MORPHOLOGICAL AND MOLECULAR DIFFERENTIATION OF THE CROATIAN POPULATIONS OF *QUERCUS PUBESCENS* WILLD. (FAGACEAE)

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ABSTRACT

Taxonomy of the genus *Quercus* L. is very complicated and often controversial because of its great variability and intense gene flow among the related species. The purpose of this research was to determine morphological and molecular variation, relationships and taxonomic status of the Croatian populations of *Quercus pubescens* Willd. using morphological analysis of the leaves and RAPD-PCR technique. The results of the morphological and molecular analyses were very similar, both showing differentiation of the southern (Mediterranean) from the northern (Continental) pubescent oak populations. These two groups were clearly separated and the estimated gene flow among the populations that belong to different groups (Nm=1.38) is significantly less than among the populations that belong to the same group (Nm=3.70). The obtained results were compared to the available studies. This study confirms a high variability of the *Q. pubescens* populations, but differences were not so big to confirm the opinion of existence of several species in this area. The conclusion is that the southern Croatian populations could be pure *Q. pubescens* populations, while the peculiarities of the northern Croatian populations originate probably from the *Q. petraea* introgression.

KEY WORDS: *Quercus pubescens*, population differentiation, morphology, RAPD, introgression, Croatia.

INTRODUCTION

Genus *Quercus* L. is characterized by high polymorphism, and complex patterns of variation (Burger 1975; Manos et al. 1999). Hybridization and introgression are often present, even between morphologically and/or ecologically clearly differentiated species. This makes the taxonomy of the genus very complex and often controversial (Burger 1975; Rushton 1993; Franjić 1996).

The results of the morphological, cytological and molecular researches of the European oaks of the section *Quercus* point to close relation and intense gene flow among different species (Dupouey and Badeau 1993; Samuel et al. 1995; Bussoti and Grossoni 1997; Aas 1998; Bruschi et al. 2000). One of the best examples of such situation is the *Quercus pubescens* Willd. Some taxonomists suppose that the *Quercus pubescens* Willd. is divided into several species – *Q. pubescens* sensu stricto, *Q. brachyphylla* Kotschy, *Q. congesta* C. Presl, *Q. infectoria* Olivier, *Q. sicula* Borzi and *Q. virgiliana* /Ten./ Ten. (Schwarz 1964; Trinajstić 1974; Pignatti 1997), whereas the others think the differences are not big enough to be recognized as different species (Uzunova et al. 1996; Bussoti and Grossoni 1997; Bruschi et al. 2000).

In fact, like many other oaks, *Quercus pubescens* hybridizes with related species (*Q. petraea* /Matt./ Liebl., *Q. robur* L.) and therefore, in areas with suitable (intermediary) ecological conditions, hybrids are frequent (Dupouey and Badeau 1993; Aas 1998; Müller 1999; Bruschi et al. 2000).

Pubescent oak is a widespread species of great ecological and silvicultural importance in South and Central Europe. Its great variability allows it to live in different climatic and edaphic environments and to adapt to extreme ecological conditions. Croatian populations of *Q. pubescens* can

be found in the central part of the distribution range, at the borderline of two different climate areas – Mediterranean and Central Europe – and their study is essential to understand the evolutionary history of this species. In Croatian literature there is a prevailing opinion that in the Mediterranean area, as well as in dry and warm expositions in the continental part of the country, grow two closely related species of pubescent oaks – *Q. pubescens* Willd. and *Q. virgiliana* (Ten.) Ten. (Trinajstić 1986). Exactly, pure stands of the *Q. virgiliana* are considered to be spread in the outermost southern part, pure stands of the *Q. pubescens* in the northern coastal area, whereas mixed populations grow between these two areas. In addition to that, *Q. brachyphylla* and *Q. dalmatica,* in the outermost southern part of the country, were described as closely related species (Bačić 1996).

