PERIODICUM BIOLOGORUM
VOL. 112, No 3, 353–362, 2010

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original scientific paper

Genetic variability of Pedunculate Oak (*Quercus robur* L.) in Bosnia and Herzegovina

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Key words: Pedunculate oak (*Quercus robur* L.), nuclear microsatellite, variability

Abstract

Background and Purpose: *The objective of this research was to determine variability of some remaining populations and groups of trees of pedunculate oak (*Quercus robur *L.) at the molecular-genetic level in Bosnia and Herzegovina. The analysis was made by means of highly polymorphic nuclear microsatellite markers, which were used in some earlier research involving pedunculate oak embryos. The obtained results will provide guidelines for proper management with and regeneration of pedunculate oak forests, and for their re-introduction. In particular, this research will contribute to further improvement and conservation of pedunculate oak using in situ and ex situ methods.*

Material and Methods: *This study analyzes molecular genetic variability of 12 natural populations of pedunculate oak (*Quercus robur *L.) in Bosnia and Herzegovina. Molecular genetic variability was analyzed on the basis of DNA by means of the following four nuclear microsatellite markers: ssrQpZAG1/5, ssrQpZAG9, ssrQpZAG36 and ssrQpZAG108. The analysis included a total of 108 alleles.*

Results and Conclusion: *Considerable differences were observed in the frequencies between populations. The same differences were confirmed with analysis of other parameters, such as the effective number of alleles, the fixation index and genetic differentiation. It should be pointed out that this research confirmed higher allele heterozygosis in the populations in relation to pedunculate oak populations in Western Europe. This result suggests that the investigated populations have not lost much of their genetic potential for adaptation. This could be attributed to the vicinity of the studied populations to their glacial refugium. This is the reason for their higher resistance and sturdiness in comparison with the populations of western and central Europe. They posses more genetic variability despite the fact that they had for centuries suffered a strong anthropogenic influence, which brought them to their survival limit and the verge of extinction. The obtained results will allow us to preserve the genetic structure of this valuable species in Bosnia and Herzegovina.*

Received June 30, 2010.

INTRODUCTION

Pedunculate oak is one of the most important forest tree species in Europe. Distributed from Spain to the Urals *(1),* it grows in highly diverse ecological conditions *(2)*. The first data on pedunculate oak distribution in Bosnia and Herzegovina were provided by Beck-Managetta *(3)*. In his survey of the dendroflora of Bosnia and Herzegovina, Fukarek *(4)* makes an infraspecific division of the species *Quercus robur*

L., where he lists the following varieties: var. *robur*, var. *cuneifolia* (Vukot.) Beck., var. *australis* (Heuff.) Simk., var. *latiloba* Lasch, var. *crassicuscula* Borbas and var. *fastigiata* (Lamk) Spach. Much later, Janjić *(5)* describes a large number of infraspecific forms of oak. Contrary to Fukarek and Janjić, Šilić *(6)* mentions only two sub-species in Bosnia and Herzegovina: *Quercus robur* L. as characteristic for the interior of the Dinaric and the Posavina regions, and *Quercus pedunculiflora* K. Koch., which occurs in the southern part (Herzegovina). Bussoti & Grossoni *(7)* report the same findings for the Mediterranean area and point out that pedunculate oak is very rare and that it has almost disappeared from the southern part of Bosnia and Herzegovina during the past hundred years. This species is also known as maritime pedunculate oak.

As a result of anthropogenic activity during the past two centuries and extensive fragmentation, pedunculate oak does not form large forest complexes in Bosnia and Herzegovina and does not represent a commercially important species. According to forest inventories conducted over large areas in Bosnia and Herzegovina, the total area of forests and forestland amounts to 2,501,465 ha, of which high forests account for 1,130,183 ha, coppices and scrub for 841,303 ha, and bare land for 529,979 ha *(8)*. High forests refer to high beech forests, pure and mixed forests of fir and spruce, mixed forests of fir, spruce and beech, forests of black and Scots pine, forests of sessile oak and other high forests. All stands of pedunculate oak belong to the category of other high forests. Based on the data from the mentioned inventory, other high forests cover an area of 32,368 ha, of which 31.7% is estimated to refer to pedunculate oak. However, another source *(9)* mentions that the total area of pedunculate oak forests is 30,000 ha.

According to the available written data, pedunculate oak used to cover large areas in Bosnia and Herzegovina, but it was cut down in a period of some 80 years, or more precisely, between 1830 and 1912. About 3,600,000 old oak trees were cut down for the purpose of obtaining French staves and 375,000 m^3 of stacked wood was cut down from coppices *(10, 11)*. Currently, the majority of pedunculate oak forests in Bosnia and Herzegovina consist of coppices *(9)*. Some of the forests are rarities, such as the well-documented forest of pedunculate oak on Glasinac plateau situated 847 m above the sea *(12, 13),* a relatively unknown forest on the Podbrdo plateau at an elevation of 727 m, and a forest on marshy peat land in Livno field (697 metres above sea level) in the sub-Mediterranean area.

