



Genetic Characterization of Common ash (*Fraxinus excelsior* L.) populations in Bosnia and Herzegovina

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Abstract

Background and Purpose: Common ash (*Fraxinus excelsior* L.) is a species with a widespread distribution in Europe. It mostly grows on mesophyll sites, mainly in karst valleys and by forest creeks, and in Herzegovina at northern expositions and higher altitudes with somewhat better types of soil.

Materials and Methods: We studied 10 natural populations. We collected dormant buds with branches from 24 trees, randomly selected, but equally distributed in the population. We used six pairs of highly polymorphic microsatellite loci for identification and verification of genetic differentiation (FEMSATL 04, 10, 11, 12, 16 and 19). We additionally analyzed integration between genetic and ecological data for the area where material was selected, and also defined genetically homogenous areas of common ash.

Results and Conclusions: The research of intra-population genetic structure pointed at the presence of a high level of polymorphism. Confirmation for such high polymorphism is in populations with approximately 10.2 alleles per locus (N) of the observed heterozygosity (H_o), while in the expected heterozygosity the values were considerably higher. Analysis of the inbreeding coefficient showed the value of 0.312, which demonstrates the presence of inbreeding in investigated populations. In this study we obtained a very low value of differentiation between populations, with the FS_T value as low as 0.023; this value measures the proportion of the total genetic diversity due to differences among populations. By correlation of the obtained microsatellite data and basic ecological parameters, climate for instance, common ash was grouped into two groups. The remaining five studied populations could not be grouped to either of these two, or a separate one, and they represent autonomous genetic units.

INTRODUCTION

Common ash (*Fraxinus excelsior* L.) is a species of a rather widespread distribution in Europe, including Bosnia and Herzegovina. It never appears in pure forest communities, but can mostly be found in small groups and as separate trees. The occurrence of the said species comprises ecologically quite differentiated forest communities, most of which are to be found in Bosnia and Herzegovina, with the exception of southern and southwestern parts of the country which are characterized by a draught that the species cannot stand. Furthermore

Common ash mostly grows on mesophyll sites, mainly in karst valleys and by forest creeks. In Herzegovina, the species is more common at northern expositions and high altitudes with better types of soil. The best known locations for ash are the areas of central Bosnia, where it forms specific phyto-associates with mountain maple, better known as Illyrian forest of ash and maple or *Aceri-Fraxinetum illyricum*, Horv., 1938. Otherwise, common ash grows scattered, and as such it can be found in the entire Bosnia and Herzegovina as an additional species in economically significant forests. Even though it is quite rare, its economic significance in forests is enormous mainly due to the high quality of wood, which makes it a valuable resource.

All changes that result from human conscious or unconscious influence in forests and forest communities also affect this valuable species. Thus, because of the value of its wood, this species has considerably declined in forests. Due to such conditions, there is an increased need to protect the gene-pool, as well as the seed and reproduction material.

One of the most promising possibilities for protection of this valuable species in changed economic and ecological situation lies in the methods of molecular-genetic identification of populations and the formation of *in situ* and *ex situ* archives, as well as in securing seeds and planting material of high genetic quality.

The purpose of this study was to analyze variation of molecular markers, namely microsatellites, in order to obtain tools for molecular identification and genetic differentiation of the populations of Common ash. Besides fundamental significance of this investigation, results of the research could also be useful for forests management, and above all for further establishment of breeding of common ash in order to control the origin of seeds and the planting material necessary for artificial rejuvenation of degraded populations, as well as for the activities related to the founding of gene banks and archives by *in situ* and *ex situ* methods.

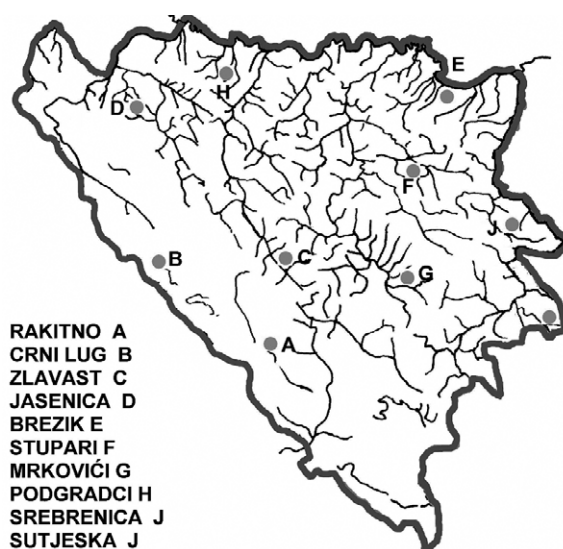


Figure 1. Geographic distribution of the studied populations of common ash.

