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Genetic Diversity and Genetic Structure of Three Sympatric Oak Species in Serbian Landscape of Outstanding Features "Kosmaj" Assessed by Nuclear Microsatellites

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ABSTRACT

Quercus petraea (Matt.) Liebl., *Q. pubescens* Willd., and *Q. frainetto* Ten. grow naturally in sympatry in the Landscape of Outstanding Features "Kosmaj" (Mt. Kosmaj) in Serbia, in the Western Balkans. The levels of genetic diversity and genetic structure in populations of these species (160 trees in total) was assessed by means of 14 nuclear microsatellites. The number of alleles detected in overall sample was 314, with the locus QrZAG90 being the most informative one in all three species (31, 35 and 36 alleles in *Q. frainetto*, *Q. pubescens* and *Q. petraea*, respectively). The levels of genetic diversity of all three species were relatively high (H_E = 0.824, 0.834, and 0.794 in *Q. petraea, Q. pubescens* and *Q. frainetto*, respectively). Low but statistically significant inbreeding was detected in all three species (F = 0.100, 0.131 and 0.065 in *Q. petraea, Q. pubescens* and *Q. frainetto*, respectively), which, however, most likely reflects population substructure, which was observed in the STRUCTURE analysis. The optimal number of genetic groups revealed by Bayesian clustering analysis did not coincide with the number of analyzed species as it amounted to four (one gene pool was observed in each of the two species, *Q. petraea* and *Q. pubescens*, while two gene pools were observed in *Q. pubescens*). Furthermore, a higher level of hierarchical genetic structure, with six gene pools was found, with *Q. frainetto* being the only oak species in which population substructure was not observed. Genetic differentiation of three sympatric oak species was relatively low but statistically significant, with the highest F_{ST} value found among *Q. petraea* and *Q. frainetto* (F_{ST} = 0.047, P = 0.001), and the lowest between *Q. petraea* and *Q. pubescens* (F_{ST} = 0.032, P = 0.001). Our results reveal rather high levels of genetic diversity in all three studied oak species, which retained their genetic integrity despite sympatric distribution, indicating low levels of interspecific hybridization, and pronounced genetic structure of *Q. pubescens* and *Q. petraea.*

Keywords: *Quercus petraea* (Matt.) Liebl.; *Quercus pubescens* Willd.; *Quercus frainetto* Ten.; nuclear SSRs (Simple Sequence Repeats)

INTRODUCTION

Genetic diversity is a key requirement for the long-term survival of species on an evolutionary time scale (Pakkad et al. 2008, Fady et al. 2022) and is usually rather high in long-lived forest tree species (Kremer et al. 2012, Rajora and Zinck 2021). Forests play important roles in ecosystem functioning, species diversity maintenance, climate regulation, and soil and water conservation. Thus, the biodiversity and stability of the entire forest ecosystems are mainly dependent on the genetic diversity of forest trees (Sandurska et al. 2019), commonly shaped by pollen and seed dispersal (Dow and Ashley 1998), demographic changes and evolutionary processes, such as genetic drift and migration, which may account for the occurrence of genetic structure in forest tree populations (Fernández-M and Sork 2006). In forest trees, gene flow is particularly important, as it has the ability to both permit local genetic differentiation and introduce variability in isolated populations (Ducousso et al. 1993) as well as to erase present genetic structure over generations.

Genetic diversity loss became particularly important in the context of climate change, relevant also for the production of well-adapted reproductive material used for reforestation (Ivetić et al. 2016, Katičić Bogdan et al. 2018, Popović et al. 2022). Information on genetic variability, genetic structure, and inbreeding is therefore required for the effective management of forest populations (Craft and Ashley 2007).

Widely known species richness of the Balkan Peninsula is a result of complex geological history, and interactions between populations, species, and ecosystems (Savić 2008). Among many forest tree species, oaks are one of the ecologically and economically most important genera in the northern hemisphere. A total of 12 oak species occur in the Balkans, including six species belonging to the *Quercus* (white oak) group (Nixon 1993). In the genus *Quercus*, several taxonomic groups are characterized by complex patterns of variation, leading to difficulties in the identification of taxa to the species level (Bruschi et al. 2000, Gömöry et al. 2001). Resolving the species boundaries in oaks can be challenging due to the presence of natural hybrids and introgressive forms, especially in contact zones where two or more species occur (Curtu et al. 2007, Viscosi et al. 2009, Yücedağ and Gailing 2013, Lyu et al. 2018). One of such sites, where three oak species, namely *Quercus petraea* (Matt.) Liebl., *Quercus pubescens* Willd., and *Quercus frainetto* Ten., grow in sympatry, is found in Serbia, in the protected zone (protection degree II) within the Landscape of Outstanding Features "Kosmaj" at the Mt. Kosmaj. This site is geographically close to the metropolitan region and it is threatened by habitat loss and degradation, mostly due to the expansion of agricultural and urban areas, illegal cutting of forests for firewood and other wood products, and excessive grazing. These autochthonous oak populations are important both from scientific and economic point of view (Jovanović et al. 2023). Studies of genetic diversity of oaks in Serbia were conducted by several authors, using both chloroplast and nuclear DNA (e.g., Milovanović 2009, Šijačić-Nikolić et al. 2009, Ballian 2010, Neophytou et al. 2010, Kesić et al. 2021, Šijačić-Nikolić et al. 2021, Popović et al. 2022). However, at the region of the Landscape of Outstanding Features "Kosmaj" no such research has been conducted so far.

