



The genetic population study of Balkan Silver Fir (*Abies alba* Mill.)

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Abstract

Background and Purpose: Silver fir (*Abies alba* Mill.) is one of the most important forest tree species in the Western Balkan area from both economic and ecological aspect. Its distribution has for years been the subject of scientific research, the reason being that silver fir from these areas displays very interesting morphological and genetic differentiation.

Material and methods: The development of modern methods and the use of *nad5-4* marker have solved the problem of contact zones of silver fir originating from the Apennine and Balkan glacial refugia in the Western Balkan area. The objective of this study was to determine the boundary of gene introgression of silver fir derived from the Apennine and Balkan glacial refugia in the western Balkan populations with the use of isoenzymes. Twenty-four selected populations from Bosnia and Herzegovina and Croatia were investigated. Nine enzyme systems were studied, polymorphic gene loci were scored and so were their allelic types. The analysis of general genetic parameters did not allow for any conclusion, and neither did the analysis of genetic distances. For this reason, the allele distribution was analyzed.

Results and Conclusions: Statistical analysis showed the separation of the populations from Croatia which belong to the group west of the contact zone. In the populations along the contact zone and in those from the eastern part, the grouping did not show any regularity or any clinal variability. The main reason for this is attributed to high ecological diversity, fragmentation and the occurrence of genetic drift. It can be concluded that silver fir originating from the Balkan refugium was more widely distributed in the past, but is now constantly retreating in relation to that originating from the Apennines. Another hypothesis is that it may have reached southern Germany through a similar gene flow. The impact of the alleles originating from the Apennine refugium is also present in the eastern parts of Bosnia.

INTRODUCTION

Much has been said about the importance of silver fir (*Abies alba* Mill.), a species naturally distributed in Central Europe and the Western Balkans area. Covering 560 000 ha in Bosnia and Herzegovina, 210,000 ha in Slovenia, and 200,000 ha in Croatia, it is one of the most important forest trees from both economic and ecological standpoint. Although silver fir in the Western Balkan area very rarely

forms pure stands, it is the most important constituent element of mixed forests of fir and beech (*Abieti-Fagetum*) and mixed forests of fir, beech and spruce. Its significance, but also its threatened status and decline caused by atmospheric and soil pollution, especially in the Slovenian Dinaric Alps, in Gorski Kotar (Croatia) and western Bosnia (1, 2, 3) has made it the subject of numerous studies. The vulnerability of silver fir in western and Central Europe and its possible disappearance has also been discussed by (4, 5, 6). Additionally, several western Balkan countries are burdened with the problem of poor system of silver fir management, which significantly contributes to its rapid decline. This is the reason that researchers stress the importance of an urgent study of healthy and undamaged fir stands (7, 8, 9, 10, 11). Such stands are still relatively abundant in the western balkans area. In order to successfully protect and preserve silver fir with *in situ* and *ex situ* methods, it is essential to have an in-depth knowledge of its genetic constitution.

During the last 13,000 years, silver fir has been subjected to a constant dynamic migration from its glacial refugia in the south of Europe towards central, western and eastern Europe. These migrations of forest trees were predominantly caused by extensive climate changes. Following the great glaciation, which lasted for about 100,000 years and reached its maximum 15,000 years ago, all forest tree populations in the temperate zone survived in several glacial refugia in the Holocene populations (12) and expanded toward north (13). The migration of silver fir followed the same pattern from its three refugia, the central Apennines, the south of the Balkan Peninsula and the central massife in France, while two refugia remained isolated, one in Calabria in the south of the Apennines and one situated in the Pyrenees. Before the research (14), the best presentation of the dynamic tree migration, including silver fir, was obtained by pollen analysis, albeit this was incomplete for the Balkan area. Pollen analysis successfully indicated general directions of forest tree migrations in Europe (15), thus indicating possible glacial refuge areas. However, pollen investigations could not clarify where and when populations and individuals from different refugia came into contact, nor could these analyses show differentiations between the species within one genus. The development of techniques and methods of biochemical and DNA marker analyses of forest trees allowed simple determination of contact zones and mixing zones of individuals and populations on their post-glacial migration routes. Research on silver fir represented a turning point in phylogenetic studies, although similar attempts had been made earlier. As phylogenetic research progressed, especially in the 1980s, several new hypotheses on the number and the location of glacial refugia and migration routes were established (7, 16). These hypotheses were successfully dealt with at molecular level (17) for one single area, and then for the entire distribution range of silver fir (14, 18, 19) for the Carpathians and the Tatra mountains (20) and for the wider area of central and eastern Europe, and for Switzerland (21).

The development of molecular genetic markers accelerated research on postglacial migrations. Postglacial migration of white oaks was successfully solved by means of cpDNA (22), however, the same method could not be applied to conifers due to the specific method of chloroplast inheritance (23) until the applicability of mtDNA analyses was discovered (24) and provided probably the best presentation of the postglacial migration synthesis of the silver fir (25).

