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RESEARCH ARTICLE

Genetic diversity of natural Silver fir populations in Bulgaria*

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** This paper is dedicated to the memory of Professor Velichko Gagov (1944-2024)*

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Abstract

Genetic diversity of eleven natural populations of Silver Fir in Bulgaria was studied by means of allozyme gene markers. Within population diversity varied among populations and was generally high, but within the range reported for other European populations of the species. Mean number of alleles per locus ranged from 2.1 to 2.4 and effective allele number – from 1.2 to 1.5. Expected and observed heterozygosities were also high (mean values 0.295 and 0.265, respectively), but there was significant heterozygote deficiency in most loci and populations, resulting in positive inbreeding coefficient (mean F_{IS}=0.049). Differentiation among populations was low (F_{ST} = 0.027), resulting probably from extensive gene flow and recent divergence. The results are discussed in the light of conservation and sustainable management of Silver Fir natural populations.

Key words

Abies alba, allozymes, variation, genetic differentiation, conservation

Introduction

Silver Fir (*Abies alba* Mill.), one of the large European conifers, is an important tree species in the temperate forests in Europe, forming pure and more frequently, mixed stands, preferably with Norway Spruce (*Picea abies* (L.) Karst.) and European beech (*Fagus sylvatica* L.). In Carpathians and in the mountains of Balkan Peninsula it is an

important part of the still existing virgin forests (Korpeľ, 1995; Kostov, 1989; Panayotov et al., 2011), representing habitats of high conservation value (Roussakova, 2009; Dimitrov, 2009). Given its fast growth and high productivity, Silver Fir is considered important from both economic and ecological point of view.

As an important forest tree species, it has attracted the attention of the experts and has been extensively studied throughout Europe. The studies encompass a broad range of issues, from phylogeny and evolution to stand management and gene conservation. Substantial part of ecological research in 1980s and 1990s were focused to Silver Fir decline (Larsen, 1986; Bergmann et al., 1990) and more recently – to the species adaptation to climate change (Bošela et al., 2016; Konôpková et al., 2019). A thorough review of the recent progress of research on the ecology and silviculture was done by Dobrowolska et al. (2017).

Genetic diversity was studied by means of phenotypic, biochemical and molecular markers. Great deal of studies used allozymes (Bergmann et al., 1990; Konnert & Bergmann, 1995; Longauer, 1996; Parducci et al., 1996; Breitenbach-Dorfer et al., 1997; Sclaltsoyannes et al., 1999, Bergmann, Gagov, 2001, etc.) and later on, new generation genetic markers, which helped to reveal new insights of species diversity and evolution (Vendramin et al., 1999; Liepelt et al., 2002, 2009 2010; Gömöry et al., 2004; Voleková et al., 2014, Teodosiu et al., 2019). Substantial amount of studies focused on the evolution and post-glacial migrations of the species (Terhürne-Berson et al., 2004; Gömöry et al., 2004, 2012; Cheddadi et al., 2014). Quantitative traits were studied not only for documenting of existing variation but also in relation to improvement activities (Sagnard et al., 2002, Mihai et al., 2014, Sancho-Knapik et al., 2014).

In Balkans and particularly in Bulgaria, Silver Fir reaches its southernmost limit of distribution and populations to further south are considered a zone of introgression between *Abies alba* and *A. cephalonica* Loud. representing the taxon *A. borisii-regis* Mattf. (Mattfeld et al., 1925; Liepelt et al., 2010; Alizoti et al., 2011). This hybridization has received considerable attention (Bella et al., 2014; Krajmerová et al., 2016, and the references therein). However, compared to the other parts of the species range, the information about the distribution of genetic diversity in Silver Fir populations in Bulgaria is relatively limited (Bergmann, Gagov 2001; Longauer et al., 2003).

Therefore, the objective of present study was to characterize the distribution of genetic diversity within and among the population of *Abies alba* in Bulgaria, representing the southeastern limit of the species range.

