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Full Length Research Paper

Sessile oak (*Quercus petraea* agg. Ehrendorfer 1967) rare haplotypes appearance in Serbia

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Sessile oak (*Quercus petraea* agg. Ehrendorfer 1967) genetic variability in Serbia was estimated applying cpDNA universal primer pairs. Five different haplotypes were detected in the analyzed sample material from populations in Serbia. The areas in West and Southwest Serbia, with all their specificities, represent an exceptional potential for the conservation of sessile oak variability, which can have a very significant role also for the enhancement of sessile oak (*Q. petraea* agg. Ehrendorfer 1967) aggregate adaptability to future global climate changes, which are apparently unavoidable.

Key words: Sessile oak, chloroplast DNA, haplotypes, conservation.

INTRODUCTION

The variability of sessile oak can be considered as the 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 the adaptive differences among individuals. It is generally accepted that the level of neutral variability points to the level of the species adaptive variability. The use of molecular markers eliminates the numerous misunderstandings on variability, which are the consequence of environmental impacts, especially in the analysis of quantitative traits, the expression of which is much more impacted by the interaction between the genetic base and the variable environmental conditions. For this reason, the molecular genetics techniques have been increasingly applied in the determination of the degree of variability.

The aim of this paper is to determine the degree of sessile oak variability in Serbia by using molecular marking of the chloroplast genome, and to declare the species conservation potential implied in rare haplotypes.

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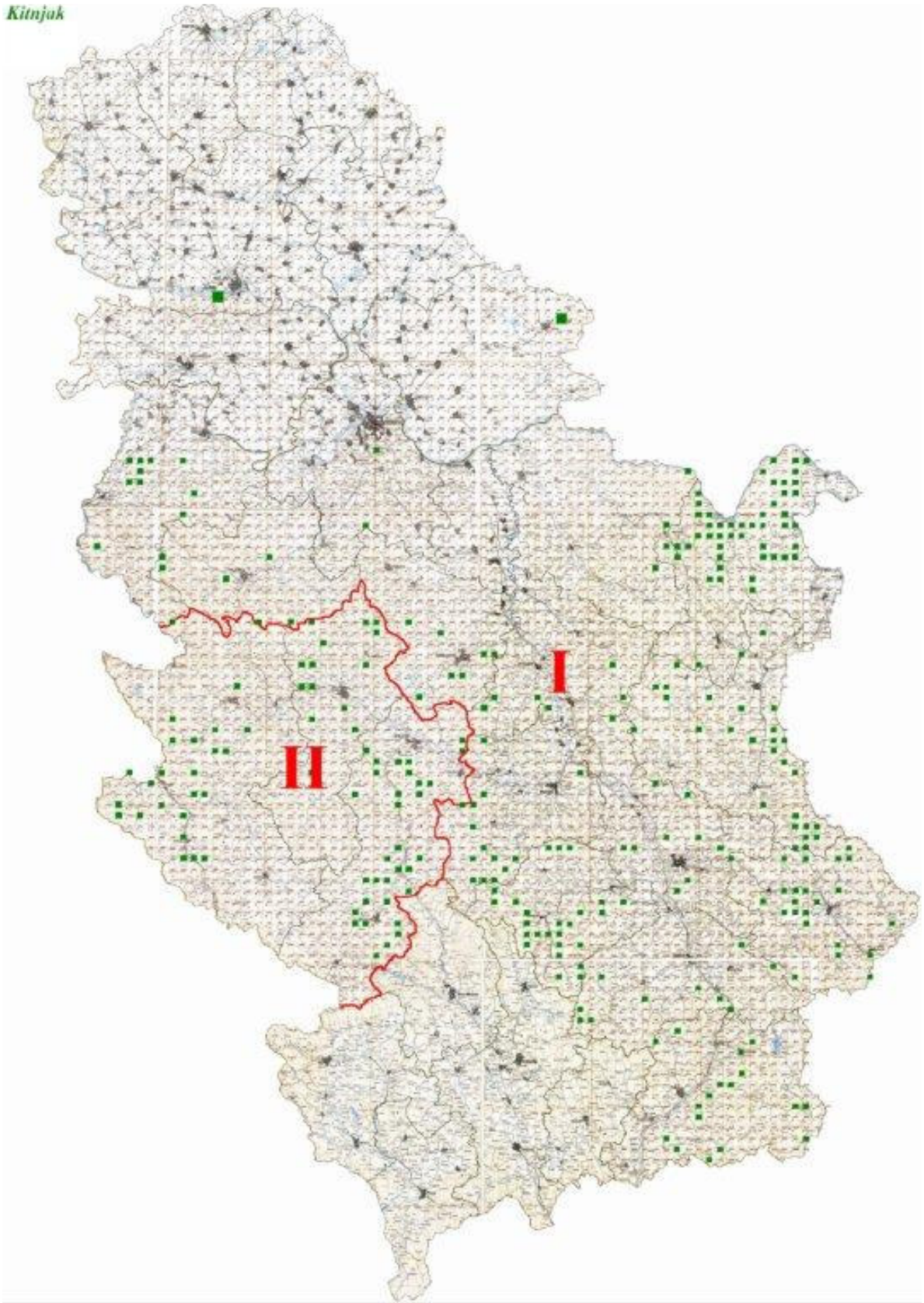
MATERIALS AND METHODS