Being largely under the impact of the Mediterranean, the Balkan Peninsula is one of the key areas for the conservation and differentiation of European genetic sources during successive glaciations in the Quaternary. In our study, we refer to the western Balkan area of silver fir distribution, which has for many years been in the focus of interest of a number of researchers dealing with silver fir (26, 27). The basic reason for this was the fact that firs from this area manifested an interesting morphological differentiation (28, 29), and genetic variability indicated by numerous provenance tests (30, 31, 32, 50). The type of genetic structure of western Balkan populations could be surmised from research with numerous genetic markers; however, since most of the research involved a relatively small number of samples, it was not possible to ascertain the real condition (14, 24, 33, 34, 35, 36, 37). The first phylogeographic research on silver fir (14) shed light on the situation; thus, it was confirmed that silver fir had five glacial refugia and that the migration and colonization of fir in Central Europe stemmed from three refugia. During these migrations firs from different refugia came into contact and mixed in introgressive zones, or contact zones between the two migration routes in Slovenia and southern Austria, and the western Carpathians and the Tatra Mountains. These were the zones in which extensive hybridization between the firs originating from different glacial refugia could be observed. In Central Europe, this problem was particularly concentrated in the area of Austria and Bavaria (20). According to these investigations the western Balkans are interesting area with considerable impacts on Central European fir populations as shown by registering certain alleles typical for the Balkan area, Central Europe and southern Bavaria.

Development of mtDNA markers (24) made it possible to successfully solve the question of contact zones between the migration routes of fir originating from the Central Italian and the Balkan glacial refugia. At the same time, DNA-specific sequences were also determined for other fir species (38). These methods and the use of *nad5-4* marker allowed (39) researchers to solve the problem of contact zones in western Bosnia and in the Central Carpathian massife, thus slightly revising the research (14) and shifting the contact zone to the west of Bosnia. However, what is yet to be solved is the extent of the impact of individual glacial refugia on both sides of the contact zone or, in other words, the size of the hybrid zone. Research with mtDNA and *nad5-4* marker shows that this is inherited through maternal lineage and is connected with seed movement. In the second case there

is rapid pollen-generated gene flow, where wind strength and frequency in the blossoming period occurs as a special vector which can be successfully identified with isoenzyme tests.

The purpose of this research was to determine the boundary of gene introgression of firs originating from the Italian glacial refugia in natural populations of silver fir in western Balkans by means of isoenzyme genetic markers. The origin of individual alleles southeast of the contact fir zone should also be determined, as well as the degree of differentiation between the studied populations. In addition, it should be ascertained whether hybridization had any effect on this. The aim of this study was to provide a fuller picture of the genetic constitution of silver fir and complement research on this species, which in earlier studies only partially comprised populations from this distribution area.

From the 1960s to 1990s, the number of produced seedlings in Croatia was about 20,000 pieces per year, and at the beginning of the 90s the production increased. The production has risen especially in the last ten years. In the beginning of 2000s, there were 634,000 seedlings of different ages in nurseries. The increased production can be explained by the growing need for fir seedlings to be used in regeneration since fir is affected by decline and desiccation. Forests are most commonly regenerated with a combination of natural and artificial regeneration. Moreover, beech coppices growing within the range of beech-fir forests have recently been converted primarily by sowing fir seeds but also by introducing fir seedlings under the crown shelter of beech stands. These reasons have led to an increased nursery production of fir in Croatia. In the distribution range in Croatia and a part of Slovenia, two haplotypes of silver fir were found according to the research results of variability of mtDNA fragment *nad5-4* (32). In northern Croatian provenances (Macelj and Trakošćan) haplotypes of Nordmann's fir (*Abies nordmanniana*/Steven/Spach) were found. It is most likely that it was introduced by planting of seedlings and that further introgression of Nordmann's fir genes into local population occurred through random cross-breeding with silver fir.

In Bosnia and Herzegovina, first known activities on selection of seed stands of silver fir began in the beginning of the fifties of the last century, and by the sixties 52 stands were selected on 2,025.2 ha. During that period, experts ambitiously planned to obtain 241,330 kg of fir seed material that was supposed to be used in direct sowing in forests, and for turning pure beech forests into mixed ones. In order to accomplish that, 13,000 ha, comprising 250 to 350 seed stands, was supposed to be selected in mixed and pure forests, which has turned out as an unrealistic goal. During the sixties, that concept was discarded, and there was stagnation in this field until the beginning of the eighties. At that time, selection of new and revision of old seed stands began. Although some activities were planned very ambitiously, they were terminated due to the last war which lasted from 1992 to 1995, and no new activities were initiated until 1999.

After the war, selection of new and revision of old seed stands have begun and in total 727.06 ha were selected in the entire Bosnia and Herzegovina. With respect to the forest reproductive material, that number cannot satisfy the needs since not all ecological and vegetation regions are represented, and it cannot be satisfactory since it might cause loss of genetic diversity.

The relation toward artificial rejuvenation of silver fir is connected to many problems, ranging from complicated production in plantations due to special ecological demands to weak presence of gene pools in produced material. Hence, while performing artificial regeneration of silver fir, special attention should be given to local populations.

Based on all the above mentioned problems, we could assume that the current number of seed stands does not match the importance of this species, and especially is not enough for sustainability of its genetic potential in the central Dinaric Alps. This area is highly specific regarding environmental conditions, encompassing a large variety of climatic, edaphic, orographic, and other factors in a very small area that are directly affecting the differentiation of locally adapted ecotypes. For that reason, it is believed that forest tree species from the area of Dinarides show a large variability in comparison to the same species from the northern area of its distribution.

The primary goal of our study was to delineate the contact zones of postglacial migration routes in the territory of Dinaric Alps, estimate the possibilities for common provenance regions and use of forest reproductive material in the region, and define any specific locally adapted and/or introduced populations of silver fir in the studied territory.

