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VARIABILITY OF THE CHLOROPLAST DNA OF SESSILE OAK (*QUERCUS PETRAEA* AGG. EHRENDORFER, 1967) IN SERBIA

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Abstract — Genetic variability of sessile oak (*Quercus petraea* agg. Ehrendorfer, 1967) in Serbia is estimated applying cpDNA universal primer pairs that were characterized by a high informative level for chloroplast genome variability assessment in previous investigations. Five different haplotypes were detected in the analyzed sample material from populations in Serbia.

Key words: Sessile oak, chloroplast DNA, haplotypes, variability, regions, Serbia

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INTRODUCTION

The complex of sessile oak forests in Serbia occupies the lower part of the submontane belt and the low montane belt, from an altitude of 120 m in the Peri-Pannonian part to above 1200 m in Southeast and Southwest Serbia (Dinić, 1997; Tatić and Tomić, 2006; Tomić, 2006; Tomić et al., 2006a, 2006b; Banković et al., 2008). Sessile oak forests in Serbia are regarded as the aggregate (*Quercus petraea* agg. Ehrendorfer, 1967) of three sessile oak species: the European [*Quercus petraea* (Matt.) Liebl.], Balkan (*Quercus dalechampii* Ten.), and cluster-fruited (*Quercus polycarpa* Schur.) sessile oaks (Jovanović, 1991). To date, there have been no in-depth taxonomic studies on the sessile oak aggregate in Serbia, although individual species have been reported by numerous authors. Also, there are no studies at the level of molecular markers and the correlation between genetic and ecological variability of the populations.

Variability of the sessile oak aggregate can be considered as a complex of two categories: adaptive variability, affected by environmental factors, and neutral variability, which is not affected. The study of adaptive variability focuses on the range of ecological conditions of the species habitat and the morphological-anatomic-phenological traits of individuals in different habitats. Neutral variability, which can be measured using DNA profiling technique, does not include adaptive differences among individuals. It is generally accepted that the level of neutral variability points to the level of species adaptive variability. The use of molecular markers eliminates numerous misunderstandings on variability, which are a consequence of environmental impacts, especially in the analysis of quantitative traits, the expression of which is much more impacted by interaction between the genetic base and variable environmental conditions. For this reason, molecular genetics techniques have been increasingly applied in determining the degree of variability.

The aim of this paper was to determine the degree of sessile oak variability in Serbia using molecular marking of the chloroplast genome, and to designate specific regions characterized by the presence of rare haplotypes as the base of planning an adaptability enhancement strategy for the species.

MATERIAL AND METHODS

The data on sessile oak distribution in Serbia are based on the National Forest Inventory of the Republic of Serbia (Banković et al., 2008). In keeping with the spatial distribution of sessile oak forests in Serbia, material for laboratory analyses was collected from eight regions: Northern Serbia (Vojvodina), Northwest Serbia, Šumadija, Northeast Serbia, Eastern Serbia, Southeast Serbia, Western and Southwest Serbia, and Central Serbia. Sessile oak forests in Serbia are found in six altitudinal zones, from 120 m to above 1000 m. Table 1 presents a survey of altitudinal zones.

The clusters from which samples were collected were located in 21 areas and six altitudinal zones (Table 2).

The plant materials for DNA isolation were fresh dormant buds. Altogether, 20 branchlets with winter buds were collected from each tree, i.e., from three trees in each cluster. This was considered an optimum based on the results of previous investigations, which showed that chloroplast DNA was highly variable among populations, but was almost fixed within populations (Petit et al., 1993). The distance between trees was 200-500 m, which was considered as a distance which makes possible the sampling of a wide range of genetic diversity and minimizes sampling of closely related, i.e., inbred, individuals (Fraxigen, 2005).

Various haplotypes of *Quercus petraea* in Serbia were identified using previously published universal primer pairs (Taberlet et al., 1991; Demesinre et al., 1995), pairs which amplify the DT and AS segments of chloroplast DNA. These segments exhibited a high informative level in previous research on chloroplast genome variability (Ferris et al., 1997; Petit et al., 1997, 2002; Ballian et al., 2007; Slade et al., 2008).