The disappearance of pedunculate oak is due not only to anthropogenic impacts but to many other reasons as well. The primary reason relates to natural hybridization with more broadly distributed sessile oak and to the occurrence of numerous hybrid swarms. This aggravates determination and discrimination of these species and contributes to the gradual disappearance of pedunculate oak. As pointed out by Krstinić *(14),* it is these processes, between pedunculate oak in the first place and sessile oak that are responsible for highly distinct inter-population and intra-population variability. According to Trinajstić *(15),* this leads to high polymorphism and variability of morphological traits of pedunculate oak. This is further accentuated by introgressive hybridization, which occurs as a result of incomplete reproduction isolation among related species of the genus *Quercus* L. Thus, according to Mátyás *(16),* apart from the hybrid *Q. robur* × *Q. petraea* in the Carpathian basin, the main interspecies hybrids of pedunculate oak are *Q.* × *csatoi* Borb. (=*Q. polycarpa* × *Q. robur*), *Q.* × *psudo-delechampii* Cretz. (=*Q. dalechampi* × *Q. robur*), *Q.* × *pendulina* (Kit.) em Maty (=*Q. robur* × *Q. virgiliana*), *Q.* × *sublanuginosa* Borb. (=*Q. pubescens* × *Q. robur*), and *Q.* × *haynaldianai* Simk. (=*Q. frainetto* × *Q. robur*). In the words of JANJIĆ (1998), two hybrid complexes formed by the pedunculate oak were detected in the surroundings of Sarajevo: *Q.* × *rosacea* Bechst. (= *Q. robur* × *Q. petraea*) and *Q. robur* L. × *Q. pubescens* Willd.

No systematic molecular research on pedunculate oak in Bosnia and Herzegovina has been done to date. Haplotype analysis registering haplotypes 4 and 5 was carried out only partially, whereas sub-haplotypes *b* and *c* were registered in haplotype 5 *(17)*. Unlike Bosnia and Herzegovina, there is intensive research of relatively high genetic variability of this important commercial species in Europe *(18, 19)*. Oak provenances were also tested *(20, 21, 22, 23, 24),* with low levels of variability. Genetic characterization of pedunculate oak (25) was also undertaken, and so was haplotypic characterization *(26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37)*.

The objective of this research was to determine variability of some remaining populations and groups of trees of pedunculate oak at the molecular-genetic level. The analysis was made by means of highly polymorphic nuclear microsatellite markers, which Steinkellener *et al. (38),* Lefort *et al. (39),* Barreneche *et al. (40),* Lexer *et al. (41)* and Wilhelm *et al. (42)* used in some earlier research involving pedunculate oak embryos. The obtained results will provide guidelines for proper management and regeneration of pedunculate oak forests, and for their re-introduction. In particular, this research will contribute to further improvement and conservation of pedunculate oak using *in situ* and *ex-situ* methods.

MATERIAL AND METHODS

Trees of pedunculate oak were selected from natural populations during January and February of 2006 (Table 1, Figure 1). Care was taken that populations of similar size and scientific interest were selected. In order to exclude inbreeding, the samples were collected from trees at least 50 to 100 m apart. To identify pedunculate oak trees in the winter dormant period we relied on our knowledge of bark structure, bud form, fruit remains, and the pedunculi and cupula under the trees.

The population in this research consisted of 20 trees, with the exception of the population of Kiseljak with 10 trees and Novi Šeher with 5 trees. The latter populations

TABLE 1

Investigated populations of pedunculate oak in Bosnia and Herzegovina.

No.	Population	Locality	Latitude	Longitude	Altitude (m)	No. of analyzed individuals
1	Cazin	Čoralić	44°58'06"	15°56'04"	352	20
2	Bosanska Gradiška		45°08'29"	17°11'38"	85	20
3	Bosanski Brod	Zborište	45°03'01"	18°00'04"	91	20
4	Orašje	Obudovac	45°01'26"	18°33'37"	80	20
5	Mrkonjić Grad	Podbrdo	44°26'21"	16°59'52"	727	20
6	Jelah	Kalošević	44°39'11"	17°57'23"	185	20
7	Žepče	Polje	44°25'46"	18°03'21"	219	20
8	Živinice	Dubrave	44°26'46"	18°40'26"	226	20
9	Livno	Crni lug	44°00'53"	16°37'48"	697	20
10	Bugojno	Kopčić	44°05'22"	17°26'08"	539	20
11	Sarajevo	Stojčevac	43°48'55"	18°16'54"	494	20
12	Sokolac	Brezik	43°55'51"	18°48'06"	847	20
	Total					240