Due to the overexploitation of forests, together with poorly understood environmental changes, which have resulted in the decline of many oak species, oaks are involved in an increasing number of studies related to gene flow and genetic structure (e.g. Ducousso et al. 1993, Lepais et al. 2009, Fortini et al. 2015, Sandurska et al. 2019, Leroy et al. 2020). Gene flow levels in oaks are high and mostly related to the life history traits of its species, such as phenology, mating system, wind pollination, acorn production, etc. (Ducousso et al. 1993). Due to high levels of intraspecific diversity and hybridization between the species which cause the boundaries of species differentiation to be less distinctive, discerning some oak species at the molecular level and obtaining species-specific diagnostic markers can be challenging (Kelleher et al. 2005). Because of the highly informative and codominant nature, hypervariability, reproducibility, and the possibility of parallel amplification nSSRs are widely used for genotyping in plant and animal species. Although the usage of nSSRs may be challenging (e.g., Kerkez Janković et al. 2019), they have been proven suitable for assessing the levels of genetic diversity in oak species (Kesić et al. 2021). Furthermore, Muir et al. (2000) have demonstrated that nSSRs may be used for differentiation of phylogenetically distant species like *Q. robur* and *Q. petraea* despite the high levels of interspecific gene flow. For phylogenetically close species, genetic diversity in populations in hybrid zones or at the distributional margins of species, EST-SSR markers are useful (Ueno et al. 2008, Aizawa et al. 2018).

The aims of this study were to assess the genetic diversity and structure of *Q. petraea*, *Q. pubescens*, and *Q. frainetto* from the Landscape of Outstanding Features "Kosmaj" (Mt. Kosmaj, Serbia) using nuclear microsatellites. Levels of inter- and intraspecific genetic variability reveled in this study can lay the groundwork for setting the guidelines for the conservation of the available gene pool and prescribing measures for future forest management.

MATERIALS AND METHODS

Study Site and Plant Material

A total of 187 trees found in the protected area of Mt. Kosmaj (44°28'17.68"N, 20°34'32.04"E) were selected for genotyping – 65 of *Q. pubescens*, 60 of *Q. petraea*, and 62 of *Q. frainetto* (Figure 1). The protection zone occupies 3514.50 ha of a forest complex surrounded by predominantly agricultural land and rural settlements. At the lower altitudes (250-400 m a.s.l.) dominant forest type is *Quercetum frainetto-cerris* Rudski, above 400 m a.s.l. *Querco-Carpinetum serbicum* Rudski and from 500 to 626 m a.s.l. *Fagetum montanum* Rudski. At Mt. Kosmaj oaks are abundantly present in sympatry, which can be seen from forest typology previously mentioned, but are negatively impacted by the lack of natural regeneration and the coppice origin.

Samples for molecular genetics analyses comprised of young leaves which were collected in May 2022. The selection of trees was based on phenotypic characteristics (trunk and crown appearance) and general health status (absence of entomological and phytopathological damage). From each selected tree up to five normally developed, healthy leaves were sampled, herbarized, dried in silica gel, and kept in a freezer prior to the analyses. Thus, only adult trees, distant at least 50 m from each other, were sampled.

DNA Extraction, PCR Amplification and Fragment Sizing

Samples were processed in Biotechnology Laboratory at the Faculty of Forestry, University of Belgrade (Serbia). For each selected tree up to 20 mg of dried leaves were homogenized with TissueLyser II (Qiagen, Valencia, CA, USA) and used for extraction of the total genomic DNA with *peqGOLD Plant DNA Mini Kit* (PEQLAB). Genomic DNA was quantified and assessed for purity utilizing NanoVue (GE Healthcare Europe, Freiburg, Germany). DNA solutions were diluted to working concentrations of 50 $ng \cdot \mu l^{-1}$.

For genotyping, 20 nuclear microsatellites were selected, 14 of which proved to be very informative and reliable after testing on a panel of 8 samples (Table 1). For the parallel amplification Type-it Microsatellite PCR Kit (Qiagen) was used. Microsatellite loci were grouped into two mixes: OM1 – PIE239, FIR004, QrZAG90, QrZAG108, MSQ13, GOT004,