Total genomic DNA was isolated from dormant buds of sessile oak using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The quality and quantity of isolated DNA were estimated by electrophoresis through 0.8% agarose gel stained with ethidium bromide.

Table 1. Altitudinal zones of the samples taken for molecular genetic analyses of DNA markers.

Altitudinal zone	Altitudes (m)
I	less than 200
II	200-400
III	400-600
IV	600-800
V	800-1000
VI	1000-1200

Table 2. Regions and areas with altitudinal zones from which the samples were collected for molecular genetic analyses of DNA markers.

	Region	Area	Altitudinal zone	Number of samples
1	Northern Serbia (Vojvodina)	Vršački Breg	II	3
		Fruška Gora	II and III	6
		Pocerina	I	3
2	Northwest Serbia	Cer	II	3
		Gučevo	IV	3
3	Šumadija	Avala	II	3
		Rudnik	III and IV	6
		Đerdap	I and II	6
4	Northeast Serbia	Majdanpečka Domena	III	3
		Negotin	IV	3
		Ozren	II and III	6
5	Eastern Serbia	Vetren	IV	3
		Babušnica	V	3
6	Southeast Serbia	Kozjak	III, IV, and V	9
		Bosilegrad	IV and VI	3
		Suvobor	III and IV	6
7	Western and Southwest Serbia	Zlatibor	IV, V, and VI	9
		Goč	III and IV	6
		Prijepolje	VI	3
8	Central Serbia	Jastrebac	III and IV	6

The PCR for DT and AS segments was performed in a final volume of 15 μl using the Expand Long Template PCR System (Roche Diagnostics GmbH, Germany). The master mix contained 1 x Expanded Buffer 2, 0.5 mM dNTPs, 0.1 μM primers, 0.45 In Exp DNA Pol mix, 0.1% (v/v) Tween 20 (Serva, Germany), and 1-5 ng of total genomic DNA. The PCR for DT and AS segments was carried out in a Progene thermal cycler (Techne, UK) under the following cycling conditions: 3 min of initial denaturation at 97°C, 37 cycles of 45 sec at 95°C, 2 min at 56°C, and 4 min at 68°C; and 10 min of final

extension at 68°C, followed by 20 min at room temperature.

Restriction fragment length polymorphism analyses were performed by restriction digestion with the appropriate restriction enzyme and subsequent denaturing on polyacrylamide gel. A 5-µl portion of the DT PCR product was digested with 25 In TaqI enzyme (Pharmacia Biotech, USA) in a final volume of 10 µl according to the manufacturer's instructions at 65°C. A 5-µl portion of the AS PCR product was digested with 2.5 In HinfI enzyme (Sigma, USA) in a final volume of 10 µl according to the manufacturer's instructions at 37°C. Analyses of restriction digestions for AS and DT segments were performed by electrophoresis through 4% denaturaturing polyacrylamide gel stained with silver (Bassam et al., 1991). The size of each discrete band (restriction fragment) was determined according to a DNA molecular weight marker (100 bp ladder, Fermantas, Germany) and was labelled relatively. The smallest detected restriction fragment was labelled 1, and so on in order. Eleven restriction fragments were noticed for the AS segment, comprising two different genotypes, while seven restriction fragments were noticed for the DT segment, comprising four different genotypes. The detected genotypes gave five different haplotypes.

Statistical processing of the data was performed using the Microsoft® software package for population genetics, Popgene Version 1.31 (Yeh et al., 1999). The values of genetic distance after Nei (1978) range from «0» (which indicates equal genotype frequencies in the study population) to «∞» (which indicates the absence of common genotypes across the study populations).

The genetic distance parameter can have different interpretations (Milovanović, 2007). From the aspect of phylogeography, a positive correlation is expected between the values of genetic and geographic distances of two populations, although such an expectation might not be grounded if there are corridors and isolated trees permitting gene flow between distant populations. The values of genetic distance can also provide significant information in the field of taxonomy. Based on the results of several isoenzyme analyses, Nei (1976) reports that species are characterized by genetic distance values of from 0.10 to 1.00, while subspecies and varieties are characterized by values of from 0.02 to 0.20.