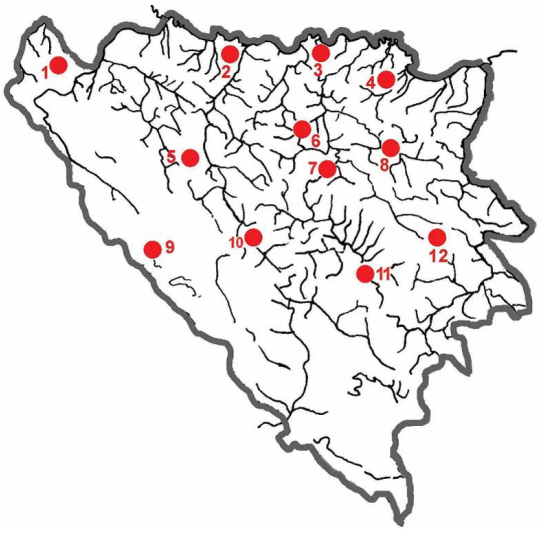

Figure 1. *Studied population: 1.Cazin, 2. Bosanska Gradiška, 3. Bosanski Brod, 4. Orašje, 5. Mrkonjić Grad, 6. Jelah, 7. Žepče, 8. Živinice, 9. Livno, 10. Bugojno, 11. Sarajevo, 12. Romanija.*

were identified but were not taken for further analysis due to the small number of samples.

The size of a population itself frequently forced us to abandon the principle of distance between the trees: a population would sometimes be very small and some old trees would be growing as much as 1 km apart.

The collected samples involved live parts of the plants, in our case dormant buds. After they were collected, the samples were stored at –20 °C until further use.

DNA was extracted from vegetative material by means of SIGMA »GenElute™ Plant Genomic DNA Miniprep Kit«. Four pairs of highly polymorphic nuclear microsatellite markers (SSR) were used for genetic analysis (Table 2), which had been used earlier by Barreneche *et al. (40),* Lefort *et al. (39),* Lexer *et al. (41)* Steinkellner *et al. (38),* Wilhelm *et al. (42)*.

In order to produce the polymerase chain reaction (PCR), we optimized DNA quantities in the solution and then applied a standardized protocol, as well as the

TABLE 2

Elementary data on primers.

Primer		Sequence 3' to 5'	Highly repetitive sequences	Annealing temp.
ssrQpZAG1/5	F	GCT TGA GAG TTG AGA TTT GT	(GT)5(GA)9	55 (58)
	R	GCA ACA CCC TTT AAC TAC CA		
ssrQpZAG9	F	GCA ATT ACA GGC TAG GCT GG	(AG)12	50 (59)
	R	GTC TGG ACC TAG CCC TCA TG		
ssrQpZAG36	F	GAT CAA ATT TGG AAT ATT AAG AGA G	(AG)19	50 (55)
	R	ACT GTG GTG GTA GTC TAA CAT GTA G		
ssrQpZAG108	F	CTA GCC ACA ATT CAG GAA CAG	(AG)13	50 (55)
	R	CCT CTT TTG TGA ATG ACC AAG		

Lefort *et al*., 1998; Wilhelm *et. al.,* 2005

thermal cycler GeneAmp®PCR System 9600, using a slightly modified procedure according to Lefort *et al. (39)* and Wilhelm *et al. (42)*.

The »DNA 4200 Sequencer LI-COR® Biotechnology« was used for PCR analysis.

Data analysis

The total number of alleles per locus (N_a) and the Polymorphism Information Content *(43)* for each microsatellite locus, as well as the average number of alleles N_{avg}, H_O and H_E in each population across loci were calculated using PowerMarker v3.25 software (44). The number of private alleles (N_{pr}) per population was assessed by MICROSAT *(45)*. GENEPOP 4.0 *(46)* was used to estimate the inbreeding coefficients, F_{IS}, and to test population genotypic frequencies across all loci for conformance to Hardy-Weinberg (HW) expectations (multi-locus test).

The population genetic structure of the overall samples was analyzed for each locus with Wright's F-statistics using Weir and Cockerham method *(47)* as implemented in FSTAT ver. 2.9.3.2 *(48)*. Genetic differentiation between all pairs of populations was measured with pairwise F_{ST} estimates. Pairwise F_{ST} and their respective *P*-values for significant differences from zero were calculated in FSTAT. Pairwise Nei's standard genetic distances *(49)* were calculated and unrooted phylogenetic tree was constructed using Neighbour-joining algorithm with 1,000 bootstraps over microsatellite loci as implemented in SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE programmes of the PHYLIP ver. 3.67 software package *(50)*.