Material and methods

Populations, sampling and electrophoresis

Eleven natural populations were selected for the study (Table 1) covering the whole area of distribution of Silver Fir in Bulgaria. The number of individuals sampled in each population varied according to their availability from 31 to 88 (mean 58). Dormant winter buds were used for the analysis. The tissue was homogenized in 0.1 M

Tris-HCl extraction buffer pH 7.3. Also, 15 mg dithiothreitol, 5 mg Na_2.EDTA, 150 mg Polyvinilpyrholidone and 300 mg saccharose were added to 10 ml buffer prior to homogenization.

Standard 12 % starch gel electrophoresis was used for separation of isozyme variants in two buffer systems: Lithium-borate – Tris citrate discontinuous buffer system pH 8.1 (Ashton, Braden, 1961) and Tris citrate continuous buffer system pH 7.4 (Shaw, Prasad, 1970). Four enzyme systems coding for 7 polymorphic loci were analyzed: Isocitrate dehydrogenase (loci Idh-1 and Idh-2), Menadione reductase (locus Mnr), 6-phosphogluconic dehydrogenase loci (6pgd-1 and 6-pgd-2) and phospho-glucose isomerase (loci Pgi-1 and Pgi-2). The electrophoretic and staining procedures and genetic control of inheritance of allozyme variants followed Hüssendörfer et al. (1995).

Data analysis

Diploid genotypes were scored from electrophoregrams. Allele frequencies, percent of polymorphic loci, observed and expected heterozygosity (Nei, 1978) were calculated using a contemporary version (v.4.7) of software Genepop (Rousset, 2008). The fit of genotypic distribution to Hardy-Weinberg expectations was tested by Fisher's combined probability test. Heterozygote deficit and excess were tested using the exact test proposed by Rousset, Raymond (1995). Linkage disequilibrium was analyzed with Fisher's exact test on contingency tables, with unbiased estimate of exact probability obtained by Markov Chain Monte Carlo (MCMC) method (Raymond, Rousset, 1995) using Genepop software.

Among-population diversity was evaluated by using two approaches: first, calculating the Nei's (1978) genetic distances (unbiased estimate) between the population pairs and second, by the measures of population differentiation (Wright's 1965 F-statistics) calculated according to Weir, Cockerham (1984). Isolation by distance was tested following Rousset (1997) by studying the regression of $F_{ST}/(1-F_{ST})$ against logarithm of the air distances between the population pairs. Migration (private allele method and F_{ST} method) was evaluated following Slatkin & Barton (1989). Analysis of F-statistics was done using software F-stat (Goudet, 1995), while all other analyses were performed on Genepop (Rousset, 2008).

Multidimensional scaling was applied for better interpretation of population differentiation based on genetic distances by software SYN-TAX (Podani, 1989).

Results

Polymorphism and diversity

Allele frequencies are presented in the Appendix 1 and the polymorphism and diversity parameters – in Table 1. Mean number of alleles per locus varied from 2.1 (Slavyanka) to 2.6 (Zhenda), with a mean value of 2.34, and the effective allele number – from 1.191 (Kresna) to 1.547 (Kipilovo) with a mean of 1.410. With three excep-

tions, all loci were polymorphic (0.95 criterion). The heterozygosity in the studied populations was high, the observed ranging from 0.161 (Kresna) to 0.336 (Slavyanka), and the expected one – from 0.187 (Kresna) to 0.358 (Kipilovo). Fixation index was positive and significantly different from 0 in 8 of 11 populations with a mean value of 0.049, and negative, but insignificant, in one population (Slavyanka).

Hardy-Weinberg equilibrium

Most populations did not show significant deviation from the expected value of equilibrium. There were 13 cases where the null hypothesis of Hardy-Weinberg equilibrium was rejected (Table 2). These concerned six loci (Idh1 and Idh2, 6pg1 and 6pg2, and Pgi1 and Pgi-2) in eight populations. Twelve significant deviations were due to heterozygote deficiency and only one was due to heterozygote excess (Slavyanka). However, the observed events of heterozygote excess and deficiency represent only small fraction of all possible cases and therefore, all loci were retained in the further analyses.