MATERIAL AND METHODS

We studied 10 natural populations (Figure 1, Table 1), which represent the diverse climatic areas where common ash grows in Bosnia and Herzegovina. All selected populations that were the subject of this study are natural, and distances among the trees that were selected were at least 50 m in order to decrease the likelihood of sampling closely related individuals. At the beginning of February 2004, we collected randomly chosen dormant buds with branches from 24 trees per population.

Extraction of DNA was performed with the SIGMA »GenElute™ Plant Genomic DNA Miniprep Kit«. Six pairs of microsatellites were used for identification and verification of genetic differentiation (FEMSATL 04, FEMSATL 10, FEMSATL 11, FENSALT 12, FEMSATL

TABLE 1

Locations of common ash populations considered in the study.

Population	Location	Latitude (°N)	Longitude (°E)	Altitude (m a.s.l.)
1 Posušje	Rakitno	43°32' 37"	17°23' 16"	940–950
2 Bosansko Grahovo	Crni lug	44°03' 37"	16°35' 22"	740
3 Bugojno	Zlavast	43°53' 35"	17°37' 15"	700–800
4 Bosanska Krupa	Jasenica	44°48' 20"	15°15' 02"	400–450
5 Sarajevo	Mrkovići	43°53' 01"	18°25' 20"	900
6 Bosanska Gradiška	Podgradci	45°03' 21"	17°03' 36"	200–400
7 Lopare-Brčko	Brezik	44°48' 56"	18°51' 55"	130–150
8 Srebrenica	Srebrenica	44°03' 39"	19°17' 07"	600–800
9 Rudo	Sutjeska	43°34' 15"	19°19' 10"	550–700
10 Kladanj	Stupari	44°18' 31"	18°42' 21"	700–800

16, FEMSATL 19). Forward primers were marked with fluorescent color (IRD 700 and IRD 800) at their 5' ends. PCR reactions were performed on a Perkin Elmer GeneAmp® PCR System 9600 thermocycler. Electrophoresis and detection of PCR products were carried on a denaturing polyacrylamide gel (6%) using a sequencer (model DNA 4200 Sequencer LI-COR® Biotechnology). Data were collected by e-Seq software (DNA Sequencing and Analysis Software).

The following software was used to process the obtained data for determining the level of intrapopulation variability and genetic differentiation: Genepop 3.4 (1), Fstat 2.9.3. (2), Gendist 3.6 (3) and Neighbor 3.6 (4).

We additionally analyzed integration between genetic and ecological data of the areas where the material was collected, and also defined genetically homogenous areas of common ash (Regions of Provenance). Climate data is listed in Table 2 (5).

RESULTS

The estimation of genetic variability of common ash populations from Bosnia and Herzegovina by means of six microsatellite loci pointed at the presence of a high level of polymorphism in all investigated populations, with the presence of high numbers of alleles in each in-

TABLE 2

Elementary climatic data of the areas characterized by the growth of populations analysed in the study.

Population	Location	Meteorology stations and altitude (m)	Logical mean annual temperature in °C	Temperature during vegetation period V-IX in °C	Days with an average temperature > 10°C	Yearly precipitation mm	Precipitation during vegetation period V-IX mm
Posušje	Rakitno	Rakitno 915	9,4	14,8	173	1983	624
Bosansko Grahovo	Crni lug	Bosansko Grahovo 861	8,2	13,9	166	1356	558
Bugojno	Zlavast	Bugojno 562	8,8	14,8	174	826	383
Bosanska Krupa	Jasenica	Bosanska krupa 176	10,3	16,2	198	1304	666
Lopare-Brčko	Brezik	Brčko 96	11,2	17,9	211	781	429
Kladanj	Stupari	Kladanj 560	8,9	15,1	183	1028	602
Sarajevo	Mrkovići	Sarajevo 630	9,7	15,5	186	946	450
Bosanska Gradiška	Podgradci	Bosanska Gradiška 95	10,6	17,2	185	831	457
Srebrenica	Srebrenica	Srebrenica 400	9,5	15,9	192	1027	579
Rudo	Sutjeska	Čajniče 816	8,1	14,6	166	1147	579

TABLE 3

Allelic diversity of the microsatellite loci scored in common ash populations.

Locus*	Repeat motif	Number of alleles	Molecular weight (range in bp)
FEMSATL 04	(CA) _n (AG) _m	20	155–197
FEMSATL 10	(CT) _n	55	149–335
FEMSATL 11	(GA) _n (TA) _m	24	179–228
FENSALT 12	(GA) _n CA(GA) _m	28	177–259
FEMSATL 16	(CA) _n CG(CA) _m	12	182–214
FEMSATL 19	(CA) _n CGGC(CA) _m	26	142–210

*(6)

vestigated locus (Table 3). The mean number of alleles per locus was on average 10.2 (Table 4); Rakitno had the lowest value (7.5) and Sutjeska the highest (12.0).