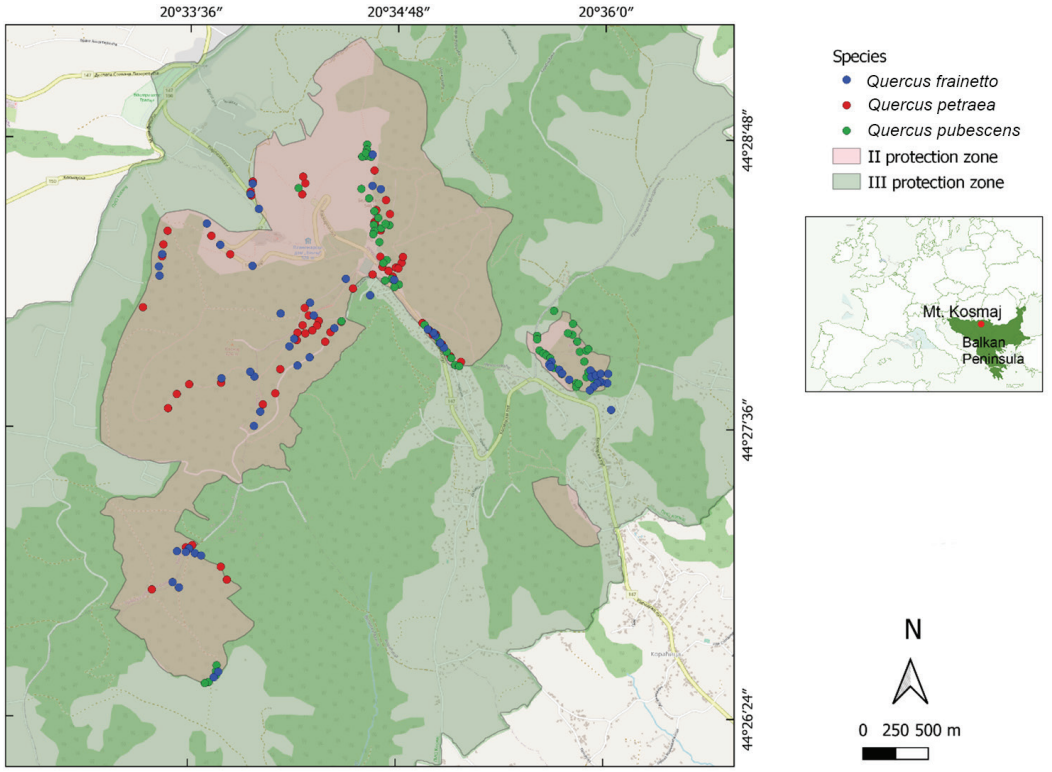

Figure 1. Distribution of the selected oak trees used in the study in the Landscape of Outstanding Features "Kosmaj", with the representation of the protection zones (II and III).

QrZAG87, QpZAG104, QrZAG11, QrZAG103, QrZAG102, and OM2 – QpZAG36, QrZAG101, MAQ4, PIE242, QrZAG20, QpZAG1/2, QpZAG58, QrZAG7, QpZAG110. Forward (F) primers from each primer pair of all selected loci were labelled with one out of the four different fluorescent dyes from the Dye set G5 (DS-33, Applied Biosystems, USA) to enable fragment sizing on an automated DNA fragment analyser. PCRs were carried out using Multigene Opti Max (Labnet International, Inc.). The PCR amplifications were performed as follows: initial denaturation at 95°C for 5 min; then 28 cycles of denaturing for 30 s at 95°C, annealing for 90 s at a 60°C, and extension for 30 s at 72°C; with a final extension at 60°C for 30 min. The PCR products were visualized on 1% agarose gels. PCR amplification products were separated commercially by Center for Forensic and Applied Molecular Genetics at the Faculty of Biology, University of Belgrade via capillary electrophoresis using 96-capillary 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Inc. USA). The lengths of PCR amplification products were assessed using GeneMapper (Applied Biosystems Inc., Foster City, USA), with the GeneScanTM-600LIZTM Size Standard (Applied Biosystems).

Data Analysis

The standard parameters of genetic diversity: number of alleles (A), number of private alleles (PA), average number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (H_O), expected heterozygosity (H_E) and coefficient of inbreeding (F), were determined using the GenAlEx 6.5 software package. The effective population size (Ne) was assessed using the NeEstimator software package (Do et al. 2014) with the rejection of alleles whose frequency was ≤0.02. Allelic richness was estimated using the HP-Rare 1.0 software package (Kalinowski 2005). Genetic structure was determined using two approaches: (1) obtained allele frequencies and genotypes generated for 14 molecular markers were used to determine the parameters of genetic differentiation expressed through the F_{ST} values, and PCoA analysis using the program package GenAlEx 6.5; obtained F_{ST} values were also used to assess levels of gene flow, Nm, according to the formula $Nm = [(1 / F_{ST}) - 1] / 4$; (2) Bayesian method implemented in the STRUCTURE 2.3.4 software package (Pritchard et al. 2000) was used to determine the optimal number of genetic groups (K) using the ΔK Evanno model (Evanno et al. 2005), and to determine the potential substructure of populations. Monte Carlo Markov Chain (MCMC) simulation had burn-in and run lengths of 700,000 iterations each. Ten independent analyzes for each assumed group K = 1-6 were performed. The model of correlated (dependent) frequencies of alleles was used as the allele frequency model, and the admixture model was used as the model of individual kinship.

Table 1. A list of 14 informative nuclear microsatellites used in the study, with primer sequences for their amplification, repeat motif, allele range available in literature, and references.

