isoenzymes and nuclear microsatellite markers and to assess their genetic diversity and differentiation.

Material and Methods

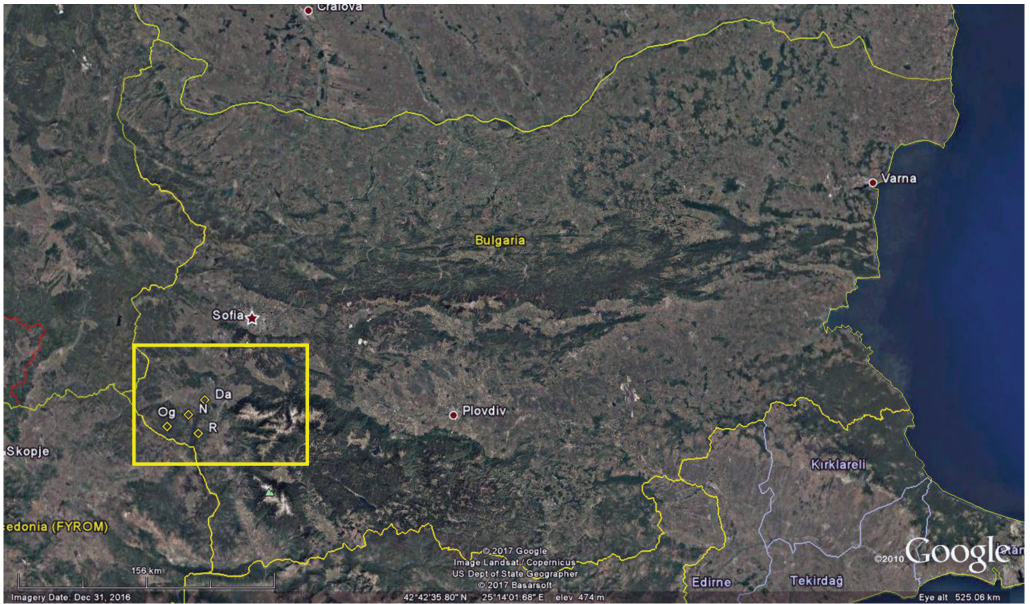
Plant material

Four plantations of Douglas-fir from the Osogovo subregion (Southwestern Bulgaria), were studied. The plantations are situated in the Forest Enterprises Rila monastery (R), Nevestino (N), Dupnitsa (Da) and Osogovo (Og) (Fig. 1). The four studied stands were planted in the middle of the 20th century with seeds of unknown origin. In total, samples from 192 individuals (Douglas-fir branches) were collected at the beginning of March 2016 (Table 1). In the stand R, the natural regeneration was also included in the study. Altogether 170 adults (stands R, N, Da, Og) and 22 young trees from natural regeneration (stand R) were analyzed (Table 1).

Laboratory analyses

The number of analyzed individuals per population is given in Table 1 for both isoenzymes and nuclear microsatellites. Isoenzyme analysis was performed using starch gel electrophoresis following the procedures described by Konnert (2004). The following 11 isoenzyme loci, representing 7 enzyme systems, were used (see Table 2): GOT-A, GOT-B, GOT-C, FEST-B, IDH-A, MDH-A, MDH-B, MDH-C, PGI-B, PGM-A, 6PGDH-A. The distinction of varieties was based on allele frequencies at the loci 6PGDH-A and PGM-A.

For nuclear microsatellite analysis DNA was extracted from all 192 samples, using the CTAB method (Wagner et al.