The taxonomy of genus *Quercus* is based on morphological traits and these traits are still crucial to differentiating species within the genus *Quercus* L. (Dupouey and Badeau 1993; Bruschi et al. 2000). However, in the past fifteen years numerous molecular techniques which enabled systematic researches have been applied (Hillis et al. 1996; Soltis et al. 1998), especially after the polymerase chain reaction (PCR) was developed (Mullis and Falcona 1987; Wolfe and Liston 1998). Many of these techniques have been applied to various taxa of genus *Quercus* (Moreau et al. 1994; Dumolin et al. 1995; Bacilieri et al. 1996; Howard et al. 1997; Bruschi et al. 2000, 2003a; Muir et al. 2000; Petit et al. 2002), but none for the taxonomy of *Quercus pubescens.*

Since analysis of the amount and distribution of morphological and genetic variations among and within populations of some species can increase our understanding about its evolutionary history and taxonomy, the purpose of this study was to determine morphological and molecular differentiation of Croatian pubescent oak populations using morphometric analysis of the leaves and RAPD-PCR technique (Welsh and McClelland 1990; Williams et al. 1990). The obtained results could be also important for future breeding and genetic resource conservation programs.

MATERIALS AND METHODS

Materials

A total of 100 samples from 10 populations were analyzed (10 trees per population). Distance among trees in population were 50-80 m. They represent the major geographical and ecological characteristics of the natural distribution of this species in Croatia. The populations originated from the next localities: Dilj (45°17' N; 18°10' E), Papuk (45°28' N; 17°38' E), Samobor (45°45' N; 15°39' E), Grobnik (45°22' N; 14°32' E), Lanišće (45°23' N; 14°07' E), Pula (44°56' N; 13°51' E), Jablanac (44°42' N; 14°54' E), Šibenik (43°46' N; 15°57' E), Imotski (43°29' N; 17°05' E) and Pelješac (42°51' N; 17°32' E) (Fig. 1).

Morphological analysis

A very high degree of variation among vegetative characters within individual oak trees is evident in many studies (Blue and Jensen 1988). So, the leaves growing in the light and in the shadow are significantly different (cf. Bru-
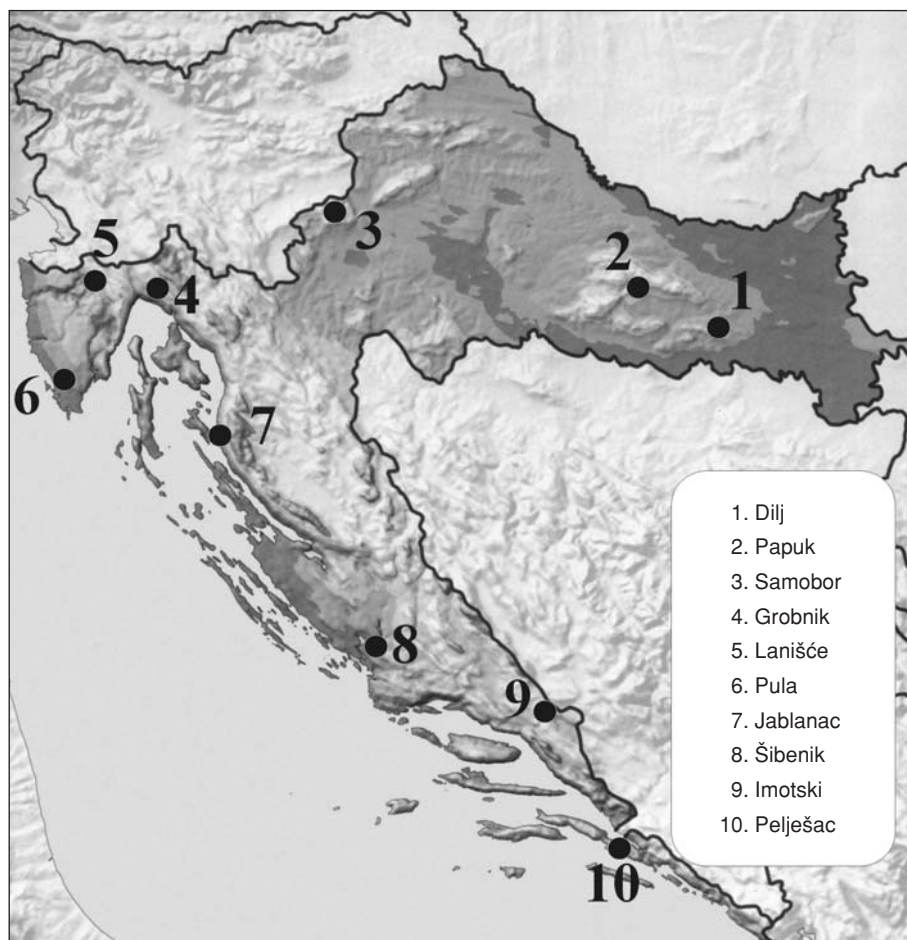