Locus	Primer sequences	Repeat motif	Allele range size (bp)	Reference
PIE239	CAACAAATGGCTCAACAGTG CCCATTTGGTAGCAAAGAGTC	$(AT)_{12}$	70-83	Durand et al. 2010
FIR004	TCTCTCTCAGGGCAGCTTCT AACCAAACTCAGATCCAGATTCA	$(CT)_{18}$	123-179	Durand et al. 2010
QrZAG90	GGAAGATAGTACC AGCTTGGTGAAT GCCTCATCTCACAGGTCACTCC	(GA)34	220-244	Kampfer et al. 1998
QrZAG108	AAGAGAGCAAATTTAGAGTGATGT GAACCTTGATCATACGTGGAGA	$(GA)_{19}(GGGA)_3$	80-108	Kampfer et al. 1998
MSQ13	TGGCTGCACCTATGGCTCTTAG ACACTCAGACCCACCATTTTTCC	(TC)	191-221	Dow et al. 1995
QrZAG87	TCCCACCACTTTGGTCTCTCA GTTGTCAGCAGTGGGATGGGTA	$(TC)_{20}$	110-131	Kampfer et al. 1998
QpZAG104	ATAGGGAGTGAGGACTGAATG GATGGTACAGTAGCAACATTC	$(AG)_{16}AT(GA)_3$	176-196	Steinkellner et al. 1997
QrZAG11	CCTTGAACTCGAAGGTGTCCTT GTAGGTCAAAACCATTGGTTGACT	$(TC)_{22}$	238-267	Kampfer et al. 1998
QrZAG101	CCTGCACAATCAAATCCTTCACTT GCCATGAACAACGG AGGTATCTAG	$(TC)_{20}(AC)_{15}$	136-160	Kampfer et al. 1998
MAQ4	TCTCCTCTCCCCATAAACAGG GTTCCTCTATCCAATCAGTAGTGAG	(AG)	203-227	Dow et al. 1995
PIE242	GGAGGGAAAAGAACAATGC TTGCAATCCTCCAAATTTAATG	$(TA)_{10}$	102-128	Durand et al. 2010
QrZAG20	CCATTAAAAGAAGCAGTATTTTGT GCAACACTCAGCCTATATCTAGAA	$(TC)_{18}$	160-200	Kampfer et al. 1998
QrZAG7	CAACTTGGTGTTCGGATCAA GTGCATTTCTTTTATAGCATTCAC	$(TC)_{17}$	115-153	Kampfer et al. 1998
QpZAG110	GGAGGCTTCCTTCAACCTACT GATCTCTTGTGTGCTGTATTT	$(AG)_{15}$	206-262	Steinkellner et al. 1997

RESULTS

Genetic Diversity

The summary of genetic diversity parameters obtained upon analyzing variability at 14 microsatellite loci in the three oak species is shown in Table 2, while the summary of genetic diversity parameters per species and in overall sample is shown in Table 3. A total of 314 alleles were detected in overall sample. The smallest number of alleles in *Q. petraea* was observed at the PIE239 locus (6), in *Q. pubescens* at QrZAG108 (6), and in *Q. frainetto* at QrZAG87 (7). The largest number of alleles for all tree species was observed at the QrZAG90 locus (A = 36, 35, and 31; Ae = 24.69, 20.64, 22.58 in *Q. petraea, Q. pubescens* and *Q. frainetto*, respectively). A total of 249 alleles were detected in *Q. petraea*, 238 alleles in *Q. pubescens*, and 213 alleles in *Q. frainetto* (Table 3). The average number of alleles per locus in the entire sample was 16.67 (SE = 1.07), the average effective number of alleles per locus 8.35 (SE = 0.87), and the average allelic richness obtained by the rarefaction method for 98 gene copies was 16.34. The number of private alleles was 29 in *Q. petraea*, 34 in *Q. pubescens* and 18 in *Q. frainetto*. The effective population size was 305.5 (190.2; 719.2) in *Q. pubescens*, 484.3 (254.8; 3374.5) in *Q. petraea*, and ∞ (1859.9; ∞) in *Q. frainetto*. The observed heterozygosity (Ho) ranged from 0.731 (SE = 0.051) (*Q. pubescens*) to 0.753 (SE = 0.053) (*Q. petraea*), with an average value of 0.745 (SE = 0.030). The expected heterozygosity (He) ranged from 0.794 (SE = 0.033) (*Q. petraea*) to 0.834 (SE = 0.027) (*Q. pubescens*), with an average value of 0.817 (SE = 0.019). A low but statistically significant excess of homozygotes was detected in the populations of all tested oak species.

Genetic Differentiation

To better understand the relationships between the three sympatric oak species, the gene flow (Nm) and genetic differentiation coefficient (F_{ST}) were estimated for all pairs of species (Table 4). The genetic differentiation between species was low but statistically significant ($P \leq 0.05$), and ranged from 0.032 among *Q. pubescens* and *Q. petraea*, to 0.047 among *Q. petraea* and *Q. frainetto*. Consequently, the highest gene flow (Nm = 7.563) was observed between *Q. petraea* and *Q. pubescens*. The lowest gene flow (Nm = 5.069) was observed between *Q. petraea* and *Q. frainetto*.