RESULTS

Restriction fragment length polymorphism analyses for the AS and DT segments of cpDNA showed a high level of informativeness and application in study of the genetic variability of *Quercus petraea* agg. Multiple restriction fragment length polymorphism analyses were done for all collected samples.

Five different haplotypes were detected in the analyzed sample material of *Quercus petraea* populations from the territory of Serbia (Table 3 and Fig. 1).

The most frequent haplotype was haplotype 1, and its presence was determined in all regions, i.e., throughout the territory of Serbia (Table 4).

Haplotype 2 differed from haplotype 1 with respect to the DT genotype, while the AS genotype was the same. Haplotype 2 individuals were recorded in Western and Southwest Serbia, i.e., on the mountains Goč and Zlatibor. Haplotype 3 differed from the previous ones in the DT genotype, while the AS genotype was the same, and individuals of this haplotype were also recorded in Western and Southwest Serbia, i.e., in the area of Prijepolje. Haplotype 4 had the same DT genotype as haplotype 1, but its AS genotype was different from the AS genotype in the previous three haplotypes, and these individuals were also found in sessile oak forests in the area of Prijepolje in Western and Southwest Serbia. Haplotype 5 had the same AS genotype as haplotype 4, but its DT genotype differed from all previously mentioned haplotypes. Its presence was characteristic of the area of Mt. Goč in Western and Southwest Serbia. The genetic similarity/distance of regions within the sessile oak range in Serbia was determined on the basis of genetic distance according to Nei (1978) (Table 5).

The values of genetic distance show theoretically absolute genetic similarity (D=0.0000) of all the study regions except Western and Southwest Serbia, where, in addition to haplotype 1 (characteristic of