1. Dilj
2. Papuk
3. Samobor
4. Grobnik
5. Lanišće
6. Pula
7. Jablanac
8. Šibenik
9. Imotski
10. Pelješac

Fig. 1. Geographical position of the analysed pubescent oak populations.

schi et al. 2003) as are the leaves in various types of shoots (cf. Trinajstić and Franjić 1996). Because of that, only healthy, undamaged and fully developed leaves from the short fertile shoots and from the part of a tree crown exposed to light have been analyzed. Each tree was represented by 50 leaves. For each leaf the following traits have been measured and evaluated – leaf blade length (LL), leaf blade width (LW), distance between the leaf base and the leaf's widest part (WP), petiole length (PL), number of lobes on the left side of the leaf (NLL) and number of lobes on the right side of the leaf (NLR). All parameters have been measured with the accuracy of 0.1 mm. To estimate measurement error, leaves was measured twice using a small subsample of 100 leaves chosen across all samples. The percentage of measurement errors were 0.9-1.6%.

Descriptive statistical methods were used to calculate arithmetic mean values ($\bar{x}$) and standard deviations (SD) (Sokal and Rohlf 1989). To determine intrapopulational and interpopulational variability of *Q. pubescens* analysis of variance (ANOVA) was used. The analyzed factors were population and tree (tree factor nested inside the population factor). All these statistical data processing methods were done in the program package SAS System for Windows 6.12 (1990). Differentiation of populations was analyzed using cluster UPGMA and the Mahalanobis' distance from the STATISTICA for Windows software (StatSoft, Inc. 2001).

DNA analysis

DNA isolation: DNAs were extracted from 100 mg of fresh leaf tissue, as described in Dumolin et al. (1995) with some modifications. These modifications included addition of 1% soluble polyvinylpyrrolidone (PVP-40) and 1% 2-mercaptoethanol in 2% (w/v) alkyltrimethylammonium bromide buffer (Sigma M 7635), two extractions with dichloromethane and precipitation of nucleic acids using 2.5 volumes of cold ethanol. The quality and concentration of the DNAs were checked by electrophoresis in 0.8% (w/v) agarose and by comparison with λ DNA as a standard (Sambrook et al. 1989). Every DNA sample was checked for its clarity and applicability for RAPD (Random Amplified Polymorphic DNA) analysis, so after finishing the DNA isolation, every sample of DNA was diluted to three concentrations (5, 25 and 50 ng/25 μl PCR mixture) and preliminary amplified by usage of the same RAPD primer. Only those DNAs which showed constant RAPD banding patterns in these three DNA concentrations were determined as DNAs of adequate purity and quality for the subsequent RAPD analysis (Liber et al. 2002).

DNA amplification and electrophoresis: In total, 27 RAPD primers (Operon Technologies) were used, 20 of which from kit B and primers OPA-06, -08, -09, -12; OPE-08, -09; OPX-04. The total reaction volume was 25 μl, and it contained 25 ng of genomic DNA, 1x PCR Buffer (Applied Biosystems®), 2.5 mM MgCl$_2$, 100 μM dATP, dCTP, dGTP and dTTP, 0.36 μM 10-base RAPD primer (Operon Technologies Inc.) and 0.75 unit of Taq DNA polymerase (Eppendorf®). The amplification profile was 40 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. All PCR reactions were done on the GeneAmp PCR System 2700 (Applied Biosystems®) with a negative control without the DNA. The amplification products were analyzed by electrophoresis in 1.4% agarose gels, and visualized by ethidium-bromide staining. The molecular weights of the RAPD fragments were determined using 100 bp PCR standard (Bio-Rad).