Principal coordinate analysis (PCoA) results, obtained by summarizing the genetic distances between genotypes within each of the populations showed that *Q. frainetto* separated from *Q. pubescens* and *Q. petraea* along the first principal coordinate, while the separation of *Q. pubsecens* and *Q. petraea* was observed along the second principal coordinate (Figure 2). The first principal coordinate (Coord. 1) explained 63.32% of the variability, and the second principal coordinate (Coord. 2) 36.68% of the variability, suggesting high reliability of the obtained results.

Table 2. Summary of the genetic diversity parameters based on genotyping with 14 nuclear microsatellite loci in the three oak species.

Species	*Q. petraea*						*Q. pubescens*						*Q. frainetto*					
Locus	N	A	Ae	H_O	H_E	F	N	A	Ae	H_O	H_E	F	N	A	Ae	H_O	H_E	F
FIR004	52	21	14.383	0.942	0.93	-0.013	53	20	14.707	0.83	0.932	0.109	55	18	11.415	0.891	0.912	0.024
PIE239	52	6	1.633	0.231	0.388	0.405	53	12	2.984	0.509	0.665	0.234	55	9	3.286	0.4	0.696	0.425
PIE242	52	15	7.48	0.865	0.866	0.001	53	13	6.12	0.811	0.837	0.03	55	16	7.544	0.891	0.867	-0.027
MAQ4	52	13	6.216	0.596	0.839	0.29	52	14	6.5	0.5	0.846	0.409	54	12	4.696	0.593	0.787	0.247
MSQ13	52	13	4.189	0.731	0.761	0.04	53	13	4.41	0.66	0.773	0.146	55	16	5.465	0.873	0.817	-0.068
QpZAG11	52	13	4.337	0.538	0.769	0.3	49	17	10.088	0.551	0.901	0.388	54	11	2.508	0.537	0.601	0.107
QpZAG104	52	28	17.731	0.923	0.944	0.022	52	25	11.938	0.846	0.916	0.076	55	27	16.22	0.945	0.938	-0.008
QpZAG110	52	19	7.501	0.865	0.867	0.001	53	18	5.958	0.83	0.832	0.002	55	13	2.307	0.582	0.567	-0.027
QrZAG7	52	19	12.126	0.692	0.918	0.245	53	17	9.753	0.925	0.897	-0.03	55	13	6.335	0.873	0.842	-0.036
QrZAG20	52	18	8.503	0.865	0.882	0.019	53	16	9.571	0.849	0.896	0.052	55	12	6.97	0.873	0.857	-0.019
QrZAG87	52	15	5.307	0.808	0.812	0.005	52	13	3.567	0.769	0.72	-0.069	55	7	4.13	0.745	0.758	0.016
QrZAG90	52	36	24.694	0.885	0.96	0.078	52	35	20.641	0.962	0.952	-0.01	55	31	22.575	0.964	0.956	-0.008
QrZAG101	52	21	11.834	0.942	0.915	-0.029	53	19	7.825	0.868	0.872	0.005	55	18	8.473	0.909	0.882	-0.031
QrZAG108	52	12	3.153	0.654	0.683	0.043	53	6	2.751	0.321	0.637	0.496	55	10	2.789	0.436	0.641	0.32
Total/ Average	52	17.786	9.22	0.753	0.824	0.100	52.429	17	8.344	0.731	0.834	0.131	54.857	15.214	7.48	0.751	0.794	0.065
SE	0	1.984	1.704	0.053	0.04	0.038	0.291	1.825	1.334	0.051	0.027	0.048	0.097	1.795	1.548	0.053	0.033	0.041

N – population size; A – number of alleles; Ae – effective number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F – coefficient of inbreeding; SE – standard error

Table 3. Standard genetic diversity parameters in populations of the three oak species.

Species	N	A	PA	Na (SE)	Ae (SE)	Ar98	Ne (95% CI)	H_O (SE)	H_E (SE)	F (SE)
Q. petraea	52	249	29	17.786	9.220	17.54	484.3	0.753	0.824	0.100
				-1.984	-1.704		(254.8; 3374.5)	(0.053)	(0.040)	(0.038)
Q. pubescens	53	238	34	17.000	8.344	16.71	305.5	0.731	0.834	0.131
				-1.825	-1.334		(190.2; 719.2)	(0.051)	(0.027)	(0.048)
Q. frainetto	55	213	18	15.214	7.480	14.76	∞	0.751	0.794	0.065
				-1.795	-1.548		(1859.9; ∞)	(0.053)	(0.033)	(0.041)
Total / Average	160	314	81	16.667	8.348	16.34	-	0.745	0.817	0.099
				-1.066	(0.872)			(0.030)	(0.019)	(0.024)

N – population size; A – number of alleles; PA – number of private alleles; Na – average number of alleles per locus; Ae – average effective number of alleles per locus; Ar98 – allelic richness according to the rarefaction method for 98 gene copies; Ne – effective population size; 95% CI – 95% confidence intervals; H_O – observed heterozygosity; H_E – expected heterozygosity; F – coefficient of inbreeding; SE – standard error

Table 4. The F_{ST} values and Nm between the pairs of populations of *Q. petraea*, *Q. pubescens* and *Q. frainetto*.