The analysis of molecular variance AMOVA *(51)* by means of ARLEQUIN version 2.000 *(52)* AMOVA was used to partition the total microsatellite diversity into (A) among and within populations components, and (B) among three altitude groups (<100 meters above sea level; 100–300 m; >300 m), among populations within groups, and within population components. The variance components were tested statistically by non-parametric randomisation tests using 10,000 permutations.

The program BOTTLENECK version 1.2.02 *(53, 54)* was used to test for evidence of recent bottleneck events on the basis of this theoretical expectation. The gene diversity observed (H_E) was compared to the gene diversity expected at mutation-drift equilibrium (H_{EQ}) and calculated from the observed number of alleles under different mutation models: Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and an intermediate Two-Phase Model (TPM). The IAM *(55)* assumes that each mutation crates a novel allele, the SMM *(56)* assumes that new alleles arise by gain or loss of one repeat unit, and the TPM *(57)* assumes that most mutations follow the SMM but allows multistep changes following a geometric distribution. The TPM model was applied assuming 30% (TPM 1) *(58, 59, 60)* and 5% multistep changes (TPM 2) *(54)*. Based on the number of loci in our dataset, the Wilcoxon sign-rank test *(61)* was chosen for the statistical analysis of heterozygote excess or deficiency as recommended by Piry *et al. (54)*.

Isolation by distance (IBD) among populations was tested using the method of Rousset *(62)*. A Mantel test *(63)* on the matrix of pairwise $F_{ST}/(1-F_{ST})$ ratios and that of the natural logarithm of geographical distances (in km) between pairs of populations was performed using 1000 permutations in NTSYS-pc version 2.10s *(64)*.

RESULTS

Polymorphism was analyzed in twelve populations of pedunculate oak in Bosnia and Herzegovina by means of four nuclear microsatellites (SSR): QpZAG1/5, QpZAG9, QpZAG36, and QpZAG108. The successfully sequenced amplified samples from the analyzed populations and statistical data processing resulted in a total of 108 different alleles (Table 3). The result indicated the presence of high polymorphism in the analyzed microsatellites, i.e., genetic diversity even in the small, isolated populations included in this research.

These results clearly show that pedunculate oak does not have any significant share of non-specific alleles in the investigation with this group of nuclear SSR markers.

According to Table 4, the average number of alleles per population is 11.83. The number of alleles in the Žepče population is 10.00 and in the western Cazin population it is 14.25, which can be attributed to stronger anthropogenic influences on the Žepče population through history. In addition, the analysis of private alleles shows that there are 20 in number, which is a very high number for one such small area under analysis.

As for real heterozygosity, it is smaller than theoretical heterozygosity in all the cases. The existing differences between real and theoretical heterozygosity shows the deviation of the real condition from the state of balance. This was to be expected due to the size of the population and the condition of all natural populations of pedunculate oak in Bosnia and Herzegovina.

In this research, the fixation index shows positive values, which indicates the presence of inbreeding (Table 5). Still, its value in the Cazin population is very low and amounts to only 0.074. The reason could be the openness of the population and its relatively well preserved condi-

TABLE 3

Allelic diversity of the microsatellite loci scored in 12 pedunculate oak populations from Bosnia and Herzegovina.

Locus	Repeat motif	Size range (bp)	N_a	PIC
QpZAG 1/5	$(GT)_5(GA)_9$	156–187	19	0.874
QpZAG 9	$(AG)_{12}$	180–209	22	0.924
QpZAG 36	$(AG)_{19}$	205–240	33	0.932
QpZAG 108	$(AG)_{13}$	186–251	34	0.942

N_a– total number of; PIC – Polymorphic Information Content

TABLE 4

Statistics of genetic variation within pedunculate oak populations at four microsatellite loci.

No.	Population	n	Latitude (N)[a]	Longitude (E)	Altitude[b]	N_{avg}	N_{pr}	H_O	H_E	F_{IS}[c]
1	Cazin	20	44.97	15.93	352	14.25	3	0.838	0.880	0.074*
2	Bosanska Gradiška	20	45.14	17.19	85	12.75	2	0.650	0.866	0.273***
3	Bosanski Brod	20	45.05	18.00	91	12.00	3	0.600	0.857	0.323***
4	Orašje	20	45.02	18.56	80	11.75	2	0.700	0.842	0.193***
5	Mrkonjić grad	20	44.44	17.00	727	10.25	1	0.563	0.851	0.362***
6	Jelah	20	44.65	17.96	185	11.75	2	0.788	0.875	0.125***
7	Žepče	20	44.43	18.06	219	10.00	2	0.625	0.826	0.267***
8	Živinice	20	44.45	18.67	226	12.75	1	0.725	0.860	0.182***
9	Livno	20	44.01	16.63	697	13.75	0	0.638	0.887	0.305***
10	Bugojno	20	44.09	17.44	539	10.50	0	0.563	0.813	0.331***
11	Sarajevo	20	43.82	18.28	494	11.75	0	0.725	0.863	0.185**
12	Romanija	20	43.93	18.80	847	10.50	4	0.700	0.844	0.196***
	Mean					11.83		0.676	0.855	0.234***