Table 1. Polymorphism and diversity in the populations studied

Population (altitude)	N	n_a	n_e	P%	Ho (s.e.)	He (s.e.)	F
Borovetz (1200)	88	2.3	1.428	100	0.278 (0.052)	0.302 (0.054)	0.079
Rakitovo (1400)	92	2.4	1.498	100	0.277 (0.035)	0.334 (0.037)	0.171*
Goce Delchev (1400)	66	2.3	1.413	100	0.273 (0.035)	0.295 (0.047)	0.075
Smolyan (1200)	31	2.3	1.386	100	0.255 (0.045)	0.283 (0.048)	0.099
Kirkovo (800)	64	2.4	1.424	100	0.260 (0.015)	0.300 (0.034)	0.133*
Kipilovo (450)	45	2.4	1.547	100	0.317 (0.043)	0.358 (0.046)	0.115*
Pamporovo (1600)	31	2.4	1.502	100	0.281 (0.046)	0.339 (0.039)	0.171*
Zhenda (900)	74	2.6	1.392	85.7	0.272 (0.051)	0.283 (0.052)	0.039
Slavyanka (1400)	48	2.1	1.463	85.7	0.336 (0.067)	0.320 (0.061)	-0.05
Kresna (1200)	50	2.3	1.191	71.4	0.161 (0.055)	0.187 (0.059)	0.139*
Osogovo (1200)	46	2.3	1.261	100	0.207 (0.043)	0.246 (0.048)	0.158*
Mean	57.7	2.34	1.410	94.8	0.265	0.295	0.049

Legend: *N* – no of individuals studied; n_a – mean no of alleles per locus; n_e – effective allele number (harmonic mean); *P%* – percent of polymorphic loci (0.05 criterion); *Ho* – observed heterozygosity; *He* – expected heterozygosity; *F* – inbreeding coefficient (*F=1-Ho/He*); *s.e.* – standard error; * significantly different from zero ($p \leq 0.05$).

Linkage disequilibrium

Significant linkage disequilibrium was found in eight populations: Borovetz (5 locus pairs), Osogovo (3 locus pairs), Rakitovo and Kirkovo (2 locus pairs) and Goce Delchev, Pamporovo, Zhenda and Slavyanka (one locus pair). When all loci and all populations were pooled, there was significant linkage disequilibrium among only five locus pairs (Table 3). Considering the correlation structure between loci, we found no necessity to remove a particular locus from further analyses.

Table 2. Departures from Hardy-Weinberg equilibrium

Locus	Population	p-value
Heterozygote deficiency		
Idh1	Rakitovo	0.0221
	Zhenda	0.0369
Idh2	Rakitovo	0.0041
	Goce Delchev	0.0493
6pg1	Rakitovo	0.0443
6pg2	Rakitovo	0.0351
Pgi1	Kirkovo	0.0090
	Pamporovo	0.0048
	Kirkovo	0.0187
Pgi2	Rakitovo	0.0057
	Kipilovo	0.0358
	Pamporovo	0.0204
Heterozygote excess		
6pg1	Slavyanka	0.0431

Table 3. Locus pairs where significant linkage disequilibrium was detected across all populations by Fisher's exact test

Two-locus combination	χ^2-*test*	DF	*p*-value
Mnr:6pg1	42.58	22	0.00532
6pg1:6pg2	32.26	20	0.04058
6pg2:Pgi1	39.62	20	0.00558
Idh2:Pgi2	39.21	22	0.01335
Pgi1:Pgi2	43.36	22	0.00426

Legend: for abbreviation of isozyme loci, see Material and methods. DF stands for degrees of freedom

Interpopulation differentiation and migration

Despite of the low level of overall genetic differentiation (F_{ST}=0.027), it was highly significant (p<0.001). It was not significant only for the loci *Mnr* and *6pg1*, which could not change the general picture of among-population diversity (Table 4).

Estimations of the number of migrants calculated by using private allele method (corrected for sample size) and F_{ST} method were fairly similar: 10.75, and 9.01, respectively.

The model of isolation by distance test provided the regression:

$F_{ST}/(1-F_{ST}) = -0.0499 + 0.0171 \ln (d)$,

which is statistically significant ($p=0.00101$).