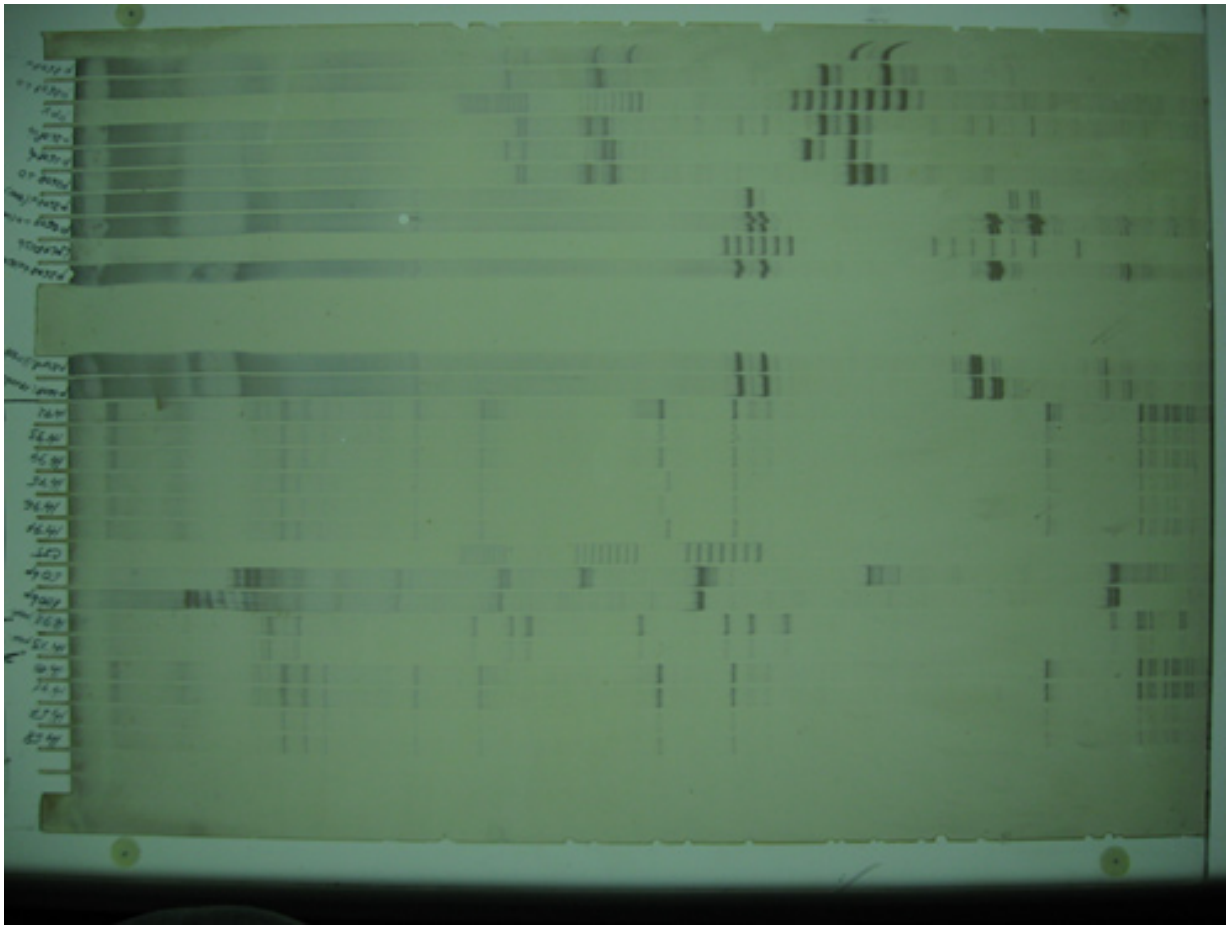

Fig. 1. Restriction fragment length polymorphism analyses of the AS and DT segments of a cpDNA on 4% denaturing polyacrylamide gel stained by silver.

all of Serbia), there were also haplotypes 2, 3, 4, and 5. The genetic distance between Western and Southwest Serbia and all other regions is characterized by a value of D = 0.1023, which represents intraspecific variability after Nei (1976). The occurrence of individuals of different and rare sessile oak haplotypes in Serbia constitutes an exceptional potential for the conservation and enhancement of genetic and adaptive variability. For this reason, the populations in Western and Southwest Serbia should be given special attention in further investigations.

DISCUSSION AND CONCLUSION

Based on the study results, it can be concluded that sessile oak is represented in eight regions in Serbia: Northern Serbia (Vojvodina), Northwest Serbia, Šumadija, Northeast Serbia, Eastern Serbia, Southeast Serbia, Western and Southwest Serbia, and Central Serbia. In the phytocenological sense, the sessile oak aggregate has a large number of communities in Serbia (Dinić, 1997; Tatić and Tomić, 2006; Tomić, 2006; Tomić et al., 2006a, 2006b).

Molecular genetic analyses show that the territories of Vojvodina, Northwest Serbia, Šumadija, Northeast Serbia, Eastern Serbia, Southeast Serbia, and Central Serbia represent a homogeneous entity, as only one haplotype (haplotype 1) was recorded in all sampled populations in these regions. The areas in Western and Southwest Serbia in which sessile oak populations were recorded can be defined as a

Table 3. Restriction fragment length polymorphism analyses of the AS and DT segments of a cpDNA and haplotype frequency per 100 individuals.

Haplotype	AS genotype	DT genotype	Haplotype /100 individuals
Haplotype 1	1,2,4,5,6,8,10,11	1,2,4,5,6	88.60
Haplotype 2	1,2,4,5,6,8,10,11	2,4,5,6	7.59
Haplotype 3	1,2,4,5,6,8,10,11	2,3,5,6	1.27
Haplotype 4	1,2,3,5,6,7,9	1,2,4,5,6	1.27
Haplotype 5	1,2,3,5,6,7,9	2,4,5,7	1.27

Table 4. Sessile oak haplotypes in the analysed clusters in Serbia.