RAPD data analysis: RAPD bands with the same molecular weight and mobility were treated as identical fragments. In the data matrices, the presence of a band was coded as 1, whereas the absence of the band was coded as 0. The analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to partition the total phenotypic variance within populations and among populations. Additional AMOVA was performed to partition the total variance into: within populations, among populations within regions and between regions. The AMOVA was performed using the GENALEX V5 program (Peakall and Smouse 2001). For each analysis 1000 permutations were performed to obtain significance levels. Nei's pairwise genetic distances among populations were calculated using POPGENE software, version 1.32 (Yeh et al. 1997). Relationships among populations were analyzed using Nei's distances and UPGMA clu-

TABLE 1. Means (x), standard deviations (SD) and ANOVA (F) for measured leaf traits.

Leaf variable		Dilj	Papuk	Samobor	Grobnik	Lanišće	Pula	Jablanac	Šibenik	Imotski	Pelješac	F [9, 110]	
												A*	W*
LL*	x (mm)	68.8	67.8	70.5	61.7	68.3	58.1	63.5	57.1	61.6	56.8	8.46	20.17
	SD (mm)	11.1	12.5	12.8	12.2	9.8	10.5	11.1	9.7	10.3	11.9		
LW*	x (mm)	46.1	46.5	46.6	41.7	47.7	41.3	44.0	40.0	42.3	43.6	3.43	17.85
	SD (mm)	8.7	10.7	9.3	9.8	9.6	8.1	10.0	8.6	8.2	10.7		
PL*	x (mm)	11.5	14.3	11.7	9.4	12.5	8.3	12.2	10.7	10.8	9.5	9.52	32.78
	SD (mm)	2.8	3.8	3.1	2.7	3.4	2.6	3.0	3.0	2.9	2.7		
WP*	x (mm)	40.1	38.8	37.5	33.1	39.9	31.8	34.6	31.1	33.5	31.8	7.73	16.51
	SD (mm)	8.8	8.0	10.5	9.2	8.3	8.0	8.4	7.8	7.9	9.2		
NLL*	x	5.7	5.1	5.1	5.2	4.8	5.0	5.1	4.1	5.0	4.8	5.87	32.55
	SD	1.0	1.0	0.9	1.0	0.8	0.9	0.9	0.8	1.1	0.9		
NLR*	x	5.8	5.1	5.1	5.3	4.7	4.9	5.1	4.0	5.0	4.8	6.87	33.17
	SD	1.0	1.0	0.9	1.0	0.8	1.0	1.0	0.8	1.1	0.9		

* LL – leaf blade length; LW – leaf blade width; WP – distance between the leaf base and the leaf's widest part; PL – petiole length; NLL – number of lobes on the left side of the leaf and NLR – number of lobes on the right side of the leaf; A – among populations; W – within populations

ster analyses from the STATISTICA for Windows software (StatSoft, Inc. 2001). From the Nei's pairwise genetic distances (Gst) gene flow between populations was calculated using the equation Nm=1/4(1/Gst-1) (Wright 1951).

RESULTS

Morphological data

The measured leaf characters were highly variable. There were significant differences among populations and among trees inside populations (ANOVA, p<0.001) for all measured leaf traits. The morphological leaf variation was higher within than among populations (Table 1). It could be seen that the continental populations (Dilj, Samobor, Papuk and Lanišće) had higher values of the variables determining the leaf size (LL, LW, PL, WP) than other populations. With respect to the number of lobes (NLL, NLR), the population Dilj was distinguished with its maximum values and the population Šibenik with its minimum ones (Table 1).

UPGMA method showed similar results as descriptive analysis – a clear differentiation of Continental (Dilj, Papuk, Samobor, Lanišće) and Mediterranean populations (Grobnik, Pula, Jablanac, Imotski, Pelješac) (Fig. 2).

Molecular data

From total of 27 RAPD primers used, seven of them produced scorable bands (Table 2). These seven RAPD pri-

mers yielded 84 polymorphic bands, which is 12 polymorphic RAPD bands per primer (Fig. 3).

AMOVA revealed 21.3% of the genetic variation among and 78.7% within populations (Table 3). In UPGMA dendrogram based on Nei's genetic distances the studied populations were grouped into two clusters. Similar to the morphological analysis, the populations from the northern part of Croatia (Dilj, Papuk, Samobor, Lanišće, Grobnik) formed one group and populations from the southern part another one (Pula, Jablanac, Šibenik, Imotski, Pelješac) (Fig. 4). Considering the results of the cluster analysis, we conducted an additional AMOVA in which the populations were arranged in these two geographical groups. In this analysis was observed 12.4% of molecular variance between the groups of populations, which supports the results of the cluster analysis. All AMOVA variations were found to be highly significant (p<0.001) (Table 3).