Table 4. Population differentiation evaluated by Wright's F-statistics

Locus	F_{IS}	F_{IT}	F_{ST}	p-value[1)]
Idh1	0.072	0.110	0.041	<0.001
Idh2	0.175	0.201	0.032	<0.001
Mnr	0.073	0.077	0.005	0.115
6pg1	-0.009	-0.008	0.001	0.352
6pg2	0.131	0.163	0.036	<0.001
Pgi1	0.131	0.168	0.043	<0.001
Pgi2	0.142	0.162	0.023	<0.001
Overall	**0.102**	**0.127**	**0.027**	**<0.001**
95 % CI[2)]	0.055-0.145	0.071-0.174	0.014-0.037	

Note: *F*IS, *F*IT, and *F*ST are *F* statistics as defined by Wright (1965) and estimated as suggested by Weir and Cockerham (1984).

[1)] *p* represents the probability of Fisher's exact test on contingency tables

[2)] 95 % confidence intervals calculated using the F-stat program (Goudet, 1995).

Genetic distances among the studied populations were of low magnitude and did not reveal particular trend of variation (Table 5). The interpretation of genetic distances was facilitated by the multidimensional scaling (Fig. 1). Relatively the most distant population were Kipilovo, situated at very low altitude, Kirkovo and Kresna. However, the differences among populations were small and no particular grouping was observed.

Table 5. Genetic distances (Nei, 1978) among the populations studied

Population	Bor	Rkt	GD	Smo	Krk	Kip	Pam	Jen	Sla	Kre
Rkt	0.004	***								
GD	0.003	0.003	***							
Smo	0.005	0.001	0.001	***						
Krk	0.029	0.014	0.019	0.010	***					
Kip	0.004	0.008	0.012	0.014	0.039	***				
Pam	0.008	0.001	0.004	0.001	0.010	0.015	***			
Jen	0.005	0.002	0.004	0.001	0.011	0.017	0.001	***		
Sla	0.005	0.001	0.005	0.001	0.018	0.017	0.001	0.001	***	
Kre	0.018	0.026	0.011	0.020	0.045	0.027	0.031	0.031	0.033	***
Osg	0.003	0.008	0.012	0.007	0.029	0.006	0.011	0.008	0.012	0.029

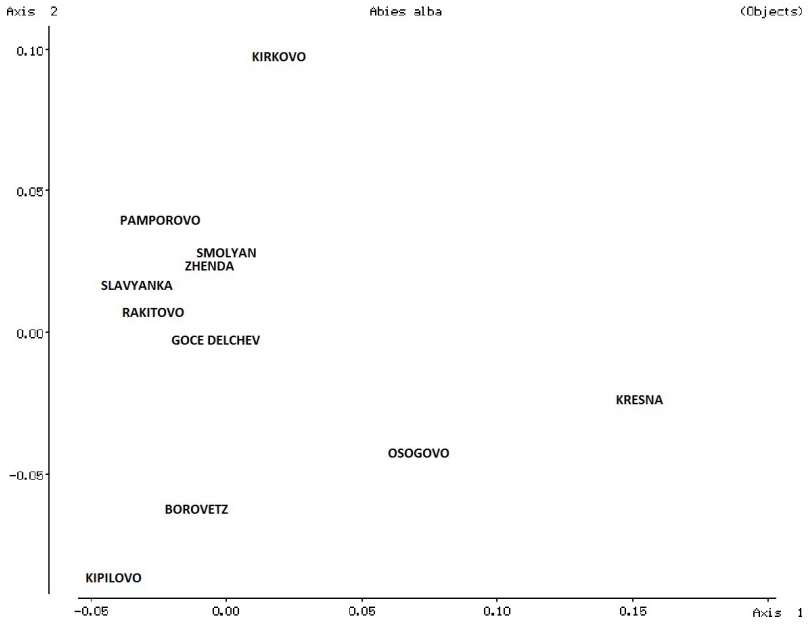

Fig. 1. Multidimensional scaling of the population coordinates based on genetic distances

Discussion

Within-population diversity

Allozymes represent class of neutral markers characterized by relatively low variability, in comparison to the new-generation markers developed for studying of population diversity, like, for example, microsatellites (Vendramin et al., 1999). However, they still can be informative and could be used successfully for characterizing the genetic variation in plant populations.

The level of diversity detected in the studied populations, measured by heterozygosity, is well within the range reported for coniferous species and particularly for species with similar life-history characteristics (Hamrick et al., 1992).