MATERIAL AND METHODS

Origin of plant material

The investigated silver fir populations originate from Bosnia and Herzegovina and from Croatia (Table 1). Twenty-four selected populations from contrasting environments were sampled (from different plant communities and geological substrates).

Methods

Dormant buds from over 80-year-old trees were collected. The distance between the sampled trees was at least 50 meters. A hundred trees were sampled in the largest population (Vranica), while in other populations the number of scored individual trees was lower (Tab. 1). Isoenzyme gene markers were used to determine the genetic structure of sampled accessions. Technical procedures and genetic interpretation of zymograms followed the proposed protocol (21, 40).

Statistical analysis

Nine enzyme systems were investigated; the polymorphic gene loci scored and the number of alleles per

TABLE 1

Some basic data on investigated populations, longitude and latitude, (n) the number of analyzed samples, position of populations in Bosnia and Herzegovina and Croatia, data in previously studied populations (sd) (a Ballian 2003; b Ballian and Kajba 2005; c Bilela and Ballian 2008; d Ballian 2009).

Population	Longitude	Latitude	n	position	sd
Fužine (CRO)	45°18'	14°41'	85	W	b
Gerovo (CRO)	45°18'	14°36'	32	W	b
Skrad (CRO)	45°26'	14°53'	32	W	b
Vrbovsko (CRO)	45°24'	15°03'	40	W	b
Oštrej (BiH)	44°28'	16°23'	50	W	d
Grmeč (BiH)	44°33'	16°39'	50	W	d
Bos. Grahovo (BiH)	44°11'	16°35'	50	W	d
Troglav (BiH)	43°57'	16°33'	50	W	d
Glamoč (BiH)	44°01'	16°50'	50	W	d
Čabalja (BiH)	43°32'	17°35'	48	SW	a
Biokovo (CRO)	43°08'	17°08'	49	SW	a
Bugojno (BiH)	44°02'	17°18'	50	C	c
Vranica (BiH)	43°56'	17°54'	100	C	a
Igman (BiH)	43°45'	18°16'	50	C	c
Vlašić (BiH)	44°19'	17°29'	50	C	c
Tešanj (BiH)	44°34'	18°00'	41	C	a
Zavidovići (BiH)	44°18'	18°15'	50	C	d
Očevija (BiH)	44°10'	18°27'	50	C	d
Jahorina (BiH)	43°44'	18°34'	50	E	c
Klis (BiH)	44°06'	18°41'	50	E	d
Knežina (BiH)	43°59'	18°45'	50	E	d
Romanija (BiH)	43°54'	18°39'	50	E	d
Kalinovik (BiH)	43°29'	18°35'	72	SE	a
Orjen (BiH)	42°38'	18°33'	49	S	a

locus are listed in Tab. 2. Based on allelic frequencies, the degree of genetic variation within and between populations was quantified using various genetic parameters, such as:

- genetic multiplicity: A/L = mean number of alleles per locus
- genetic diversity: (n_c) = multilocus diversity; v_{gam} = hypothetical gametic multilocus diversity (41); heterozygosity (H_b = observed level of heterozygosity and H_e = expected level of heterozygosity (42); – subpopulation differentiation (Dj), (43). The computation was carried out employing SAS macros (MACGEN – STAUBER AND HERTEL 1997: <http://www.mol.schuttle.de/wspc/genetik1.htm>).

Cluster analysis was made on the basis of allele frequencies and binary allele distribution by means of the statistical program MVSP, version 3.13 – Kovach Computing Services.

RESULTS

Basic genetic indicators

The analysis comprised 24 natural silver fir populations. Nine enzyme systems were used and 16 gene loci analyzed with a total of 47 registered alleles (see Tab. 1 and 2). Only the gene locus Fest-B was monomorphic in all populations. Nine gene loci had a clear minor polymorphism with one or two rare alleles beside the frequent one (examples of rare alleles: Got – B1 and B4, Got – C4, Idh – B2 and B5, 6Pgdh – A1, Pgi – B1, Pgm – A1 and A4, Sdh – A3). It should be pointed out that all gene loci were included in the calculation regardless of whether they manifested polymorphism or monomorphism.

The values calculated for the genetic variation within populations are given in Tab. 3. The mean number of alleles per locus ranged from 1.56 in a population from Očevija which was formed by the systematic removal of beech over many centuries, to 2.25 in the population Vranica which showed the highest degree of vitality.

TABLE 2

Enzyme systems, E.C. referential number, scored loci and the number of alleles.

Enzyme systems	E.C. Number	Scored loci	Number of alleles
Phosphoglucose isomerase	5.3.1.9	<i>Pgi-A, -B</i>	2,3
Phosphoglucomutase	2.7.5.1	<i>Pgm-A, -B</i>	4,2
Isocitrate dehydrogenase	1.1.1.42	<i>Idh-B,</i>	4
Menadion reductase	1.6.99.2	<i>Mnr-B</i>	2
Shikimate dehydrogenase	1.1.1.25	<i>Sdh-A</i>	3
Leucinaminopeptidase	3.4.11.1	<i>Lap-A, -B</i>	3,4
Fluorescent esterase	3.1.1.1	<i>Fest-A, -B</i>	2,1
Glutamate oxalacetate transaminase	2.6.1.1	<i>Got-A, -B, -C</i>	3,4,4
6Phosphogluconate dehydrogenase	1.1.1.44	<i>6-Pgdh-A, -B</i>	3,3
Total	9	16	47

The genetic diversity parameters, v_{gam} and n_e , showed increased fluctuation between populations. By far the highest diversities were found in the population Vranica ($v_{gam} = 42.36$, $n_e = 1.226$) and Skrad ($v_{gam} = 41.47$, $n_e = 1.212$). Noticeably less diverse were the populations Očevija ($v_{gam} = 13.23$, $n_e = 1.135$) and Zavidovići ($v_{gam} = 14.11$, $n_e = 1,148$). The highest single locus diversity was manifested by the gene locus Lap-B with 3.41. At this gene locus, we identified 3 to 4 alleles to be nearly equally distributed.