Maximum genetic distance (Gst) and minimum gene flow (Nm) were between populations Dilj and Imotski and minimum genetic distance and maximum gene flow were between populations Papuk and Samobor. Mean Gst among all analyzed populations was 0.14 while mean Nm was 2.39. Mean Gst within both geographical groups which were supported by the cluster analysis was 0.08 respectively, while mean Gst between these groups was 0.20. Mean Nm within geographical groups was 4.21 (north populations) and 3.19 (south populations), while mean Nm among these groups was 1.38 (Table 4).
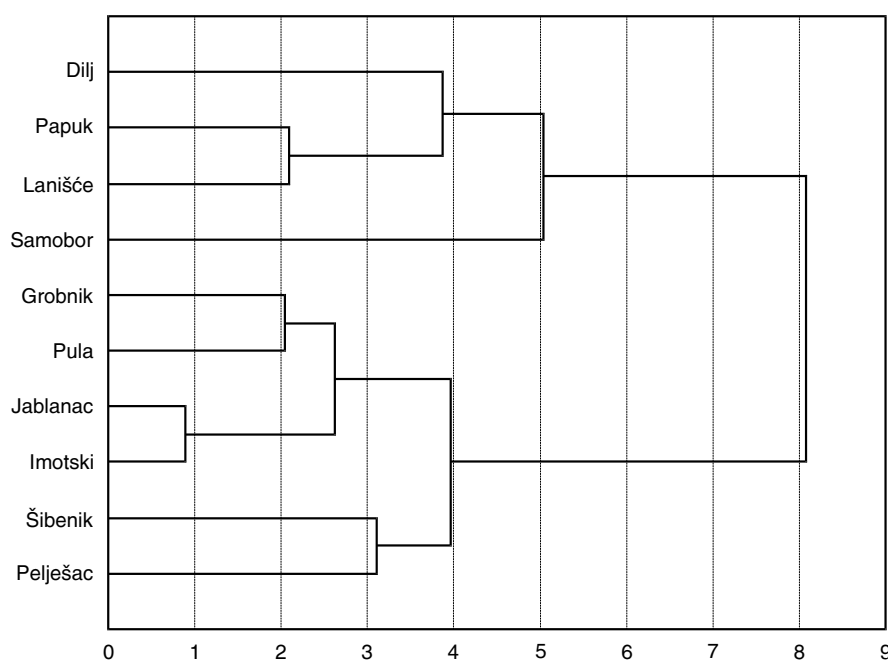

Fig 2. Cluster analysis (UPGMA method) of 10 populations of *Quercus pubescens* based on morphology of leaves and Mahalanobis' distance.

TABLE 2. RAPD primers that produced useful polymorphic bands among *Q. pubescens* accessions.