Different studies on the within-population genetic diversity of *A. alba* based on allozymes report values within the range 0.1-0.4 for the expected and observed heterozygosities. For example, Fady et al. (1999) and Sagnard et al. (2002) reported for the populations in Southeastern France values from 0.114 to 0.213 with a reference population from Pyrenees with gene diversity as low as 0.063. In other parts of Europe, the highest gene diversity was reported for the Silver Fir from Switzerland and Austria – effective allele number 1.22 to 1.29 in the Swiss populations and observed heterozygosity 0.163 to 0.222 in the Swiss (Hüssendorfer, 1999), and 0.313 to 0.474 in the Austrian populations (Breitenbach-Dorfer et al., 1997). Many other studies reported intermediate results: Parducci et al. (1996), Scaltsoyiannes et al. (1999), Lewandowski et al. (2001), Mejnartowicz (2003), Korshikov et al. (2005), Ballian et al. (2012).

Observed heterozygosity was higher than the expected one only in the population Savyanka, which is hypothesized to represent the taxon *Abies borisii-regis*. The positive values of the inbreeding coefficient in most populations reflect the small to moderate deviations from Hardy-Weinberg expectations, due to heterozygote deficiency. This phenomenon is not unprecedented in conifers in general, and in *A. alba*, in particular (e.g., Parducci et al., 1996), but in most studies was reported to be of minor importance, with small differences between the observed and expected heterozygosities (Lewandowski et al., 2001; Ballian et al., 2012; Longauer et al., 2003). For the southern populations of *A. alba* (including Balkans) Longauer et al. (2003) reported heterozygosities ranging from 0.14 to 0.19, and outlined the fact already pointed by Bergmann, Gagov (2001) that there were many rare and region-specific alleles, and some of these alleles were different in the geographically adjacent North Macedonia and Bulgaria.

Additional informative measure of diversity is the effective allele number (the reciprocal of homozygosity), which in the general case is equivalent to Gregorius' (1987) measure of diversity, ***v***. In most studies the reported values were similar to or lower than the values in the present study. Bergmann et al. (1990) reported values from 1.2 to 1.32 gathered from a study of 48 populations from different parts of Europe. Similar results were documented by Hüssendorfer et al. (1999), Vicario et al. (1999), Longauer et al. (2003), Ballian et al. (2012).

The rare significant cases of linkage disequilibrium in some populations in most cases involved the loci Idh-1, Idh-2, 6pg1 and 6pg2. Significant linkage among these locus pairs were found by Schroeder (1989), while Hüssendorfer et al. (1995) reported a linkage between Idh2 and aminopeptidases, which were not included in the present study. Given the modest percentage of linkage disequilibrium in single populations, it was not possible to test whether it results from epistatic selection or random genetic drift (Ohta, 1982).

Population differentiation.

The differentiation among populations, as evaluated by F_{ST}, was low (0.027), indicating that less than 3 % of the total diversity is due to its among-population component. Low differentiation among populations is a common trend for the conifer species. Most studies on *A. alba* employing isozyme gene markers report values of F_{ST} (or its analog, G_{ST}) below 0.1 (Parducci et al., 1996; Breitenbach-Dorfer et al., 1997; Lewandowski et al., 2001; Longauer et al., 2003; Korshikov et al., 2005). Vicario et al. (1999) reported a slightly higher value for the Italian populations, but only when *A. nebrodensis* was included in the dataset.

Low population differentiation has been documented for many wind-pollinated tree species. It is at least partly due to the extensive migration typical for such species. Migration estimates by using two different indirect methods were fairly similar (9.01 and 10.75). Even though there are substantial arguments against F_{ST} method (Whitlock, McCauley, 1999), our estimates show at least that the migration rate is sufficient to compensate the differences caused solely by genetic drift (Mills, Allendorf, 1996).