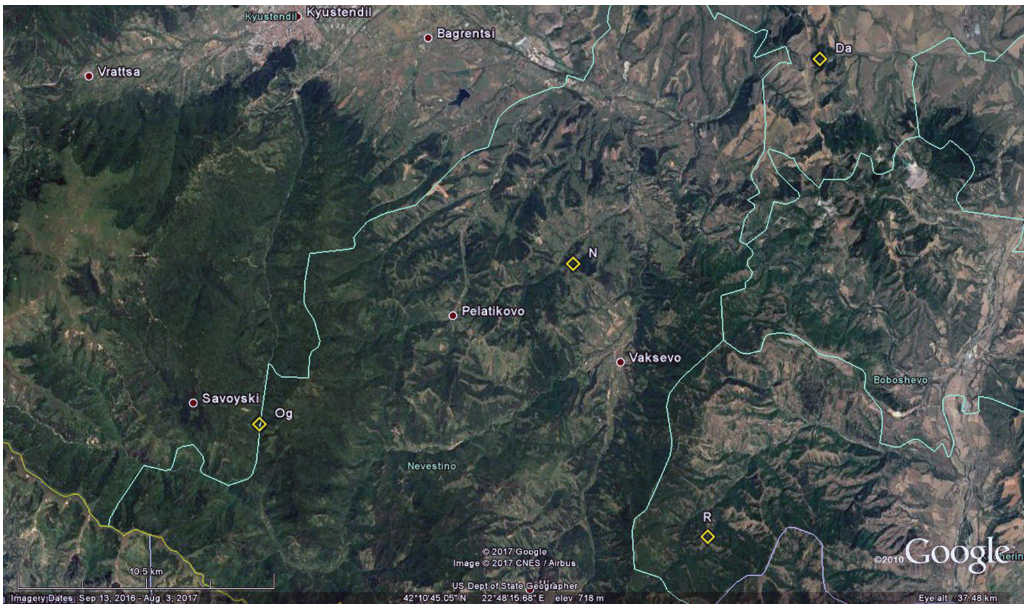


Fig. 1. Location of the studied Douglas-fir stands.

Table 1. Description of plant material for the investigated Douglas-fir stands in Southwestern Bulgaria.

Population abbr.	Location	Latitude	Longitude	Altitude, m, a.s.l	Age, years	Exposure	Soil		Collective	Material	No of indiv.
							depth	moisture			
R	Rila monastery	42°5'34.29" N	22°54'4.95" E	900	35	East	Moderately deep	Mesic	Adult trees Natural regeneration	Needles/buds Needles/buds	20 22
N	Nevestino	42°11'39.84" N	22°50'01.68" E	650	40	South - East	Moderately deep	Mesic	Adult tree	Needles/buds	50
Da	Dupnica	42°16'15.73" N	22°57'29.9" E	600	35	North - East	Moderately deep	Mesic	Adult tree	Needles/buds	50
Og	Osogovo	42°8'5.85" N	22°40'40.98" E	1300	40	East	Moderately deep	Mesic	Adult tree	Needles/buds	50
Total											192

1987). Ten to twelve needles of each individual were cut in half and put in two DNA-pellets. The samples were further dissolved in 10 µl 1xTE Buffer and the pellets were stored overnight in a fridge. DNA extracts were quantified (Gene Quant Pro, Amersham Bioscience) and adjusted to 20 ng·µl^{-1}. All 192 individuals were genotyped at nine highly polymorphic nuclear microsatellite loci, namely: PmOSU_3F1, PmOSU_2G12, PmOSU_3B2, PmOSU_3G9, PmOSU_4A7, PmOSU_1F9, PmOSU_2D4, PmOSU_1C3, PmOSU_2D6 (Slavov et al. 2004, Krutovsky et al. 2009).

A Multiplex PCR with two versions of PCR–program was applied – 'Kombi A' and 'Kombi B'. Multiplex PCR reaction was performed using fluorescent labelled primers in a mixture of 10 µl total volume containing 1 X reaction buffer (Qiagen), 0.2 µM of each primer and about 20 ng template DNA. The program for amplification was optimized using gradient PCR-conditions. The final PCR program started with initial denaturation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 sec, 54 °C for 1.30 min for 'Kombi A' and 60 °C for 1.30 min for 'Kombi B', 72 °C for 30 sec and a final elongation step at 60 °C for 30 min. The length of the PCR fragments was determined by using an automated sequencer (CEQ 8000 Beckman Coulter) and analyzed by using an internal size standard. Fragment length determination and allele assignment were carried out using the fragment analysis tool of CEQ 8000 (Beckman Coulter).

Data analysis

For isoenzymes SAS 9.4 Software (SAS/STAT Software) and for nuclear microsatellites GenAlEx 6.41 (Peakall and Smouse 2006) were used to calculate

relative allele frequencies per each population, the mean number of alleles (N_A), gene diversity (N_E), effective number of alleles (Brown and Weir 1983), observed heterozygosity (*H*o, Hartl and Clark 1997), expected heterozygosity (H_E, Hartl and Clark 1997) and inbreeding coefficient (F_{IS}, Hartl and Clark 1997). By using the FSTAT software (Goudet 1995), inbreeding coefficients (F_{IS}) for each locus and population was calculated. For nuclear microsatellites possible scoring errors and occurrence of null (non-amplifiable) alleles were checked by using the software Microchecker (Van Oosterhout et al. 2004). Based on this software, scoring errors, like large allele drop-off (poor amplification of large-sized alleles) and error due to stuttering, both leading to homozygote excess, were proved. In order to detect deviations from Hardy-Weinberg-equilibrium due to null alleles, the software conducts simulations within each population. The adjusted frequencies accounting for null alleles were then used for further statistical analysis. The pairwise genetic distance, a measure of differentiation between populations, was calculated for isozyme data according to Gregorius (1974), with the SAS 9.4 Software. For nuclear microsatellites the software GenAlEx 6.41 (Peakall and Smouse 2006) was used to estimate genetic distance between populations following Nei (1972).