n – sample size; N_{avg} – average number of alleles; N_{pr} – total number of private alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient.
[a]N – North; E – East; Coordinates are in degree decimal format. [b]In meters above sea level. [c]Probabilities of heterozygote deficiency: »***« corresponds to significance at the 0.1% nominal level, »**« corresponds to significance at the 1% nominal level, »*« significance at the 5% nominal level and »ns« depicts non-significant values

tion, as well as possible gene flow with Croatian populations from the Pokuplje area. The highest values are exhibited by small and isolated populations of Bosanski Brod, Livno, Bugojno and Mrkonjić Grad. Since Bosanski Brod belongs to the Posavina area and it is divided from the renowned Slavonian forests of pedunculate oak only by the River Sava, this is an unexpected value. This can largely be attributed to the reduced number of trees in the area of Bosanski Brod and to significant historical influences, as documented by Begović *(10, 11)*. In contrast to these populations, the Romanija population shows an unexpectedly small value in view of the fact that it is relatively isolated, small and devoid of any possibility of gene flow. The final result is that the average index value is positive, with the value of Fit = 0.234 (Table 4). This is the consequence of considerable fragmentation of pedunculate oak. The same insights were obtained when the fixation index was analyzed per loci (Table 5), since they all showed positive values and inbreeding. The Bosnian-Herzegovinian populations show relatively high intra-population diversity, with the average for all loci Fis = 0.273, and low inter-population differentiation in all the investigated loci, with Fst = 0.051 (Table 5).

The analysis of genetic distances according the NEI *(49)* among the studied populations is given in Table 6. The results can be explained by small geographical distances among the analyzed populations. However, despite relatively small geographical distances, the obtained values are very important, and even small differentiation has high significance.

In our research, genetic distance of 1.149 (Table 6) according to NEI *(49)* is the highest between the Orašje and Romanija populations in view of the relatively small geographical distance. The smallest genetic distance of 9.411 was observed between the populations of Bosanska Gradiška and Živinice, which was expected since Bosanska Gradiška is situated in the north of Bosnia and is related to Slavonian populations, while the Živinice population is separated from Posavina only by Mount Majevica. Still (Nevertheless), the Živinica population should be closer to the Orašje population. These results show that the three populations of pedunculate oak from the area of Bosanska Posavina: Orašje, Bosanski Brod and Bosanska Gradiška, deviate from one another, although

TABLE 5

F-statistics for genetic diversity and differentiation among 12 pedunculate oak populations from Bosnia and Herzegovina.

Locus	F_{IT}	F_{ST}	F_{IS}
QpZAG1/5	0.227	0.043	0.192
QpZAG9	0.199	0.069	0.139
QpZAG36	0.299	0.018	0.286
QpZAG108	0.362	0.072	0.312
Multilocus estimates	0.273	0.051	0.234
P-value	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

F-statistics was calculated using Weir and Cockerham method: F_{IT} – overall inbreeding; F_{IS} – average inbreeding coefficient; F_{ST} – differentiation among populations

TABLE 6

Nei's standard genetic distance (upper diagonal) and pairwise F_{ST} values (lower diagonal) among 12 pedunculate oak populations from Bosnia and Herzegovina.

No.	Population	1	2	3	4	5	6	7	8	9	10	11	12
1	Cazin		0.824	0.540	0.997	0.596	0.570	1.002	0.684	0.498	0.471	0.849	0.865
2	Bosanska Gradiška	0.048***		1.043	0.655	0.782	0.645	0.773	0.411	0.425	0.649	0.598	0.625
3	Bosanski Brod	0.031***	0.064***		1.046	1.038	0.629	0.681	0.712	0.787	0.585	1.087	0.805
4	Orašje	0.067***	0.047**	0.074***		0.770	0.794	0.825	0.596	0.803	1.030	0.674	1.149
5	Mrkonjić grad	0.036**	0.051***	0.068***	0.059***		0.674	0.709	0.985	0.536	0.615	0.778	0.832
6	Jelah	0.031***	0.037**	0.038***	0.056***	0.043***		0.622	0.680	0.476	0.677	0.654	0.765
7	Žepče	0.072***	0.060***	0.055***	0.072***	0.058***	0.048***		0.782	0.612	0.692	0.799	0.728
8	Živinice	0.042**	0.021*	0.048***	0.045***	0.066***	0.043***	0.064***		0.455	0.758	0.620	0.971
9	Livno	0.020ns	0.015ns	0.043**	0.051**	0.027**	0.019*	0.042***	0.020ns		0.439	0.425	0.608
10	Bugojno	0.038**	0.054***	0.050***	0.090***	0.054***	0.056***	0.069***	0.066***	0.030*		0.691	0.679
11	Sarajevo	0.051***	0.036*	0.068***	0.050***	0.052***	0.040***	0.064***	0.041***	0.017ns	0.060***		1.081
12	Romanija	0.059***	0.044***	0.059***	0.086***	0.062***	0.053***	0.064***	0.070***	0.037***	0.064***	0.074***	