We could not make a valid conclusion about population grouping from analyses of the genetic variability and diversity. The same holds for observed and expected heterozygosity.

The highest observed heterozygosity was registered in the Skrad population with 0.183, followed by the Vranica population with 0.172 and the Čabulja population with 0.171. However, in terms of expected values, the populations Skrad and Vranica changed places. Heterozygosity was lowest in the populations Biokovo and Bos. Grahovo with only 0.131.

The fixation index calculation shows that the total inbreeding coefficient (F_{IT}) in western Balkan populations is 1.46%, whereas in terms of populations it ranges from -82% (excess of heterozygotes) in the Očevija population, which has been regenerated naturally following mass beech felling in the last 2000 years (34), to 7.5% (excess of homozygotes) in the Biokovo population, which is small and extremely isolated.

Total differentiation of the investigated populations (δ) amounts to 4.9%. The highest differentiation values were shown by the group of populations from western Croatia (Dj between 6.6 and 8.2%), lying west of the migration contact zone, whereas the lowest Dj value was obtained for the population from Bugojno with only 2.8%. This population is situated in Central Bosnia, in the main range of silver fir.

Cluster Analysis

Several analyses were undertaken, 1000 bootstraps, on the distances and Tanimoto distances calculated from the presence/absence of alleles. As there are no significant differences between the results of the first two methods, we will present a method based on genetic distances according to Tanimoto.

The obtained dendrogram (Fig. 1) derived from genetic distances according to Gregorius showed the grouping of populations from western Croatia (Gerovo, Fužine, Skrad, Vrbovsko), which was to be expected. These

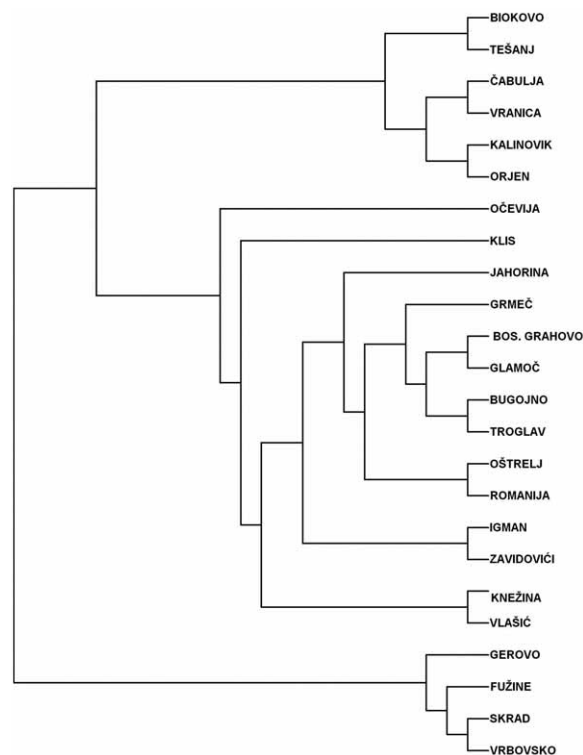


Figure 1. Dendrogram based on genetic distances.

TABLE 3

Average number of alleles (A/L), multilocus (V_p) and gene pool (V_{gen}) diversity, observed (H_o) and expected (H_e) heterozygosity, inbreeding coefficient (F_{IS}) and differentiation according to Gregorius (D_j).

Population	Position	A/L	V_p	V_{gen}	H_o	H_e	F_{IS}	D_j
Fužine (CRO)	W	2.00	21.01	1.172	0.141	0.147	0.003	0.076
Gerovo (CRO)	W	1.87	25.14	1.179	0.154	0.152	-0.004	0.082
Skrad (CRO)	W	1.12	41.47	1.212	0.184	0.175	-0.020	0.080
Vrbovsko (CRO)	W	1.75	25.90	1.186	0.142	0.157	0.026	0.063
Oštrej (BiH)	W	2.25	27.99	1.185	0.153	0.156	0.024	0.036
Grmeč (BiH)	W	1.75	22.63	1.163	0.133	0.140	0.039	0.038
Bos. Grahovo (BiH)	W	1.37	20.83	1.163	0.131	0.140	0.049	0.035
Trogjav (BiH)	W	1.37	31.92	1.192	0.152	0.161	0.031	0.040
Glamoč (BiH)	W	2.87	26.78	1.186	0.144	0.157	0.031	0.032
Čabulja (BiH)	SW	2.62	27.69	1.196	0.171	0.164	-0.028	0.046
Biokovo (CRO)	SW	1.12	17.85	1.168	0.131	0.144	0.075	0.066
Bugojno (BiH)	C	2.25	30.83	1.191	0.145	0.160	0.008	0.028
Vranica (BiH)	C	2.50	42.37	1.226	0.172	0.184	0.051	0.053
Igman (BiH)	C	2.00	26.10	1.184	0.141	0.156	0.023	0.038
Vlašić (BiH)	C	1.37	28.49	1.185	0.154	0.156	-0.005	0.047
Tešanj (BiH)	C	2.00	18.87	1.171	0.139	0.146	0.086	0.062
Zavidovići (BiH)	C	1.50	14.11	1.148	0.153	0.129	-0.068	0.054
Očevija (BiH)	C	1.62	13.23	1.136	0.153	0.119	-0.082	0.045
Jahorina (BiH)	E	1.75	29.35	1.180	0.156	0.159	-0.001	0.039
Klis (BiH)	E	2.62	27.22	1.190	0.144	0.160	0.072	0.054
Knežina (BiH)	E	2.00	24.90	1.177	0.153	0.151	-0.013	0.049
Romanija (BiH)	E	1.75	20.89	1.164	0.145	0.141	-0.010	0.035
Kalinovik (BiH)	SE	2.87	19.17	1.169	0.123	0.144	0.061	0.041
Orjen (BiH)	S	2.62	26.74	1.194	0.153	0.162	0.046	0.038