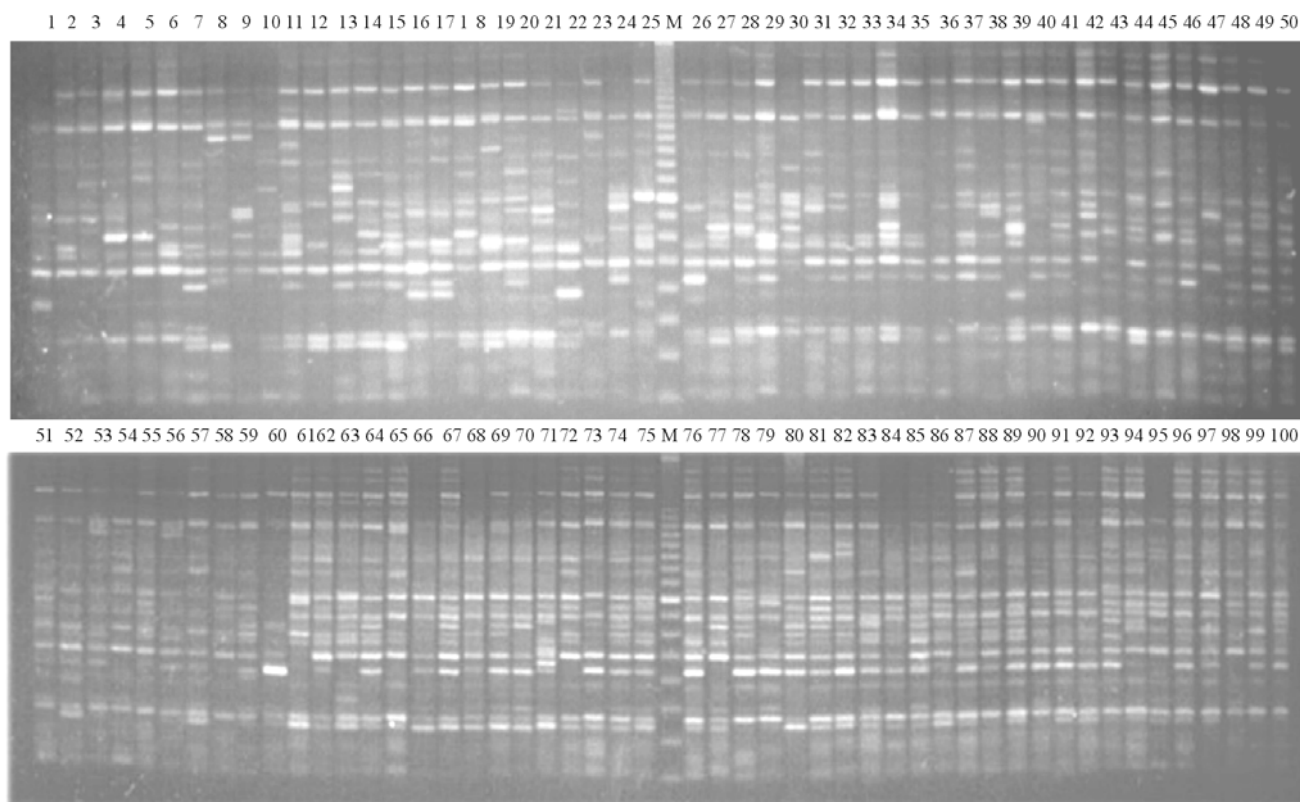| RAPD primer	Number of polymorphic bands	Size of polymorphic bands (bp)
OPB-02 (5'-TGATCCCTGG)	8	500-2100
OPB-04 (5'-GGACTGGAGT)	12	400-1950
OPB-10 (5'-CTGCTGGGAC)	15	500-2300
OPB-11 (5'-GTAGACCCGT)	8	550-1800
OPB-17 (5'-AGGGAACGAG)	17	300-2000
OPB-18 (5'-CCACAGCAGT)	14	450-1750
OPX-04 (5'-CCGCTACCGA)	10	400-2500

Fig. 3. RAPD profiles from *Quercus pubescens* s. l. accessions generated by OPB-17 primer: M = 100 bp PCR Molecular Ruler (BIO-RAD®); lanes 1-100 accessions from populations: Dilj (1-10), Papuk (11-20), Samobor (21-30), Grobnik (31-40), Lanišće (41-50), Pula (51-60), Jablanac (61-70), Šibenik (71- -80), Imotski (81-90), Pelješac (91-100).

TABLE 3. Summary of analysis of molecular variance

Level of variation	SS	MS	%	*p*
Without population grouping				
Among populations	82.350	9.150	21.3	<0.001
Within populations	221.900	2.466	78.7	<0.001
Populations grouped in two groups				
Between groups	27.470	27.470	12.4	<0.001
Among populations within groups	54.880	6.860	13.2	<0.001
Within populations	221.900	2.466	74.4	<0.001

SS – sums of squares; MS – mean squares; % – proportion of genetic variability; *p* – level of significance

DISCUSSION

The results of ANOVA and AMOVA, like in the other studies of long-lived woody perennials and wind-pollinated species (e.g. Hamrick and Godt 1990; Franjić 1996; Baliuckas et al. 1999; Rehfeldt 1999; Kovačić and Šimić 2001; Bruschi et al. 2003), show higher morphological and genetic variation within populations than among themselves (Table 1 and 3). The 21.3% of differentiation among populations and 78.7% within populations is similar result to other RAPD studies of outcrossing species (e.g. Reisch et al. 2005; Krüger et al. 2002; Nebauer et al. 1999) or to *Q. petraea* microsatellites study (Bruschi et al. 2003b).