P-values as obtained by randomizations: »***« corresponds to significance at the 0.1% nominal level, »**« corresponds to significance at the 1% nominal level, »*« significance at the 5% nominal level and »ns« depicts non-significant values

these populations were expected to show the smallest genetic distance.

In terms of pairwise F_{ST} values, there is minimal value between the populations of Bosanska Gradiška and Livno and maximal value between the populations of Orašje and Bugojno, as expected.

The first analysis (A), in which within population and among population variability by altitude was included, showed differentiation. Although most of the genetic diversity was attributable to differences among individuals within a population (92.49%), the significant ϕ_{ST} value among populations ($\phi_{ST} = 0.075$; $p < 0.0001$) suggested the existence of genetic differentiation (Table 7).

In the second analysis (B), which also included variability among the populations and within the groups, some of the components did not show any differentiation. A two-way nested AMOVA analysis was used to further partition the total genetic variance among three altitude groups (<100 meters above sea level; 100–300 m; >300 m), among populations within groups, and within population components. The among-group variance component was not significant ($\phi_{CT} = 0.001$; $p = 0.392$), suggesting that straightforward classification of populations into altitude groups could not explain the observed inter-population variability.

Our research was faced with the problem of the small number of loci analyzed, which was due to objective reasons. This is also the basic reason for which we could not make any definite conclusions. Still (However), the obtained results suggest gene diversity excess as a sign of gene bottleneck. Yet, owing to the small number of ana-

TABLE 7

AMOVA analysis for the partitioning of microsatellite diversity (A) among and within pedunculate oak populations and (B) among three altitude groups (<100 meters above sea level; 100–300 m; >300 m), among populations within groups, and within populations.

Analysis	Source of variation	df	Variance components	Percentage of variation	ϕ-statistics	P(ϕ)
(A)	Among populations	11	0.034	7.51	$\phi_{ST} = 0.075$	< 0.0001
	Within populations	228	0.416	92.49		
(B)	Among groups	2	0.0005	0.10	$\phi_{CT} = 0.001$	0.392
	Among populations within groups	9	0.033	7.44	$\phi_{SC} = 0.074$	< 0.0001
	Within populations	228	0.416	92.45	$\phi_{ST} = 0.075$	< 0.0001

P(ϕ) – ϕ-statistics probability level after 10,000 permutations

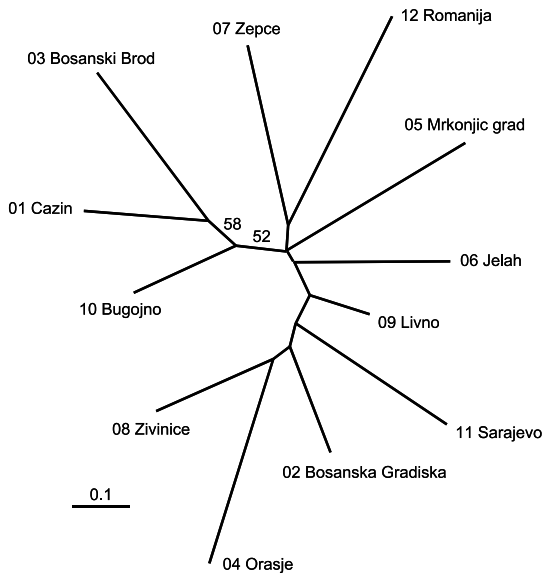

Figure 2. *Unrooted Neighbour joining tree based on Nei's standard genetic distance between 12 pedunculate oak populations from Bosnia and Herzegovina. (Numbers above branches indicate bootstrap support percentage over 50% in 10,000 pseudoreplicats).*

lyzed loci, nothing significant was obtained at the $P<0.01$ level. Further research concerning pedunculate oak should include a larger number of loci (Table 8).