populations are located west of the contact zone. On the other hand, populations from Bosnia and Herzegovina did not manifest any grouping patterns, the reason being a strong and irregular gene flow from the contact zone eastwards, and vice versa. Mention should also be made of the distinctly present anthropogenic impact in the central Bosnia.

The second analysis was made on the basis of Tanimoto distances (Fig. 2), calculated from present and absent alleles. The grouping of populations from Western Croatia was also present, and so was the grouping of the Očevija population and an independent position of the Orijen population in relation to the rest of Bosnian-Herzegovinian populations. This was expected for the Očevija population due to the fact that this population suffered a strong anthropogenic impact for over 2,000 years. On the other hand, the Orijen population is isolated in the southernmost part of the study area, with the lowest impact of gene flow from the west. As a result, it contains the largest number of alleles characteristic for the Balkan glacial refugium. Other populations are under a stronger or weaker influence of the gene flow from the west. Thus, the grouping of populations in this den-

drogram based on the presence and absence of alleles is similar to that obtained on the basis of genetic distances (Fig. 1).

Analysis of hybrid zone according to investigated alleles

Statistic analysis conducted on the matrix with all gene loci could not yield valid results related to regular clinal variability for some alleles. The specific features of the Dinaric Alps prevented us to obtain the expected groupings and differentiation. It is a well known fact that the Dinaric Alps, in terms of environmental conditions, display high diversity of climatic, edaphic, orographic and other factors at distances of as short as 10 km. These factors can have a direct effect on the genetic structure and differentiation of different ecotypes (33).

This survey will only present those gene loci in which it was possible to register a hybrid impact area.

The 6Pgdh – B1 genetic allele is characteristic of the southern Balkan area (Fig. 3.). It should be present up to the boundary of the distribution range of silver fir originating from the Balkan glacial refuge. The situation

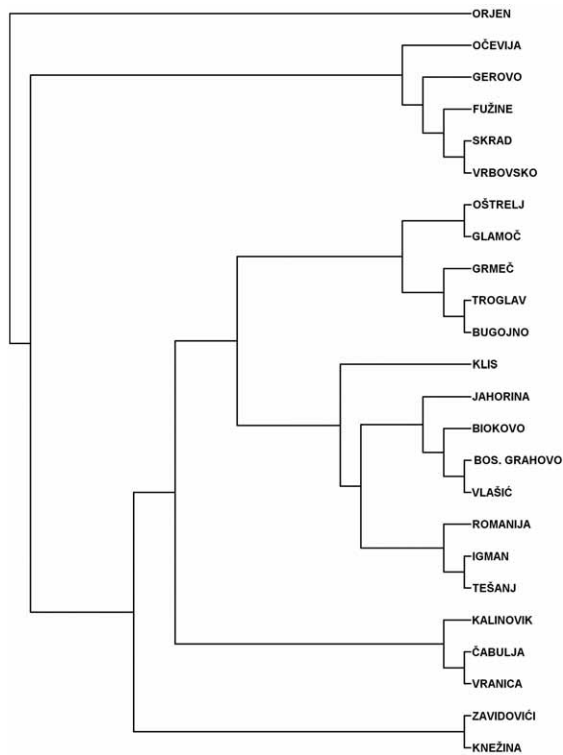


Figure 2. Dendrogram based on binary matrix according to Tamoto's distances.

here is very interesting because this allele is present along the southern Dinaric Mountain (39) and passes west of the boundary obtained on the basis of haplotype analysis *nad5-4* (Fig. 3). Thus, it was registered in the Fužine population in Gorski Kotar, in northern Italy, in the Tatra Mountains, and in the central massife in France (14), as well as in the Alps (20). Further analysis revealed that this allele was not registered in a group of populations distributed north and northeast from the distribution zone of the B1 allele (Fig. 3). In the research (14) it was not registered in the population from Bosnia and Herzegovina (Donja Stupčanica = Klis), because this population belongs to a group of northeastern populations.

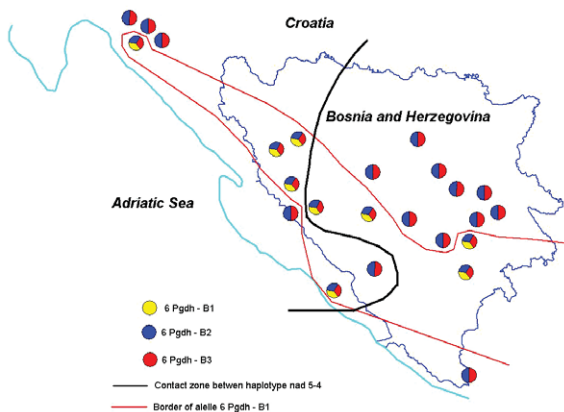


Figure 3. Distribution of gene locus 6Pgdh-B.