Results and Discussion

Variety identification

Allele frequencies for the four Douglas-fir populations and the 11 investigated isoenzyme gene loci are listed in Table 2. No variation was detected at the loci FEST-B and GOT-A. All other gene loci were polymorphic. Differences between populations in the allele frequencies were low. An exception was the allele GOT-C_1, with 6.2 % in the populations Da and N. In the other two populations – Og and R – the frequency of GOT-C_1 was substantially higher with 14.6 % and 14.5 %, respectively. A similar situation was observed at the locus 6-PGDH. The frequencies of allele 6-PGDH-A_6 was in two populations – Da and N – around 6.0 %, while in the other two populations it was significantly lower: for Og – 1.2 %, for R – 1.1 %. (see Table 2).

Allele frequencies for all nine investigated nuclear microsatellite loci are available from authors upon request. All observed gene loci were polymorphic in at least one of the studied populations. Similar to the results obtained with isozymes, differences in allele frequencies between populations were low with a few exceptions: thus, for example, the allele 215 at the gene locus PmOSU_3F1 ranged between 4.9 % in population R and 16.3 % in population Og. The frequency of allele 231 at the same locus varied between 3.1 % in population Og and 12.2 % in populations R. There was also a difference in allele frequencies at the gene locus PmOSU_2G12 with the allele 274 varying between 1.0 % for the Og stand and 14 % for the Da stand.

Here we will focus only on the microsatellite loci PmOSU_3B2 and PmOSU_4A7, which together with the two isozyme 6-PGDHA and PGM-A loci were reported to be the most efficient for variety identification (Fussi et al. 2013). For these four loci the following variety-specific alleles were identified: 6-PGDH-A_6, PGM-A_4, allele 96 at the locus PmOSU_3B2 and allele 242 at the locus PmOSU_4A7. Fussi et al. (2013) reported that in populations with known

Table 2. Allele frequencies at isozyme loci for the investigated Douglas-fir populations.

Locus	Allele	Population			
		Da	N	Og	R
FEST-B	B_2	1.000	1.000	1.000	1.000
GOT-A	A_2	1.000	1.000	1.000	1.000
GOT-B	B_0	0.020	0.000	0.010	0.000
	B_1	0.102	0.030	0.051	0.071
	B_2	0.020	0.010	0.030	0.036
	B_3	0.806	0.900	0.879	0.869
	B_5	0.051	0.060	0.030	0.024
GOT-C	C_0	0.031	0.031	0.042	0.012
	C_1	0.062	0.062	0.146	0.145
	C_2	0.887	0.907	0.812	0.795
	C_3	0.020	0.000	0.000	0.048
IDH-A	A_0	0.000	0.000	0.010	0.000
	A_2	0.090	0.120	0.131	0.119
	A_3	0.010	0.010	0.020	0.024
	A_4	0.890	0.870	0.808	0.845
	A_5	0.000	0.000	0.020	0.000
	A6	0.010	0.000	0.010	0.012
MDH-A	A_2	0.040	0.010	0.000	0.024
	A_3	0.880	0.900	0.850	0.905
	A_4	0.070	0.090	0.130	0.060
	A_5	0.010	0.000	0.020	0.012
MDH-B	B_2	0.920	0.990	0.970	0.941
	B_3	0.080	0.010	0.030	0.060
MDH-C	C_1	0.020	0.000	0.000	0.000
	C_2	0.800	0.810	0.790	0.810
	C_3	0.180	0.190	0.210	0.191
PGI-B	B_0	0.000	0.000	0.010	0.000
	B_1	0.080	0.070	0.010	0.036
	B_2	0.910	0.920	0.950	0.929
	B_3	0.010	0.010	0.030	0.024
	B_4	0.000	0.000	0.000	0.012
PGM-A	A_2	0.110	0.170	0.090	0.155
	A_4	0.830	0.770	0.790	0.738
	A_6	0.060	0.060	0.120	0.107
6-PGDH-A	A_0	0.000	0.000	0.010	0.000
	A_1	0.000	0.020	0.010	0.000
	A_3	0.960	0.960	0.919	0.976
	A_6	0.040	0.020	0.061	0.024