The results of this study confirm, with no doubt, a high variability of the *Q. pubescens* in this area, but these differences can not confirm the views on existing of several species in this area, as some studies have stated so far (Trinajstić 1986; Bačić 1996). Contrary to that, morphological and molecular analyses divide all the Croatian populations

of pubescent oak only into southern (Mediterranean) and northern (Continental) groups (Figs 2 and 4). These two groups are separated so strongly that, for example, the estimated gene flow among them (Nm) is significantly lower than among the populations of the same group (Table 4). Although Nm is only estimation based on Gst, it reflects the degree of population differentiation.

There is a remarkable congruence between the results of this study and the results of the most recent researches that deny the existence of several species within the complex of *Q. pubescens* (Bussotti and Grossoni 1997; Uzunova et al. 1997) and affirm differentiation between the Central and South European *Q. pubescens* populations (Müller and Aas 1997; Müller 1999).

Comparing the results of this study with the results of the recent researches of the *Q. pubescens* in Central Europe (Samuel et al. 1995; Müller and Aas 1997; Aas 1998; Müller 1999, Bruschi et al. 2000, 2003a, b; Škvorc et al. 2005) we can conclude that the differences found between
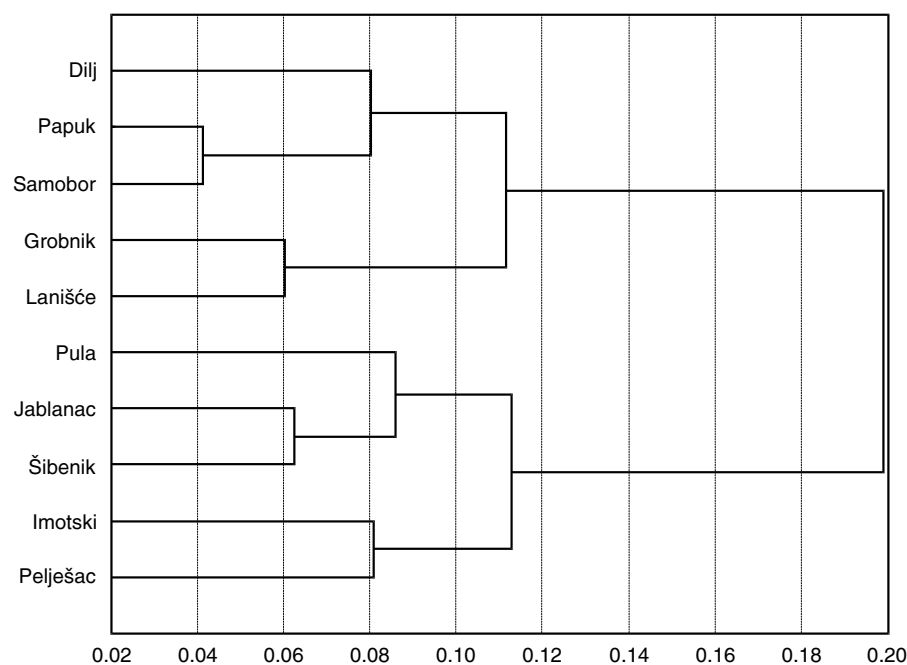
Fig. 4. Cluster analysis (UPGMA method) of 10 populations of *Quercus pubescens* based on RAPD data and Nei's genetic distance.

TABLE 4. Pairwise Nei's genetic distances (Gst – below diagonal) and gene flow among populations (Nm – above diagonal).

Population	Dilj	Papuk	Samobor	Grobnik	Lanišće	Pula	Jablanac	Šibenik	Imotski	Pelješac
Dilj	****	2.71	6.69	2.46	1.33	1.63	0.77	1.04	0.52	0.70
Papuk	0.08	****	14.37	2.69	2.24	1.61	0.88	0.85	0.69	0.81
Samobor	0.04	0.02	****	4.10	4.05	2.02	1.24	1.46	0.81	0.95
Grobnik	0.09	0.09	0.06	****	1.50	2.04	1.79	1.29	0.97	0.81
Lanišće	0.16	0.10	0.06	0.14	****	1.98	1.37	3.39	1.83	2.15
Pula	0.13	0.13	0.11	0.11	0.11	****	3.01	4.08	1.88	2.53
Jablanac	0.25	0.22	0.17	0.12	0.15	0.08	****	5.28	2.12	1.52
Šibenik	0.19	0.23	0.15	0.16	0.07	0.06	0.05	****	3.92	3.60
Imotski	0.32	0.27	0.24	0.20	0.12	0.12	0.11	0.06	****	3.91
Pelješac	0.26	0.24	0.21	0.24	0.10	0.09	0.14	0.06	0.07	****