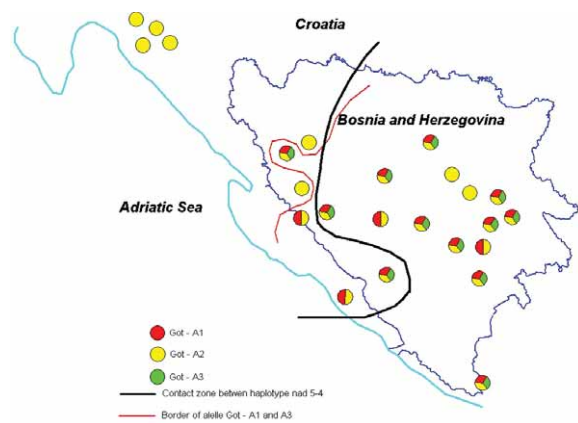


Figure 4. Distribution of gene locus Got-A.

In the Got-A gene locus, the distribution range of the A1 and A3 alleles reaches east of the haplotype boundary (Fig. 4.). Even though these alleles can also be found slightly to the west of the mentioned boundary, they were not registered in Gorski Kotar in Croatia, which confirms their Balkan affiliation. The A2 allele is also widely distributed east of the haplotype boundary.

The gene locus Got-B is characterized by the fact that the B1 allele is found in the populations from the Tatra Mountains, the Carpathians and Bulgaria. In this research, it was registered in two populations in eastern Bosnia where its distribution boundary is west-oriented (Fig. 5.). In this gene locus, the occurrence of the B3 allele is interesting because this allele is characteristic of western European populations but was not registered in the populations of southern Italy (14). Still, it was registered in the populations of Gorski Kotar and in only three populations in Bosnia where it penetrates east of the haplotype boundary, which is in western Bosnia.

A characteristic trait of the gene locus Got-C (Fig. 6.) is that it manifests homogeneity for three alleles, whereas the C4 allele occurs in the central part and is slightly more distributed west of the haplotype boundary. Thus, it is present in two populations in the east, and in only

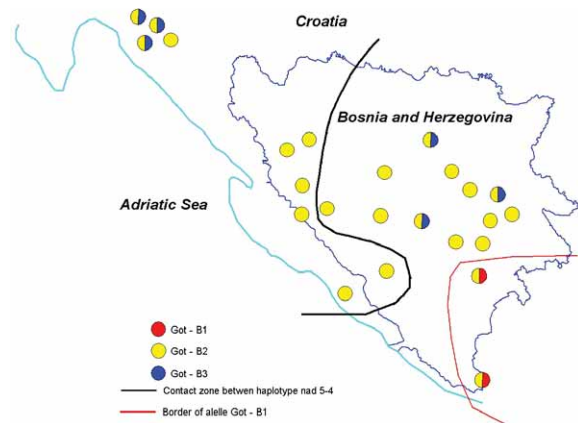


Figure 5. Distribution of gene locus Got-B.

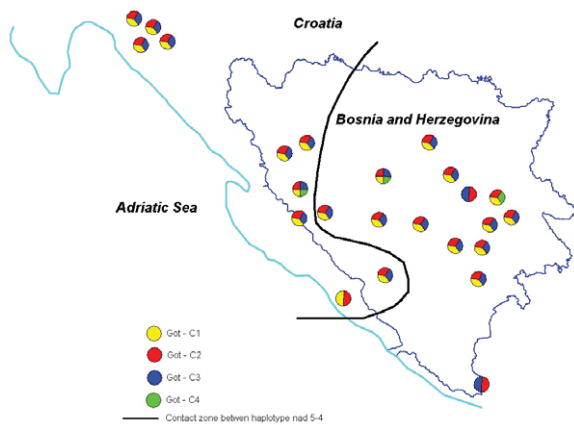


Figure 6. Distribution of gene locus *Got-C*.

one in the west, immediately along the boundary. Therefore, we can presume that this allele is characteristic of the populations originating from the Balkan glacial refuge, albeit being relatively rare.

The gene locus *Lap-A* is characterized by the occurrence of the A5 allele which was registered in the western part of the silver fir distribution range included in this study. This allele is distributed from the west to the east of Bosnia, where it disappears. However, it was not registered in southeastern and southern areas, or in several northern populations (Fig. 7.). Thus, this allele reaches eastern populations of silver fir across the haplotype boundary in western Bosnia.

In the gene locus *Mnr-B*, only the B2 and B3 alleles were registered. They were registered in practically all populations, except for the absent B2 allele in seven populations (Fig. 8.). Otherwise, the B2 allele is characteristic of the population from the south of the Apennine Peninsula where it was registered (14), as well as of the investigated populations.