Species	Q. petraea	Q. pubescens	Q. frainetto
Q. petraea	0.000	Nm = 7.563	Nm = 5.069
Q. pubescens	F_{ST} = 0.032	0.000	Nm = 6.160
Q. frainetto	F_{ST} = 0.047	F_{ST} = 0.039	0.000

PCoA results obtained by summarizing the genetic distances between genotypes within each of the individuals of the three species (Figure 3) showed the same grouping pattern as in population analysis (Figure 2) despite the small percentage of the total variability explained by the first two principal coordinates (12.27%, Coord. 1 = 6.84%, Coord. 2 = 5.43%).

Genetic Structure

The optimal number of genetic groups in the study sample, obtained by the ΔK Evanno model, was four (Figure 4). However, a higher level of hierarchical structure, with six genetic groups, was observed as well.

Results of the STRUCTURE analysis with four (K = 4) and six (K = 6) genetic groups in *Q. petraea*, *Q. pubescens*, and *Q. frainetto* are shown in Figures 5 and 6.

The STRUCTURE analysis showed that *Q. frainetto* represents a coherent and distinct genetic group that was not substructured, i.e., in which a large number of individuals had a high proportion of assignment to the unique gene pool. In contrast to *Q. frainetto*, the populations of *Q. petraea* and *Q. pubescens* were substructured, i.e., comprised individuals strongly assigned (qi > 0.80) to distinct gene pools. The population of *Q. petraea* in the area of Mt. Kosmaj consisted of two separate genetic groups. The substructure has also been observed in the population of *Q. pubescens* (Figure 6). The results of the STRUCTURE analysis under the assumption of six genetic groups (K = 6) showed that *Q. pubescens* population comprised individuals strongly assigned (qi > 0.80) to three distinct gene pools.

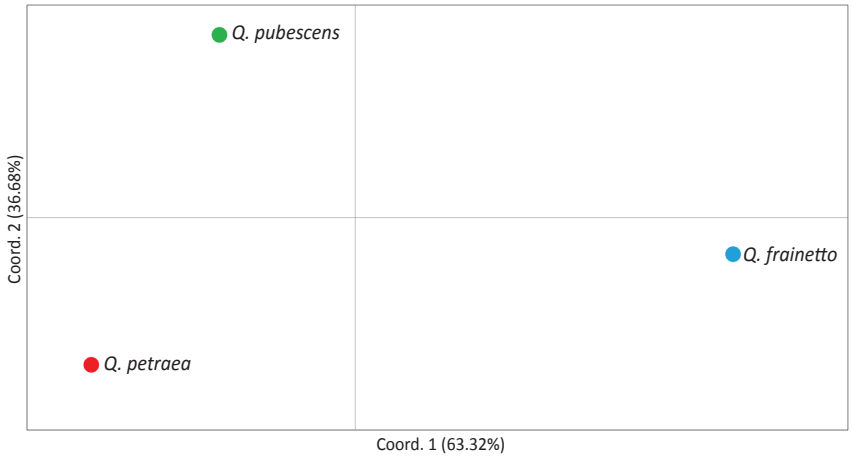

Figure 2. Scatterplot of *Q. petraea*, *Q. pubescens*, and *Q. frainetto* populations based on PCoA analysis obtained by the genetic distances.

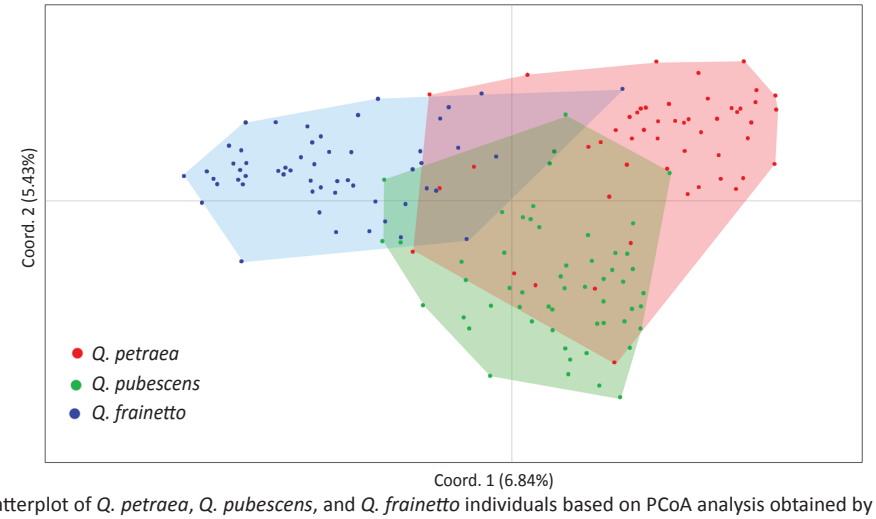

Figure 3. Scatterplot of *Q. petraea*, *Q. pubescens*, and *Q. frainetto* individuals based on PCoA analysis obtained by the genetic distances.