The obtained bootstrap values in this case are necessarily very low. The reason is the small number of analyzed loci (only four), so each locus has a large influence on the calculation of genetic distance and the shape of the obtained tree (Figure 2). More distinct differentiation of the populations from Livno, Romania and Mrkonić Grad was expected as regards the obtained picture, the condition in the field and ecological conditions. No grouping was observed in terms of geographic affiliation, river catchments, and logical gene flow, either. This could be attributed to small populations, which were formed randomly from the remaining trees, and to their relative isolation, i.e. gene flow interruption. Since this research was conducted in a small country, even small deviations among the populations could be significant. On the other hand, high ecological diversity of the central Dinaric massif would suggest higher differentiation than was actually obtained in this research.

DISCUSSION

The use of genetic markers (QpZAG1/5, QpZAG9, QpZAG36, QpZAG108) in this research reveal and clarify differences among populations or within populations. Some possible causes for these differences, apart from natural selection, can primarily be attributed to strong anthropogenic impacts, developmental factors and processes of adaptation to changed ecological conditions in the past 150 years. In Bosnia and Herzegovina, these ecological conditions have been distinctly hostile to pedunculate oak.

TABLE 8

Test for mutation-drift equilibrium at polymorphic loci in 12 populations of *Quercus robur*. The gene diversity observed (H_E) was compared with the gene diversity expected at mutation-drift equilibrium (H_{EQ}) and calculated from the observed number of alleles under four mutation models: IAM = infinite allele model; TPM 1 = two-phase model with 30% multiple-step mutations; TPM 2 = two-phase model with 5% multiple-step mutations; SMM = stepwise mutation model (Cornuet and Luikart 1996).

No.	Population	IAM				TPM1				TPM2				SMM			
		D	P(D)	E	P(E)	D	P(D)	E	P(E)	D	P(D)	E	P(E)	D	P(D)	E	P(E)
1	Cazin	1	0.969	3	0.063	2	0.156	2	0.906	3	0.094	1	0.938	3	0.063	1	0.969
2	Bosanska Gradiška	0	1.000	4	0.031	1	0.969	3	0.063	1	0.906	3	0.156	2	0.563	2	0.563
3	Bosanski Brod	0	1.000	4	0.031	3	0.438	1	0.844	3	0.156	1	0.906	3	0.063	1	0.969
4	Orašje	0	1.000	4	0.031	2	0.906	2	0.156	2	0.156	2	0.906	3	0.094	1	0.938
5	Mrkonjić grad	0	1.000	4	0.031	0	1.000	4	0.031	0	1.000	4	0.031	1	0.969	3	0.063
6	Jelah	0	1.000	4	0.031	0	1.000	4	0.031	1	0.938	3	0.094	2	0.906	2	0.156
7	Žepče	1	0.969	3	0.063	1	0.938	3	0.094	1	0.844	3	0.438	1	0.844	3	0.438
8	Živinice	1	0.969	3	0.063	2	0.438	2	0.844	3	0.094	1	0.938	3	0.094	1	0.938
9	Livno	0	1.000	4	0.031	0	1.000	4	0.031	2	0.844	2	0.438	2	0.563	2	0.563
10	Bugojno	1	0.969	3	0.063	1	0.844	3	0.438	2	0.563	2	0.563	2	0.438	2	0.844
11	Sarajevo	1	0.969	3	0.063	1	0.844	3	0.438	2	0.563	2	0.563	2	0.438	2	0.844
12	Romanija	0	1.000	4	0.031	1	0.938	3	0.094	2	0.563	2	0.563	2	0.438	2	0.844

D – Number of loci exhibiting gene diversity deficiency; P(D) – probability of gene diversity deficiency ($H_E < H_{EQ}$) on average across all polymorphic loci using Wilcoxon's test (*53*, *54*) – sign of population expansion; E – Number of loci exhibiting gene diversity excess; P(E) – probability of gene diversity excess ($H_E > H_{EQ}$) on average across all polymorphic loci using Wilcoxon's test (*53*, *54*) – sign of population bottleneck

The specific features of the central Dinaric massif, with its variety of climatic, edaphic, orographic and other factors over a small area *(65),* directly affect population differentiation, and consequently genetic differentiation. According to a number of experts, forest tree species from the Balkan area, to which the Dinaric range belongs, show high variability compared to the same species from central, western and eastern Europe *(66)*.

The influence of orographic factors, i.e. genetic adaptation of pedunculate oak to altitude and the formation of ecotypes can be seen in physiological-genetic research by Stojković *(67),* Gračan *et al. (22)* and Gračan *(23)* in neighbouring Croatia. In terms of genetic analyses, we can refer to research conducted by Slade *et al. (17),* who confirmed findings by Petit *et al. (35, 36)* on a secondary glacial refugium in Dalmatia and on considerable numbers of haplotypes existing in white oak populations; in turn, these could significantly affect genetic variability of populations and their differentiation. In this research, the high values of alleles and their effective number identified in the Cazin and Livno populations, which are close to the assumed refugia *(17, 35, 36),* can confirm the above findings, in view of the fact that they did not lose much of their initial genetic potential.