Region		Area	Altitudinal zone	Haplotype
1	Vojvodina	Vršački Breg	II	1
		Fruška Gora	II and III	1
2	Northwest Serbia	Pocerina	I	1
		Cer	II	1
		Gučevo	IV	1
3	Šumadija	Avala	II	1
		Rudnik	III and IV	1
4	Northeast Serbia	Đerdap	I and II	1
		Majdanpečka Domena	III	1
		Negotin	IV	1
5	Eastern Serbia	Ozren	II and III	1
		Vetren	IV	1
		Babušnica	V	1
6	Southeast Serbia	Kozjak	III, IV, and V	1
		Bosilegrad	IV and VI	1
7	Western and Southwest Serbia	Suvobor	III and IV	1
		Zlatibor	IV, V, and VI	1, 2
		Goč	III and IV	1, 2, 5
		Prijepolje	VI	1, 3, 4
8	Central Serbia	Jastrebac	III and IV	1

Table 5. Genetic distance among sessile oak regions after Nei (1978).

Region	1	2	3	4	5	6	7	8
1	****		1.0000	1.0000	1.0000	1.0000	0.9027	1.0000
2	0.0000	****	1.0000	1.0000	1.0000	1.0000	0.9027	1.0000
3	0.0000	1.0000	****	1.0000	1.0000	1.0000	0.9027	1.0000
4	0.0000	0.0000	0.0000	****	1.0000	1.0000	0.9027	1.0000
5	0.0000	0.0000	0.0000	0.0000	****	1.0000	0.9027	1.0000
6	0.0000	0.0000	0.0000	0.0000	0.0000	****	0.9027	1.0000
7	0.1023	0.1023	0.1023	0.1023	0.1023	0.1023	****	0.9027
8	0.0000	0.1023	0.0000	0.0000	0.0000	0.0000	0.1023	****

special entity, as in addition to the widely represented haplotype 1, they also support the rare haplotypes 2, 3, 4, and 5.

This conclusion is also confirmed by ecological characteristics of the sessile oak sites in this area, which is characterized by a specific bedrock of ultrabasic eruptives (serpentinite, serpentinized peridotite, and peridotite), as well as by three different types of climate (humid climate of coppice forests, humid climate of high forests, and wet perhumid climate).

Thus, the region of Western and Southwest Serbia is specific in all basic characteristics and offers obvious proof of the complex cause-and-effect interactions between genotype and environment. The spatial specificity of this region is also indicated by the study of sessile oak haplotypes in Bosnia and Herzegovina, where the highest variability was recorded in the meeting place of Illyrian and Moesian floristic groups, i.e., climate zones (Ballian et al., 2007). The answer to the question as to whether the specific environment caused changes in the gene pool population or whether a specific genotype colonized the specific environment requires considerably more complex research, which should also include different types of genetic markers and progeny tests.

The areas in Western and Southwest Serbia, with all their specificities, represent an exceptional potential for the conservation of sessile oak variability, which can also have a very significant role for the enhancement of sessile oak (*Quercus petraea* agg. Ehrendorfer, 1967) aggregate adaptability to future global climate changes, which are apparently unavoidable. In these areas, all populations with rare haplotypes should be designated and incorporated into a program of static and dynamic conservation *in situ* and *ex situ* (establishment of seed banks; conservation of individual trees, tree groups, or entire populations; performance of progeny tests in different environmental conditions), since the central issues of conservation are evolutive processes that change and enhance genetic diversity, and not efforts for conservation of the exclusively current distribution of variability as the final form (Namkoong et al., 1997; Namkoong, 2001; Šijačić-Nikolić and Milovanović, 2007).

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ВАРИЈАБИЛНОСТ ХЛОРОПЛАСТНЕ ДНК ХРАСТА КИТЊАКА (*QUERCUS PETRAEA* AGG. EHRENDORFER, 1967) У СРБИЈИ

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Генетичка варијабилност храста китњака (*Quercus petraea* agg. Ehrendorfer, 1967) у Србији утврђивана је применом универзалних прајмерских парова хлоропластне ДНК, карактерисаних високим степеном информативности за процену варијабилности хлоропластног генома у претходним истраживањима. Пет различитих хаплотипова је детектовано у анализираном материјалу из популација у Србији.