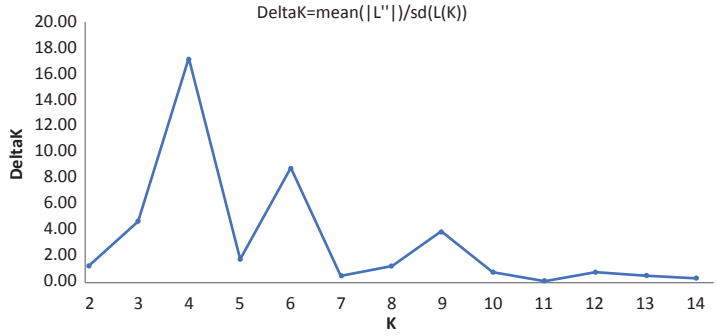

Figure 4. The optimal number of genetic groups in the studied oak populations determined by the ΔK Evanno model.

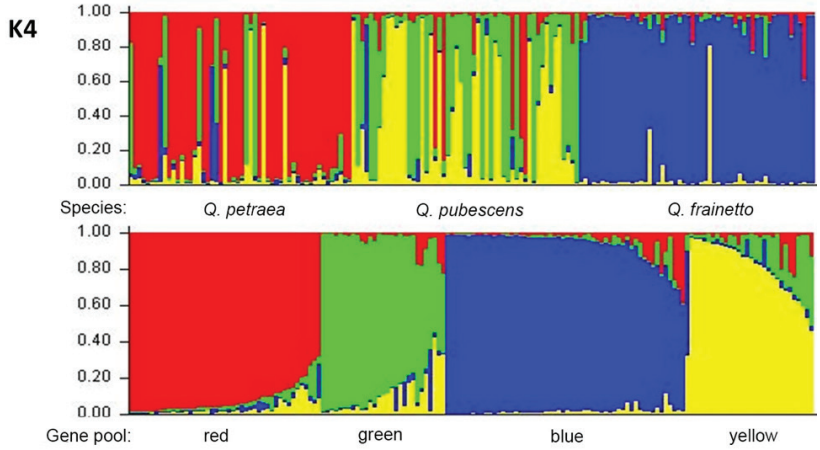

Figure 5. The results of the STRUCTURE analysis under the assumption of four genetic groups (K = 4). The proportion of each cluster group for each individual is shown by the color code.

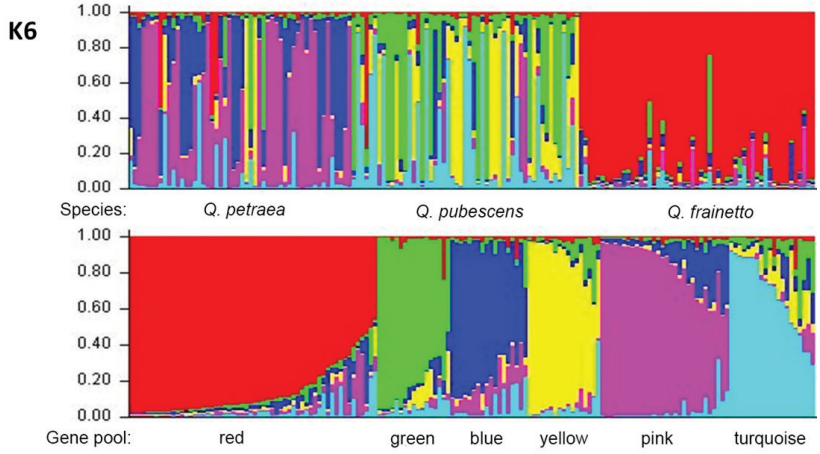

Figure 6. The results of the STRUCTURE analysis under the assumption of six genetic groups (K = 6). The proportion of each cluster group for each individual is shown by the color code.

DISCUSSION

For the conservation of forest genetic resources and sustained forest management, knowledge of the levels and distribution of genetic variation is crucial, especially if it is related to genetic processes taking place during reproduction and stand establishment, i.e. during developmental stages that are under high anthropogenic influence (Liesebach and Zaspel 2004). However, complex patterns of genetic diversity and characteristic phenotypic plasticity of oaks hampered the efforts to manage their gene pools and accurately assess the conservation status of most of the oak taxa (Sullivan et al. 2013). While oaks are capable of high rates of local adaptation, their long generation times and immobility make them vulnerable to rapid environmental changes (Yan et al. 2019). The high levels of genetic diversity favor rapid adaptation, and the result of this local adaptation is the development of a phenotype that optimizes the response to environmental pressures, and is associated with the highest fitness (Kremer 2010).

Q. petraea, *Q. frainetto* and *Q. pubescens* belong to the *Quercus* (white oak) group (Nixon 1993) and their populations are rather common in Serbia, in the Western Balkans, where they occasionally occur in sympatry. This is the case with the site at Mt. Kosmaj, which acquired the status of the Landscape of Outstanding Features, due to its species richness and diversity, in addition to cultural, historical, and geological features. *Q. petraea*, *Q. frainetto* and *Q. pubescens* are autochthonous in this area (Stajić et al. 2019), and have not been studied to date at the molecular level. We analyzed 160 individuals of these three species with 14 nuclear microsatellites in order to assess the levels of genetic diversity in their populations and their genetic structure, needed for the formulation of conservation strategies and management practice.