The results of this research suggest that there are certain differences among populations from different ecological niches. In other words, it is very probable that genetic differentiation among populations was caused not only by strong anthropogenic impacts through history but also by important differences in site ecology and by historical migration processes, as discussed by a number of authors *(26, 35, 36)*. It is also interesting to note that the studied populations have been daily exposed to strong selection impacts by man in the past 200 years. Such influence includes inadequate management *(10, 11),* waterway regulations and field irrigation, and in the last 60 years severe air, water and soil pollution. Illegal cutting operations in the last decade are one of the main causes for the disappearance of the last large and old oaks. A combination of the above factors has resulted in a visible reduction in the number of individuals in populations. Populations have virtually been brought to the verge of extinction; gene flow is poor or almost non-existent; and the occurrence of genetic drift will turn the populations into a completely different direction. This is particularly evident in the population from Mrkonjić Grad, but also in other investigated populations from Bosnia and Herzegovina. The extent of anthropogenic impact on forests is best illustrated by research conducted by Ducci *(68)* in the Apennines. Therefore, the values of distances among the populations obtained in this research should be viewed conditionally, because they may not only be the consequence of natural processes and genetic differentiation itself.

Variability obtained in this research is relatively high in relation to that obtained in central and Western Europe, as reported by Yakovlev and Kleinschmidt *(69)*. In an extensive European study of oak, in which migration routes were analyzed using a large number of haplotypic markers, it was confirmed that variability decreased from the south to the north *(26, 27, 28, 29, 30, 31, 32, 34, 35, 36, 70, 71)*. In Bosnia and Herzegovina, only two haplotypes (4 and 5) were found in five analyzed populations of pedunculate oak with a total of 15 trees, with haplotype 5 manifesting two subhaplotypes *(17)*. Five haplotypes were identified in neighbouring Croatia *(17)* but the analyzed sample was much larger. If the number of analyzed populations in Bosnia and Herzegovina had been larger, a higher number of different haplotypes would probably have been obtained.

Genetic variability based on microsatellite analyses was reported by Steinkellner *et al.*, *(38),* Lefort *et al. (39),* Barreneche *et al. (40),* Lexer *et al. (41),* Wilhelm *et al. (42)*. They analyzed different developmental stages of pedunculate oak and its embryos. No relationship between the studied microsatellite regions and certain adaptive potential of pedunculate oak was identified in this research, although some research with other marker types do confirm this relationship *(18)*. Based on research undertaken in the west of Europe, where pedunculate oak has relatively lower variability *(38, 40),* in relation to the results of this research the populations are also relatively fragmented, but, according to their conclusions, relatively stable. Since our investigated populations are near glacial refugia, they probably did not lose much of their adaptive potential and should manifest a much higher degree of resistance in comparison with west European ones. Population heterzygosity is also largely influenced by the type of management applied in certain areas over a longer period, which has turned diversity into different directions. We should point out that Bosnian-Herzegovinian populations of pedunculate oak are seriously devastated and fragmented, that systematic negative selection has disturbed their genetic structure and that they do not show continuous variability, which is otherwise characteristic of pedunculate oak with its large distribution range.

The obtained results and a deeper knowledge of the genetic variability of pedunculate oak in Bosnia and Herzegovina will contribute to plans for its regeneration, reintroduction and conservation of genetic diversity by means of *in situ* and *ex situ* methods.

Populations with few trees or individual trees, as well as poor and sparse tree distribution may lead to constant inbreeding and to the occurrence of inbreeding depression, which may have a negative effect on the next generation. This could play a very important role in some of our populations (Sarajevo, Mrkonjić Grad, Bugojno, Sokolac), as confirmed by this research. In order for a population to adapt and survive *in situ*, we should take into account the fact that survival also depends on basic life factors and on the individual, which is the carrier of genetic resource. In other words, an individual must have the ability to pass the genetic resource to the next generation (vitality, fructification, resistance, etc.). Therefore, in addition to understanding the genetic structure obtained by means of genetic markers, we should also know the basic ecological factors reigning in these sites. The re-

sults derived from statistical analyses should be taken with reserve because, despite their exactness, proper results may remain elusive and hidden in a multitude of data.

Acknowledgement: *This research has been carried out within the framework of joint projects of Regione Lombardia and Bosnia-Herzegovina, financed by the ERSAF – Regione Lombardia. The authors wish to thank Mioč D., Čavkunović J., Tuzlak M., Bašić N., Začević T. and Ilić N. for the collection of plant material.*

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