The gene locus *Pgi-A* is characterized by the presence of the A1 allele in southern populations of the Balkan Peninsula. In our study it was also registered slightly more westwards from the haplotype boundary, but it was

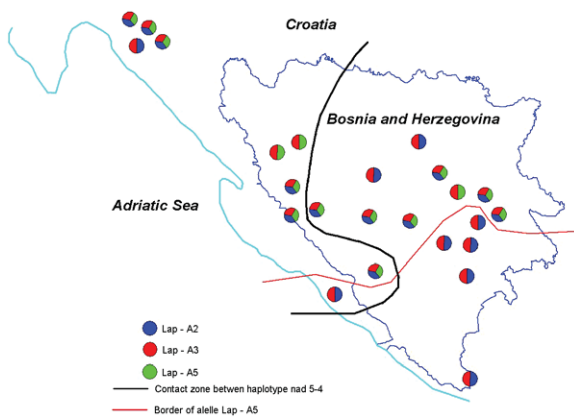


Figure 7. Distribution of gene locus *Lap-A*.

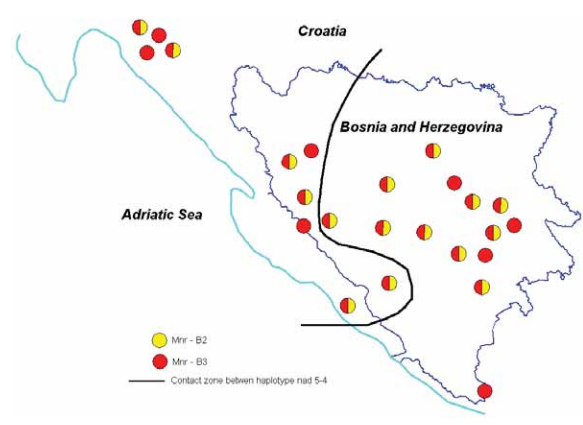


Figure 8. Distribution of gene locus *Mnr-B*.

not registered in the populations from Gorski Kotar. This confirms its distribution from Bulgaria and Macedonia up to western Bosnia (Fig. 9.). The second allele, the A2, is characteristic of all populations, and especially those from the west.

In Bulgaria, the alleles B1 and B3 were registered in the gene locus *Pgi-B* (14). They were also registered in our study, but only as rare alleles (Fig. 10.). Thus, the B1 allele was found in only two populations, the westernmost being in central Bosnia (the Vranica population), and the second in the far south (the Orjen population). The situation is somewhat different for the B3 allele as it is present in three populations. The westernmost population is situated immediately along the haplotype boundary (the Glamoč population), the second one in central Bosnia (the Vranica population) and the third one in eastern Bosnia (the Knežina population).

The A2 allele in the gene locus *Pgm-A* was found in all populations, but with increased frequency in western, northern and southern populations, while the A3 allele was found only in the central part of the Dinaric Alps. It expands slightly more to the west from the haplotype boundary (Fig. 11.). This gene locus is also characterized by the occurrence of rare alleles such as

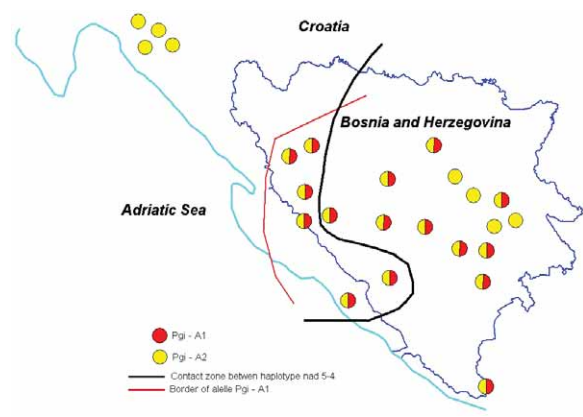


Figure 9. Distribution of gene locus *Pgi-A*.

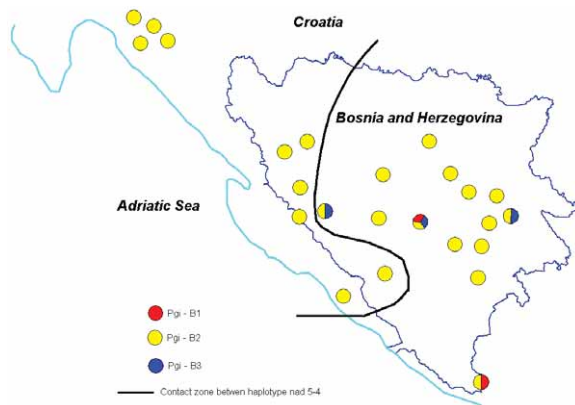


Figure 10. Distribution of gene locus Pgi-B.

the A1 and A4, which are found in the eastern part of Bosnia.

The gene locus Sdh-A shows a distinct area containing homozygotic A3 allele in eastern Bosnia, and the A2 allele in Croatia (Fig. 12.). The A1 allele was found slightly more to the west from the haplotype boundary, while the A2 allele reaches central Bosnia and the southern Orjen population possibly as the consequence of its migration to east and south. There is also a very rare A1 allele in this gene locus in central Bosnia. This allele is in the mixing zone of the A3 and A2 alleles, located in the very centre of the Dinaric area.

The analysis of the Balkan migration route of the silver fir has established the contact zone between silver firs originating from two glacial refugia (39). Nevertheless, this study failed to determine the extent of the influence of gene pools from both refugia. Our study supports the established impact of the Apennine pool east of the contact zone. Even though the analysis of general genetic parameters treated in this research, or the analysis of genetic distances presented through a dendrogram, could not provide a valid conclusion, we obtained the expected selection and grouping of populations from western Croatia to belong to the group of popula-

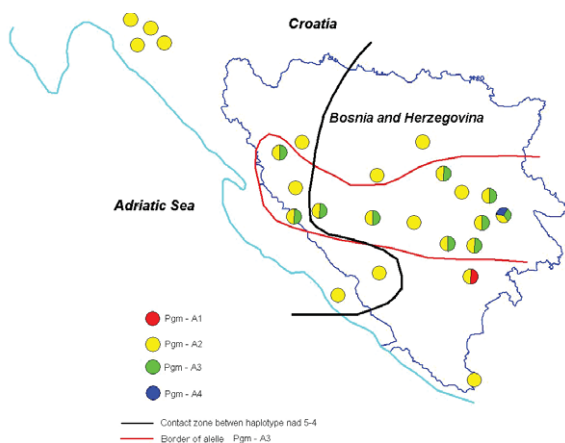


Figure 11. Distribution of gene locus Pgm-A.