northern and southern Croatian populations exist probably due to introgression of the *Q. petraea* (Matt.) Liebl. into the genome of the *Q. pubescens*. In other words, *Q. pubescens* in Central Europe was pushed out to very small and isolated areas of southern expositions where they exchange territory with *Q. petraea*. Due to the favourable ecological conditions, hybrids between those two species often manage to survive, so what is thought to be *Q. pubescens* in the Central Europe area (and likely in northern Croatia) in a certain way represents the transition form between *Q. pubescens* and *Q. petraea* (Samuel et al. 1995; Müller 1999). Because of the completely different ecological conditions *Q. petraea* occurs only as a rarity in southern Europe (Trinajstić 1974, 1986; Bruschi 2003b) and hybrids between *Q. pubescens* and *Q. petraea* are really rare in that region (Trinajstić 1974; Müller 1999; Škvorc et al. 2005). Since the mentioned hybrids have no chance to survive due to strong selection pressures that favour the genome of the more thermophilic species, the group of the southern Croatian populations probably represents pure *Q. pubescens* populations. Finally, the higher gene flow among Continental than Mediterranean populations noticed in this study (Table 4), in spite of the fact that Continental populations are smaller and more scattered, could be also outcome of *Q. petraea* introgression.

The difference between morphological analysis of leaves and RAPD analysis is most obvious in the Grobnik population which is sorted into the southern group by the morphological analysis, while the molecular analysis sorts it into the northern group of populations (Figs 2 and 4, Tables 1 and 4). Since the results of some previous studies connected the morphological traits of leaves with latitudinal and altitudinal range of populations, but also with different climatologic factors (e.g. Kleinschmit 1993; Škvorc et al. 2005), the settlement of Grobnik population, in morphological analysis in the southern group, can be explained by similar stand characteristics. Contrary to that, the results of molecular analysis can be connected to the fact that Grobnik population (unlike the other southern populations) is situated in contact area with the *Q. petraea* populations (like the other northern populations). This fact and the results from some previous studies, that try to delimit *Q. petraea* and *Q. pubescens* in morphological and molecular way (e.g. Bruschi et al. 2000; Škvorc et al. 2005), lead to the conclusion that RAPD profile of Grobnik population indicates possible *Q. petraea* introgression into the genome of *Q. pubescens*. The main difference between Grobnik population and the other continental populations in this research is that the possible *Q. petraea* introgression in Grobnik population is obvious only through molecular but not through

morphological characteristics. Due to the evident transitional characteristics of the Grobnik population and partly of Istrian population Lanišće (Figs 2 and 4; Tables 1 and 4), this region (Fig. 1) could be a place where *Q. petraea* introgression into the genom of the *Q. pubescens* gradually fade away towards the South.

From the conservation perspective the high variability and high level of gene flow among populations is encouraging (Tables 1, 3 and 4). Although continental populations are small and scattered, because of the intensive gene flow there is no risk of extinction (Table 4). Moreover, *Q. pubescens* could be the source of genes necessary for the survival of oaks in Middle Europe. It is especially important referring to the eventual climate changes connected to global warming and different anthropogenic impacts (Aas 1998; Müller 1999). The results of this research confirm that, in the conservation strategy, an oak species must not to be observed as isolated and static species but as a complex system where populations of other oak species can change its genome (Harrison and Hastings 1996).

In order to make the final confirmation of the stated theory about the introgression of the *Quercus petraea* populations, it is necessary to find the molecular markers specific to the *Q. petraea* (Samuel et al. 1995; Bruschi et al. 2000), and then to detect them in the Croatian *Q. pubescens* populations. If such specific molecular markers could be found, it would be possible to connect ecological conditions with the genetic constitution of each individual. These results would be of great benefit to future researches, as well as management and conservation of these species.

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