The parameters of genetic diversity obtained in our study are consistent with previous reports in which oak species were analyzed with the same type of molecular markers (e.g., Muir and Schloetterer 2005, Salvini et al. 2009, Neophytou et al. 2010, Alberto et al. 2010, Katičić Bogdan et al. 2018). The values of expected heterozygosity for the three oak species were also recorded by different authors across Europe – e.g., ranging from 0.781 to 0.815 in *Q. petraea* from central Europe and Balkan Peninsula (Neophytou et al. 2010), from 0.251 to 0.890 in *Q. pubescens* populations from Italy (Di Pietro et al. 2020), or from 0.701 to 0.929 in *Q. pubescens* and from 0.181 to 0.922 in *Q. frainetto* from Romania (Curtu et al. 2011). As our study obtained the values of expected heterozygosity of 0.824 in *Q. petraea*, 0.834 in *Q. pubescens*, and 0.794 in *Q. frainetto*, it can be concluded that the obtained values are in accordance with the values obtained in previous studies. Thus, we found that all three analyzed species are characterized by rather high levels of genetic diversity, which indicates good prospects for their long-term survival, especially in conditions of changing climate and habitat degradation.

Despite living in sympatry, all three species of oaks have retained their genetic integrity, which is a rather important finding relevant for the conservation and management practice. Low but statistically significant genetic differentiation, expressed via F_{ST} values, was observed (0.047 among *Q. petraea* and *Q. frainetto*, 0.039 among *Q. pubescens* and *Q. frainetto*, and 0.032 among *Q. petraea* and *Q. pubescens*). Furthermore, *Q. frainetto* was clearly separated from the other two oak species in PCoA analysis along the first coordinate, while *Q. petraea* and *Q. pubescens* were clearly separated in the PCoA analysis, along the second coordinate. Also, in the case of *Q. frainetto*, almost all individuals were strongly assigned to one gene pool, while individuals belonging to *Q. petraea* and *Q. pubescens* were strongly assigned to two or three distinct gene pools, indicating complex substructure of their populations. Population substructure is commonly associated with limitations to the gene flow (Sork 2016), and introgression, which is rather common in oaks, known for interspecific hybridization (e.g., Curtu et al. 2007, Salvini et al. 2009, Neophytou et al. 2010, Ortego and Bonal 2010). The highest gene flow and the lowest genetic differentiation were observed between *Q. petraea* and *Q. pubescens*, and the lowest gene flow and the highest genetic differentiation between *Q. petraea* and *Q. frainetto*. These results were expected because lower genetic differentiation and higher levels of gene flow are commonly observed among species that are more closely related, such as *Q. petraea* and *Q. pubescens*, than those that are more distantly related, such as *Q. petraea* and *Q. frainetto* (Curtu et al. 2007). A similar pattern has been previously observed in Italian (Salvini et al. 2009, Fortini et al. 2015) and Romanian (Curtu et al. 2007, Curtu et al. 2011) populations of oak species that are more or less related. The substructure in *Q. petraea* and *Q. pubescens* could exist due to higher gene flow and potential presence of introgressive forms in both species.

An important finding is rather high effective population size in all three oak species, which was the highest in *Q. frainetto* (∞, 1859.9; ∞) and the lowest in *Q. pubescens* (305.5, 190.2; 719.2). This finding suggests a rather high number of parent's contribution to the formation of the next generation, which is important for the maintenance of high levels of genetic diversity in next generations. Nevertheless, low but statistically significant inbreeding was observed in all examined oak populations in the study area. The observed excess of homozygotes, however, most likely reflects population substructure, i.e., Wahlund effect (Wahlund 1928). It is well-known that the variation of allelic frequencies among subpopulations may create a heterozygote deficiency at the scale of the whole population (Bacillieri et al. 1994). This is supported by the outcomes of the STRUCTURE analysis which revealed population substructure in populations of *Q. petraea* and *Q. pubescens*, which comprised individuals strongly assigned to two or three distinct gene pools. It is worth mentioning that the lowest F value was observed in the population of *Q. frainetto* (F = 0.065, SE = 0.041), for which population substructure was not found in the STRUCTURE analysis.

CONCLUSIONS

Results of this study showed that the populations of *Quercus fraineto, Q. petraea* and *Q. pubescens* in the area of the Landscape of Outstanding Features "Kosmaj" are characterized by rather high levels of genetic diversity, which is a prerequisite for their long-term survival, especially in terms of climate change. Also, despite the presence in sympatry, all three species of oaks have retained their genetic integrity. However, a pronounced genetic structure, with the existence of a substructure of the populations of *Q. petraea* and *Q. pubescens*, was observed. These findings are essential for the formulation of conservation strategies, which should include the establishment of *in situ* conservation units as a first step towards the conservation and directed utilization of genetic resources of these three native oak species.

Author Contributions

MŠN (project manager) and JM organized the study, MŠN and IKJ sampled the leaves, IKJ and JMA performed laboratory and statistical analyses. IKJ and MJ drafted the manuscript, JMA, JM and MŠN improved the manuscript draft. All authors discussed the results and contributed to the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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