The complex of sessile oak forests in Serbia occupies the lower part of the submontane belt and the low montane belt, from the altitude of 120 m in the Perio-Pannonian part, to above 1,200 m in the Southeast and Southwest parts (Dinić, 1997; Tomić, 2006; Tomić et al., 2006a, b; Tatić and Tomić, 2006; Banković et al., 2008). Sessile oak forests in Serbia are regarded as the aggregate of three sessile oak species (*Q. petraea* agg. Ehrendorfer 1967): the European (*Q. 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.

The data on sessile oak distribution in Serbia are based on the National Forest Inventory of the Republic of Serbia (Banković et al., 2008). Based on the analyses of spatial distribution of sessile oak forests in Serbia (the whole territory of Serbia is covered with 4 x 4 km network of sample plots-clusters, consisted of four sample circles 200 m distant), the material for laboratory analyses was collected from eight regions: North Serbia (Vojvodina), Northwest Serbia, Šumadija, Northeast Serbia, East Serbia, Southeast Serbia, West and Southwest Serbia and central Serbia.

Samples were collected in 20 populations within the sessile oak regions in Serbia (Map 1). The plant materials for DNA isolation were fresh dormant buds. Altogether 20 branchlets with winter buds were collected from each tree; that is from three trees in each cluster (some locations have been covered with more than one cluster). This was considered an optimum based on the results of previous researches, which pointed out that chloroplast DNA was highly variable among the populations, but it was almost fixed within the populations (Petit et al., 1993). The distance between trees was



Map 1. Clusters (green points) from which the samples were collected for molecular genetic analyses and provenance regions of sessile oak in Serbia (I and II).

Table 1. 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

200-500 m, which was considered as the distance which enables the sampling of a wide range of genetic diversity and minimises the sampling of closely related individuals, i.e. inbreeding (Fraxigen, 2005).

Previously published universal primer pairs (Taberlet et al., 1991; Demesure et al., 1995), which amplify DT and AS segments of the chloroplast DNA, were used for haplotype determination. These segments showed high informative level in previous research of 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 sessile oak dormant buds using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. Quality and quantity of isolated DNA was estimated by electrophoresis through 0.8% agarose gel stained by ethidium bromide.

The PCR for DT and AS segments was performed in a final volume of 15 µl using Expand Long Template PCR System (Roche Diagnostics GmbH, Germany). 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 total genomic DNA. PCR for DT and AS segments was carried out in a Progene thermal cycler (Techne, UK) according to the following cycling conditions: 3 min of initial denaturation at 97°C, 37 cycles of 45 s at 95°C, 2 min at 56°C, and 4 min at 68°C; and 10 min of final extension at 68°C, and 20 min at room temperature.