The observed heterozygosity was on average 0.543 and ranged from 0.433 in the population of Podgradci, to 0.639 in the Mrkovići population (Table 4). The mean expected heterozygosity was higher, ranging from 0.761 (Mrkovici) and 0.830 (Sutjeska), with an average value of 0.789. Consequently, a general excess of homozygotes was found in every population, as confirmed by the positive values assumed by fixation index (0.312 on average).

Genetic diversity between 10 studied populations of common ash in Bosnia and Herzegovina was rather high, yet the genetic differentiation among them was low: only 2.3 % of the total genetic diversity resulted from the actual differences among populations (mean $F_{ST} = 0.023$) (Table 5). Both in the case of genetic diversity and inbreeding, we could consider the small number of samples and trees in the stands as a possible cause, leading to weak gene flow between the populations and most prob-

TABLE 4

Statistics of genetic variations within populations at six microsatellite loci. N, average number of alleles per locus; H_o , average observed proportion of heterozygotes; H_e , average gene diversity; F_{IS} , average inbreeding coefficient.

Population	N	H_o	H_e	F_{IS}
Rakitno	7.5	0.528	0.788	0.317
Crni lug	11.3	0.513	0.789	0.350
Zlavast	11.2	0.459	0.774	0.407
Jasenica	10.7	0.635	0.801	0.208
Brezik	10.8	0.541	0.822	0.342
Stupari	9.3	0.567	0.781	0.275
Mrkovići	10.7	0.639	0.761	0.159
Podgradci	10.2	0.433	0.778	0.444
Srebrenica	8.3	0.473	0.774	0.389
Sutjeska	12.0	0.637	0.830	0.233
Mean	10.2	0.542	0.789	0.312

TABLICA 5

F-statistics at the six microsatellite loci considered in the study. F_{IT} , overall inbreeding; F_{IS} , differentiation within populations; F_{ST} , differentiation among populations.

Locus	F_{IT}	F_{IS}	F_{ST}
FEMSATL 04	0.401	0.393	0.013
FEMSATL 10	0.401	0.371	0.047
FEMSATL 11	0.335	0.328	0.010
FEMSATL 12	0.441	0.426	0.025
FEMSATL 16	0.184	0.156	0.033
FEMSATL 19	0.176	0.167	0.011
Multilocus estimate	0.336	0.320	0.023

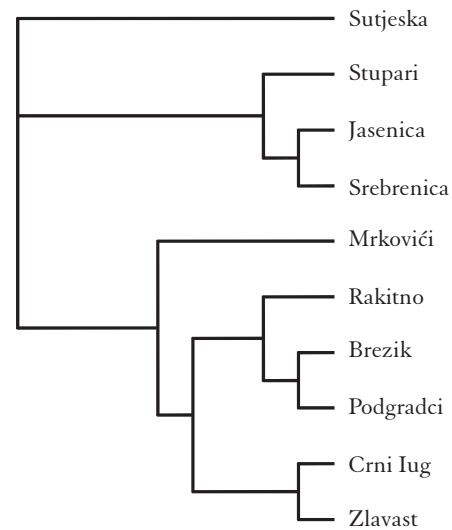


Figure 2. A dendrogram based on genetic distances between ten ash populations, obtained with the UPGMA (unweighted pair-group method based on arithmetic averages).

ably the inclination to inbreeding of individuals that comprise a population, as well as a possible anthropogenic influence along with irregular cut and unplanned forest management of this tree species.

By correlation of the obtained microsatellite data and basic ecological parameters, climate being one of them, common ash populations were grouped into two groups; however, a correlation with the type of soil could not be clearly established. The first group is the Pannonian group, with two populations located in the Pannonian basin (Podgradci and Brezik), and the second one the Mežan group, with three populations of Eastern Bosnia (Sutjeska, Srebrenica and Stupari). The remaining five studied populations could not be grouped to either of these two, or even form a separate one, and they represent autonomous genetic units.

The obtained dendrogram shows that populations are genetically differentiated, and that each of them represents a specific population. Based on results obtained in this study (Figure 2), the populations can be grouped into three groups, where the population of Sutjeska autonomously builds one of the clusters (or groups), the population of Stupari, Jasenica and Srebrenica the second cluster, and the remaining populations form the third cluster which can be further divided into two new clusters, and one of those can be further divided into lower clusters. The interesting point is that the first two clusters group three populations from eastern Bosnia, which also group in correlation to climatic parameters.