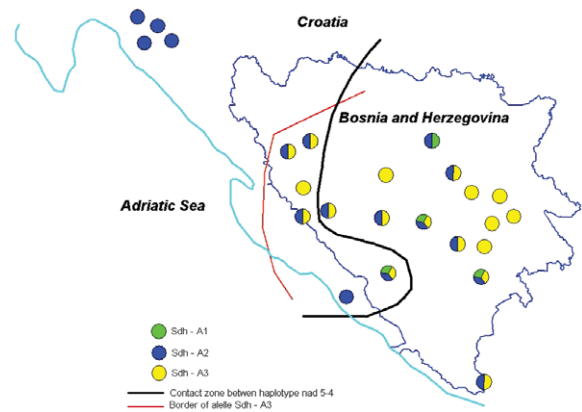


Figure 12. Distribution of gene locus Sdh-A.

tions west of the contact zone. Grouping in the populations located along the contact zone and in the populations located east of the zone did not manifest any regularity, nor any distinct clinal variability. Contrary to this research, clinal variability was also found to be affected by different factors and adaptability along the ecological gradient of one species, or along its migration route from the glacial refugia (20). Consequently, the basic reason for not obtaining clinal variability in this study can be based on the fact that there are no significant continuous ecological gradients in the central Dinaric Alps (western Balkan area). However, there were certain traces of clinal variability in some gene loci. The main reasons for this are highly heterogeneous ecological conditions in the Dinaric Alps area (44) where distinct ecological contrasts exist at distances of only several kilometers, as well as fragmentation and the occurrence of genetic drift (45, 46). This is also visible in sudden changes of forest vegetation and forest types (44). Therefore, there is no question of any large continuous ecological gradient of a particular species, as is the case in central and Eastern Europe, or in the Alps and the western Carpathians in the case of silver fir.

In Bosnia and Herzegovina and in Croatia, silver fir does not form large coherent complexes (populations) (47, 48). Its populations are divided by geographic barriers, such as river valleys and mountain massifs. Its distribution is not continuous; instead, it forms bigger and smaller, relatively fragmented units, and numerous small isolated populations in northern and southern parts of Bosnia and Herzegovina and Croatia. These islands are usually burdened with genetic drift, as reported for the Mediterranean area in general (33, 34, 35, 45, 46). With reference to distribution, we should also indicate strong anthropogenic impacts that have left severe traces on silver fir distribution in central and northern Bosnia and have led to the disappearance of large complexes of fir forests (49).

Much has already been said on the migration route of silver fir in the western Balkans (14, 39). Yet, there are still some unsolved issues concerning the arrival of silver fir from the Apennine glacial refuge, whose mtDNA with

nad5-4 marker was registered all through to the lower course of the river Neretva, i.e. the Biokovo population (24) and Čabulja (author's note). A question here is whether the Apennine fir arrived before or after the fir from the Balkan glacial refuge. We could even speculate on a secondary glacial refuge of the silver fir, since secondary refuge was reported for white oaks in central Dalmatia (22). In addition, if we perform a detailed analysis of migration maps of silver fir for the estimated distribution 7,500 years ago, there is enough ground for speculation (15).

CONCLUSIONS

The results of this study show that some alleles characteristic of the Balkans occur in central Europe, and *vice versa*. Hence, we can conclude that silver fir derived from the Balkan refuge may have been more widely distributed in the past, but is now constantly retreating, while the Apennines-derived silver fir takes its place, or, on the other hand, it may have reached southern Germany through a gene flow similar to the central Dinaric Alps.

In order to reach a conclusion, it is necessary to include certain ecological factors, in particular the role of wind as the main migration vector and an invaluable factor of seed dispersal and gene flow. The analysis of our research area, the central Dinaric Alps shows high participation of western winds along the Dinaric Mountain range, followed by southern and southwestern, as well as northern winds, while eastern and southeastern winds are relatively rare. Such wind distribution and mountain massif orientation allow the species arriving from the western quadrant to expand much more rapidly than those arriving from the eastern and southeastern quadrant, where the central Balkan glacial refuge was located. This may be one of the basic reasons for lower distribution of fir originating from this glacial refuge. In addition to the already mentioned distribution of mountain massifs, we should once again take note of the direction of the Dinaric Alps, which run from the west – northwest to the southeast. These massifs and their favorable distribution do not prevent the movement of air masses from the western quadrant. This might be the reason for the silver fir from the Apennine glacial refuge, which arrived in the south Alps earlier, to have had a distinct advantage in its expansion both to central Europe and to the south, the western Balkan area. In terms of the second migration route of silver fir from the Balkan refuge across the Carpathians, the situation is similar. The fir derived from the Balkan refuge reached only the central part of the Ukrainian Carpathians, although its influence can be felt much further along the Carpathian massif all the way to Slovakia.

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