origin representing the coastal Douglas-fir variety the frequencies of 6-PGDH-A_6 are higher than 90 %, compared to populations of interior Douglas-fir with frequencies around 50–60 %. A similar tendency was observed for the allele PGM_A_4 with frequencies generally higher than 80 % in coastal populations and only 50–60 % in interior Douglas-fir. In addition, in the coastal variety the allele 96 at the locus PmOSU_3B2 and the allele 242 at the locus PmOSU_4A7 are absent or present only in very low proportions, whereas in the interior populations frequencies of the mentioned alleles were higher than 20 % (allele 96 at locus PmOSU_3B2) respectively around 15 % (allele 242 at locus PmOSU_4A7).

Similar results were obtained by Konnert and Ruetz (2006) who investigated older Bavarian Douglas-fir stands with isozyme markers. It was already known that the stands belong to the coastal Douglas-fir variety. In these stands, frequencies for 6-PGDH-A_3 varied between 90.7–96.2 % and for PGM-A_4 between 81.9–88.5 %.

For the investigated stands from Bulgaria the values for the mentioned variety-specific alleles are introduced in Table 3. Comparing the values in the table with the mentioned findings we concluded that all four investigated stands represent the coastal variety. Both marker systems give rise to a similar conclusion.

Table 3. Allele frequencies at two isoenzyme (6-PGDH, PGM) and two nuclear microsatellite loci PmOSU_3B2 and PmOSU_4A7 for the four investigated Douglas-fir populations.

Locus	Population			
	Da	N	Og	R
6-PGDH - A_6	0.960	0.960	0.919	0.976
PGM-A_4	0.830	0.770	0.790	0.738
PmOSU_3B2 Allel 96	0.050	0.073	0.071	0.038
PmOSU_4A7 Allel 242	0.010	0.000	0.010	0.024

Genetic diversity of the investigated Douglas-fir plantations

The mean number of alleles (N_A), analyzed with isoenzymes varied between 2.55 and 3.09, the effective number of alleles (N_E) between 1.18 and 1.24 (Table 4). The observed (H_O) and expected (H_E) heterozygosity ranged from 0.153 to 0.196 and from 0.154 to 0.193, respectively, resulting in inbreeding coefficients (F_{IS}) varying from -0.0482 to 0.0300, which were close to zero (Table 4). Positive F_{IS} values as in populations Da and N show that individuals in the populations are more related than expected. The opposite holds for populations Og and *R* with negative F_{IS} values. Given the significantly higher polymorphism at microsatellite gene loci all diversity values were also higher for all four analyzed populations. The mean number of alleles (N_A), based on nuclear microsatellites was clearly higher compared to values based on isozymes and varied between 24.6 and 26.9, the effective number of alleles (N_E) lies between 15.3 and 16.6 (Table 4). The observed (H_O) and expected (H_E) heterozygosity ranged from 0.617 to 0.961 and from 0.926 to 0.933, respectively, resulting in significantly high

inbreeding coefficients (F_{IS}) varying from 0.256 to 0.337 for each population over all loci (Table 4). The observed (H_O) and expected (H_E) heterozygosity have almost the same values – 0.961 (H_O) and 0.927 (H_E). The values of F_{IS} are high and are not significant for the four investigated populations, so for each locus separately within populations were calculated F_{IS} values (Table 5).

Table 4. Mean diversity values based on isozyme gene markers (ISO) and nuclear microsatellites markers (nSSR) over all loci for the four investigated Douglas-fir populations.

Population	N_A		N_E		H_O		H_E		F_{IS}	
	ISO	SSR	ISO	SSR	ISO	SSR	ISO	SSR	ISO	SSR
Da	2.909	26.1	1 219	15.4	0.1691	0.663	0.1796	0.926	0.0300	0.284n.s
N	2.546	26.9	1 182	16.6	0.1527	0.647	0.1542	0.933	0.0058	0.307n.s.
Og	3.091	24.6	1 239	15.3	0.1964	0.617	0.1928	0.927	- 0.0209	0.337n.s.
R	2.818	24.6	1 229	15.6	0.1948	0.961	0.1866	0.927	- 0.0482	0.256n.s.