DISCUSSION

The area of Dinaric Alps is very specific with regard to environmental conditions, since in a rather small area there is a great variety of climatic, edaphic, orographic conditions and other factors that directly influence the

differentiation of several ecotypes. Various experts, therefore, consider that the types of forest trees from the area of Dinaric Alps and Southeastern Europe show greater variability in comparison with the same forest trees from the North, as estimated by allelic richness (6, 7, 8, 9). This investigation thus confirms the existence of high polymorphism, which was also confirmed by studies on common ash in the area of Bulgaria (10).

Since the populations of common ash in Bosnia and Herzegovina belong to the Balkan area, it would be interesting to compare them with reports from the studies that were carried out with the populations from Bulgaria (10). What is significant for the area of the Balkans is that being far south, it represented during the glaciation period one of the refugial areas (11), thus allowing the persistence of great genetic variability located in the western parts of the Balkans. The area of Bosnia and Herzegovina also represents a contact zone of different genetic groups originating from different glacial refugial stands, as confirmed by the studies on silver fir (12, 13, 14), oaks (15, 16), and common ash (9, 17).

Hence, the two different haplotypes of common ash in the area of Bosnia and Herzegovina (17), one typical for Central Europe, and the other for the Balkans, which in turn shows that this is a contact zone. This can be one of the explanations directly correlated to high polymorphism in the studied populations.

The studied populations of common ash in Bosnia and Herzegovina show genetic differentiation between them. In these studies, however, the results could not be confirmed (10), which points to a relatively high genetic variability of common ash and low coefficient of inbreeding for the area of Bulgaria. In our study, we found high positive values of inbreeding, close to the results reported in studies of common ash population in the area of southern France (18). Positive values obtained for inbreeding coefficient can be interpreted as a result of technical problems (non-amplifying alleles), small number of samples, as well as a low gene flow, and most likely the inclination of common ash to inbreeding. And what about the presence of the confirmed null alleles? Also, the individuals that build a population, possibly due to constant anthropogenic influence, provided a specific genetical structure within investigated populations. Thus, in Italy (19), common fir in small isolated populations was influenced by anthropomorphic changes within the period of last 2000 years.

In this study, we obtained a very low value of differentiation between populations, with values of $F_{ST} = 0.023$, which is a lower value than that obtained in the study in Bulgaria (10) ($F_{ST} = 0.087$). In beech (8), the following results were found: $F_{ST} = 0.030$ south Europe and $F_{ST} = 0.040$ for central Europe.

The values that are higher than the values obtained with common ash point to weaker barriers to gene flow, (20), and are directly influenced by different process of postglacial recolonization of common ash.

Thus, in a study of common ash in Bulgaria, the author (10) reported data from a whole sequence of studies carried out on the species of, *Juglans regia*, *Castanea sativa*, *Ulmus minor*, which are also rare, and suggested a need to carry out a research related to pollen dispersion in the species of these valuable deciduous trees, including common ash.

The presented low genetic differences between or within populations were quite apparent. Possible causes for these differences, apart from natural selection and anthropogenic influence, are developmental factors or specific processes of accommodation to certain ecological conditions.

When methodic effects cannot be excluded (such as the number of individuals in a sample), nor developmental and anthropogenic factors, the obtained differences between populations point to a significant role of adaptation processes in all investigated populations. Therefore we can present the following standpoints based on the obtained results:

- The obtained variability is not so typical for western and southeastern Europe, but is more or less typical for the western area of the Balkans, taking into account the size of the territory.
- Grouping of populations from eastern and northern Bosnia, with two climatically different areas. Pannonian and Mezan areas, when calculation includes climatic changes, include only five populations, while the other five are indifferent and confer a particular gravity to this study.

Based on the above details we can conclude that there are differences between populations, and that low genetic differentiation is caused by an entirely different process of postglacial migration, i. e. possibly the specific adaptability of certain gene types to some stands where selection processes were taking place.

CONCLUSION

Common ash in this study, which involved ten natural populations in Bosnia and Herzegovina, showed great genetic variability.

All populations showed permanent presence of a high number of homozygotes, and we favor a high level of inbreeding as the most likely explanation, with the presence of null alleles that was much higher than expected in mostly outbreeding species.

The distribution of genetic variability obtained in this study is only partially related to geographic distance between the studied populations.

Total genetic differentiation is rather low and coincides with that found in similar studies in Europe, and is most probably related with rather weak barriers in gene flow between populations.

The soil did not prove to be a crucial factor in influencing the genetic variability and grouping of the population, while climatic factors proved to be decisive.

The methodology applied in this study showed to be very appropriate, and could be used in the process of forest breeding, management and foresting in general, in seed and seedling production and in planning activities for conservation of common ash both by *in situ* and *ex situ* methods.

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