Restriction fragment length polymorphism analyses (RFLP) were performed by a restriction digestion with the appropriate restriction enzyme and by subsequent analyses on a denaturaturing polyacrilamide gel. 5 µl of the DT PCR product was digested with 25 In TaqI enzyme (Pharmacia biotech, USA) in final volume of 10 µl according to the manufacturer's instruction at 65ºC. 5 µl of the AS PCR product was digested with 2.5 In HinfI enzyme (Sigma, USA) in final volume of 10 µl according to the manufacturer's instruction at 37ºC. Analyses of restriction digestions for AS and DT segments were performed by electrophoresis through 4% denatureturing polyacrilamide gel stained by silver (Bassam et al., 1991). The size of each discrete band (restriction fragment) was determined according to DNA molecular weight marker (100 bp ladder, Fermantas, Germany) and was labelled relatively. The smallest detected restriction fragment was labelled with 1, and so on in order.

RESULTS

Multiple restriction fragment length polymorphism analyses were done for all collected samples from *Q. petraea* agg. populations. 11 restriction fragments were noticed for the AS segment, comprising 2 different genotypes, while 7 restriction fragments were noticed for the DT segment, comprising 4 different genotypes. The detected genotypes gave 5 different haplotypes (Table 1). The most frequent haplotype is haplotype 1 and its presence was determined in all regions, that is, throughout the territory of Serbia.

Haplotype 2 differed from haplotype 1 according to the DT genotype, while the AS genotype was the same. Haplotype 2 individuals were recorded in West and Southwest Serbia; that is on the mountains Goč and Zlatibor (Map 1, Region II). Haplotype 3 differed from previous ones by the DT genotype, while the AS genotype was the same, and the individuals of this haplotype were also recorded in West and Southwest Serbia, that is in the area of Prijepolje (Map 1, Region II). 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 West and Southwest Serbia. Haplotype 5 had the same AS genotype as haplotype 4, but its DT genotype differed from all previously quoted haplotypes. Its presence was characteristic for the area of Mt. Goč in West and Southwest Serbia (Map 1, Region II).

DISCUSSION

In the phytocoenological sense, sessile oak aggregate has a great number of communities in Serbia (Dinić, 1997; Tomić, 2006; Tomić et al., 2006a, b; Tatić and Tomić, 2006) represented in eight regions. However, the molecular genetic analyses show that the territories of Vojvodina, Northwest Serbia, Šumadija, Northeast, East, Southeast 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 West and Southwest Serbia in which sessile oak populations were recorded can be defined as a 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 the ecological characteristics of sessile oak sites in this area, which is characterised by a specific bedrock of ultrabasic eruptives (serpentinite, serpentinised peridotite and periodtite), as well as by three different types of climate (humid climate of coppice forests, humid climate of high forests

and wet perhumid climate). 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 that is climate zone (Ballian et al., 2007).

The areas in West and Southwest Serbia, with all their specificities, represent an exceptional potential for the conservation of sessile oak variability, which can have a very significant role also for the enhancement of sessile oak (*Q. 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 in the programme of static and dynamic conservation *in situ* and *ex situ* (seed banks, conservation of individual trees, tree groups or the entire populations, progeny tests in different environmental conditions), as the central issues of conservation are evolutive processses, which change and enhance the genetic diversity, and not the efforts for the conservation of the exclusively current distribution of variability as the final form (Namkoong et al., 1997; Namkoong, 2001; Šijačić-Nikolić and Milovanović, 2007).

According to the OECD Scheme of Forest Reproductive Material Transfer in International Trade and the Law on Forest Trees Reproductive Material ("The Official Gazette of the Republic of Serbia" number 135/04, 8/05, 41/09), production of selected and reproductive material of known origin imposes the need for provenance region definition. The provenance means one or several regions with similar ecological conditions and similar phenotypic and genetic variability level of the species. In that sense, the research results contributed to the process of sessile oak provenance regions establishing in Serbia ("The Official Gazette of the Republic of Serbia" number 91/08). Two sessile oak provenance regions (Map 1) have been pronounced according to the rare haplotypes appearance.

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