Note: N_A – mean number of alleles; N_E – effective number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient; Significance of F_{IS} values: non-significant (n.s.), *$p < 0.01$, **$p < 0.001$.

Table 5. Diversity statistics for the nine analyzed nuclear microsatellite loci.

Population	Locus	N	N_A	N_E	A	H_O	H_E	F_{IS}	
D	PmOSU_3F1	48	22.000	14.817	20.316	0.750	0.933	0.196	***
	PmOSU_2G12	50	19.000	8.104	17.185	0.680	0.877	0.224	***
	PmOSU_3B2	50	30.000	18.657	26.689	0.880	0.946	0.070	ns
	PmOSU_3G9	43	19.000	8.348	17.771	0.488	0.880	0.445	***
	PmOSU_4A7	49	27.000	11.916	23.598	0.918	0.916	-0.003	ns
	PmOSU_1F9	50	37.000	25.907	32.403	0.860	0.961	0.105	ns
	PmOSU_2D4	46	23.000	14.797	21.669	0.348	0.932	0.627	***
	PmOSU_1C3	40	26.000	17.204	25.201	0.450	0.942	0.522	***
	PmOSU_2D6	47	32.000	19.126	28.704	0.596	0.948	0.371	***
	Mean	47	26.1	15.4	23.7	0.7	0.9	0.284	
N	PmOSU_3F1	49	25.000	15.391	22.692	0.735	0.935	0.214	***
	PmOSU_2G12	49	21.000	9.681	19.174	0.878	0.897	0.021	ns
	PmOSU_3B2	48	33.000	22.154	29.647	0.750	0.955	0.215	ns
	PmOSU_3G9	48	18.000	9.481	16.700	0.500	0.895	0.441	***
	PmOSU_4A7	49	28.000	15.901	25.129	0.837	0.937	0.107	***
	PmOSU_1F9	48	34.000	26.331	31.374	0.792	0.962	0.177	*
	PmOSU_2D4	43	25.000	15.473	23.909	0.349	0.935	0.627	***
	PmOSU_1C3	39	27.000	16.355	26.009	0.385	0.939	0.590	***
	PmOSU_2D6	45	31.000	18.837	28.344	0.600	0.947	0.366	***
	Mean	46.4	26.9	16.6	24.8	0.6	0.9	0.307	
O	PmOSU_3F1	49	21.000	12.313	19.271	0.612	0.919	0.334	***
	PmOSU_2G12	49	19.000	10.671	17.605	0.776	0.906	0.144	***
	PmOSU_3B2	49	28.000	18.685	25.675	0.857	0.946	0.094	***
	PmOSU_3G9	48	17.000	7.706	15.928	0.417	0.870	0.521	***
	PmOSU_4A7	49	30.000	18.053	26.302	0.796	0.945	0.157	*
	PmOSU_1F9	47	32.000	20.938	29.048	0.809	0.952	0.151	***
	PmOSU_2D4	43	20.000	11.206	18.718	0.302	0.911	0.668	***

Population	Locus	N	N_A	N_E	A	H_O	H_E	F_{IS}	
	PmOSU_1C3	40	22.000	14.545	21.396	0.350	0.931	0.624	***
	PmOSU_2D6	46	32.000	23.910	30.252	0.630	0.958	0.342	***
	Mean	46.7	24.6	15.3	22.7	0.6	0.9	0.337	
R	PmOSU_3F1	41	19.000	13.394	18.515	0.829	0.925	0.104	ns
	PmOSU_2G12	42	20.000	10.376	18.866	0.881	0.904	0.025	ns
	PmOSU_3B2	40	26.000	15.385	24.921	0.700	0.935	0.251	***
	PmOSU_3G9	35	14.000	7.164	14.000	0.429	0.860	0.502	***
	PmOSU_4A7	42	26.000	13.213	24.305	0.905	0.924	0.021	*
	PmOSU_1F9	41	34.000	25.470	32.055	0.805	0.961	0.162	***
	PmOSU_2D4	39	24.000	16.095	23.149	0.462	0.938	0.508	***
	PmOSU_1C3	36	30.000	19.786	29.636	0.583	0.949	0.386	***
	PmOSU_2D6	40	28.000	19.277	26.684	0.625	0.948	0.341	***
	Mean	39.6	24.6	15.6	23.6	0.7	0.9	0.256	