Multidimensional scaling based on genetic distances did not reveal particular geographic trend of variation. Even though the differences among populations were

small, Kirkovo, Kipilovo and Kresna could be noted to deviate from the relatively homogeneous group of the other populations. A common characteristic of these three populations is their isolation. Additionally, Kirkovo is the southernmost population, and Kipilovo is situated at low altitude (450 m) in the Eastern Balkan range. These details could partly explain the picture of differentiation. Slavyanka, representing *A. borisii-regis*, did not differ from most of the other populations. Similar results were reported by Krajmerová et al. (2016) based on mitochondrial and microsatellite (i.e., maternally and biparentally inherited) markers. Therefore, we can state that the observed grouping provided by the multidimensional scaling is not due to the limitations of the allozyme markers, characterized by much lower variability than DNA-based markers, and hence, by lower resolution power. These patterns are in line with the conclusions of Krajmerová et al. (2016) that *A. borisii-regis* is a result of recent introgression and is not a stabilized hybridogenous species.

Implication for gene conservation

Usually, the distribution of gene diversity among populations of a given species is considered an important information for the gene conservation (Jost et al., 2018). More distinct populations or these containing rare and unique alleles are considered as deserving priority attention and conservation measures. This is directly related to selecting gene conservation units and designing conservation and management plans. In the present study the most differentiated populations were Kirkovo, Osogovo and Kresna, and these with the highest within-population diversity were Slavyanka and Kipilovo. Despite of the level of diversity, population Slavyanka is always a subject of particular interest and conservation measures, due to its hybrid origin, which classifies it to *A. borisii-regis* (Krajmerová et al., 2016). Fortunately enough, almost all of populations that can be considered important for conservation, are included in protected territories of different status – national parks, nature reserves and at least Natura 2000 zones. This is a good prerequisite for dynamic conservation of *Abies* genetic resources in Bulgaria, and for advanced management of the rich *ex situ* collections of the species, including next-generation seed orchard (Gagov et al., 2019).

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Appendix 1. Allele frequencies in 7 isozyme gene loci in the populations studied

Locus	Alleles	Populations										
		Bor	Rkt	GD	Smo	Krk	Kip	Pam	Jen	Sla	Kr	Osg
Idh-1	A	0.216	0.212	0.098	0.145	0.078	0.344	0.177	0.155	0.198	0.019	0.325
	B	0.665	0.696	0.765	0.774	0.844	0.578	0.742	0.689	0.677	0.972	0.675
	C	0.119	0.092	0.136	0.081	0.078	0.078	0.081	0.155	0.125	0.009	0.000
Idh-2	A	0.653	0.701	0.621	0.726	0.797	0.589	0.758	0.770	0.729	0.474	0.760
	B	0.000	0.000	0.000	0.000	0.062	0.000	0.000	0.007	0.000	0.000	0.000
	C	0.347	0.299	0.379	0.274	0.141	0.411	0.242	0.223	0.271	0.526	0.240
Mnr	B	0.136	0.179	0.182	0.207	0.109	0.190	0.258	0.135	0.167	0.094	0.111
	C	0.864	0.821	0.818	0.793	0.891	0.810	0.742	0.865	0.833	0.906	0.889
6pgdh-1	B	0.239	0.159	0.174	0.150	0.117	0.189	0.194	0.176	0.229	0.138	0.184
	C	0.761	0.841	0.826	0.850	0.883	0.811	0.806	0.824	0.771	0.862	0.816
6pgdh-2	A	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.943	0.902	0.932	0.933	0.797	0.856	0.935	0.953	1.000	0.981	0.941
	C	0.057	0.098	0.068	0.050	0.203	0.144	0.065	0.047	0.000	0.019	0.059
Pgi-1	A	0.136	0.250	0.212	0.250	0.359	0.122	0.290	0.257	0.313	0.075	0.078
	B	0.864	0.750	0.788	0.750	0.641	0.878	0.710	0.743	0.688	0.925	0.912
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
Pgi-2	A	0.040	0.054	0.045	0.050	0.056	0.078	0.129	0.034	0.052	0.075	0.060
	B	0.074	0.130	0.030	0.000	0.000	0.033	0.048	0.027	0.104	0.009	0.010
	C	0.886	0.804	0.924	0.950	0.839	0.778	0.806	0.926	0.844	0.915	0.930
	D	0.000	0.011	0.000	0.000	0.105	0.111	0.016	0.014	0.000	0.000	0.000