Note: N – number of analyzed individuals; N_A – actual number of alleles; N_E – effective number of allele; A – allelic richness; H_O – observed heterozygosity; H_E – expected heterozygosity; fixation index (F_{IS}:* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

For the locus PmOSU_4A7 results are significant in three populations – N, Og and R (values between 0.021 and 0.157). For the locus PmOSU_3G9 all populations showed significant deviation from HWE (range 0.441 to 0.502). All nine analyzed loci are significant for Og plantation. Compared to other studies based on the same nSSR loci genetic diversity values in Bulgarian populations are at the upper limit. Thus for example Fussi et al. (2013) reported the following values: N_A = 19.3 – 30.5; H_O = 0.734 – 0.826; H_E = 0.897 – 0.919 whereas Krutovski et al. (2009) found heterozygosity values between 0.639–0.783 (H_O) and 0.935–0.936 (H_E). For isozyme gene loci the investigated Bulgarian Douglas-fir stands have similar levels of genetic diversity with observations reported for native populations of coastal Douglas-fir (Moran and Adams 1989, Li and Adams 1989, El Kassaby and Ritland 1996, Krutovsky et al. 2009). Thus for example El Kassaby and Ritland (1996) have studied 49 coastal low-elevation Douglas-fir populations in SW British Columbia and NW Washington State at 20 isozyme loci. The determined a mean number of alleles (N_A) per locus of 2.14 (1.75 to 2.35), mean values of expected (H_E) heterozygosity of 0.163 (0.122 to 0.198). Fussi et al. (2013) applied for six planted stands in Bavaria the same isozyme loci and calculated a mean number of alleles (N_A) of 3.0 (2.9–3.2) and mean values of expected (H_E) heterozygosity of 0.159 (0.132–0.183). One more study (Krutovsky et al. 2009) at 18 isozyme loci in 20 costal Douglas-fir populations at north- and south-facing slopes in SW Oregon shows the similar results. The mean number of alleles (N_A) is 2.32 (2.11–2.55), the mean values of expected (H_E) heterozygosity is 0.160 (0.128–0.189). In naturally regenerated stands at 1,048 locations from western Oregon and Washington the mean number of alleles (N_A) was 3.02 and the heterozygosity values 0.203 (H_O), respectively 0.206 (H_E).

The extent of genetic variation between populations is measured by the genetic distances between populations. Results for genetic distances between the four Douglas-fir populations investigat-

ed based on isozymes and microsatellite markers are given in Table 6. For isozyme markers the Gregorius distance was calculated, because it reflects better the differences in the genetic structure of populations. Gregorius distance shows which amount of alleles has to be replaced to equalize the genetic structures of the compared populations. Gregorius distances, based on isoenzymes, ranged from 0.034 (population Da to population N) to 0.053 (population Da to population Og).

Table 6. Pairwise Population Matrix of Nei Genetic Distance for the investigated Douglas-fir populations, using isoenzyme (ISO) and microsatellite (SSR) markers.

Population	Da		N		Og		R	
	ISO	SSR	ISO	SSR	ISO	SSR	ISO	SSR
Da			0.034	0.218	0.053	0.240	0.041	0.250
N					0.045	0.239	0.037	0.285
Og							0.037	0.338

The highest genetic distances were found between the population pairs Og vs. Da 0.053 and Og vs. N 0.045. This means that the highest genetic distances are between the population with the highest genetic diversity (Og) and the two populations with the lowest genetic diversities (N, Da).

Nei's genetic distances, based on microsatellite markers, between all populations are clearly higher and range between 0.218 and 0.338. The highest genetic distance was found between populations Og and R (0.338). Values over 20 % can be considered extremely high and indicate probably a different origin of the four populations within the distribution range of 'coastal' Douglas-fir.

Conclusions

Provenance trials and comparative field studies in Bulgaria including coastal and interior provenances of Douglas-fir (Popov 2010, Popov 2014, Petkova et.al. 2014, Petkova et.al. 2015) demonstrated the superiority of the coastal variety in growths performance, survival, stability and a lower susceptibility to fungal diseases. Our results demonstrated that the four investigated Douglas-fir plantations are of coastal variety and thus can be recommended as seed sources for future afforestation in Bulgaria.

The comparatively high level of genetic variation within and between investigated stands is a good prerequisite for adaptation. At the same time it shows that during the artificial installation of these stands by planting no genetic bottlenecks appeared. These are good news for artificial regeneration and management of Douglas-fir in Bulgaria, as this species will become of increasing importance for Bulgarian forestry under climate change.

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