The Chloroplast DNA Polymorphisms of White Oaks of Section *Quercus* in The Central Balkans

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Abstract

A total of 444 oak trees from 110 populations from a previously under-sampled area in the central Balkans were analysed using four primer/enzyme combinations which amplified and restricted four, largely non-coding regions of the maternally inherited chloroplast DNA. Using the nomenclature of PETIT et al. (2002 a) to classify the haplotypes and lineages, the seven haplotypes that were found in Croatia, Bosnia and Herzegovina, Montenegro, Albania, Macedonia and southern Kosovo consisted of haplotypes 2, 4, 5, 6, 7, 17, 31, as well as the subtypes of haplotypes 4 (a), 5 (a, b, c), and 17 (a). Five of these haplotypes belong to lineage A. One of these, haplotype 5, is present throughout the sampled area. The distributions of the other haplotypes from this lineage are more geographically structured. The other two haplotypes, haplotype 2 and haplotype 17, belong to lineages C and E, respectively. The data are combined with previous data by PETIT et al. (2002 b) to provide more detailed information of the postglacial routes of colonisation taken by oaks in south-eastern Europe.

Key words: cpDNA polymorphism, *Quercus*, central Balkans, colonisation, glacial refugia, haplotypes.

Introduction

Because chloroplast DNA (cpDNA) is maternally inherited in oaks, it reflects the process of colonisation by seeds (acorns). Considerable research has already been done to trace the colonisation routes of oaks (FERRIS et al., 1993, 1995, 1998; PETIT et al., 2002 a, b; LUMARET et al., 2002) as well as other tree species in Europe (DEMESURE et al., 1996, FINESCHI et al., 2000). FERRIS et al. (1993, 1995, 1998) described two main groups of haplotypes, one which exhibited a western distribution, and another which occurred largely in the east of Europe. Later, using the same method as FERRIS et al. (1993), PETIT et al. (2002 a) found three main groups of haplotypes. In addition to cpDNA variation, evidence from the fossil pollen data has been used to reconstruct the changes in the geographical distribution of oaks during the period of recolonization (BREWER et al., 2002). The pollen data suggest the occurrence of different glacial refugia located at three southern European peninsulas (Iberian, Apennine and Balkan). However, there was a shortage of samples from south-eastern Europe for these previous cpDNA analysis, and given the likelihood that there were glacial refugia in the Balkan region there is a need for a more detailed sampling for oaks in this region. A more detailed knowledge of the geographic distribution of haplotypes within the region would help to explain more precisely the origin of European oaks, their migration routes and the location of the glacial refugia. The necessity for cpDNA information from this area is even more important in view of the lack of fossil pollen data corresponding to the early postglacial period (15.000–12.000 B.P.) within the south-west Balkans.

Unlike BORDÁCS et al. (2002), who analysed only the area of the northern Balkans, more precisely the north and north-west of Croatia, the samples for this analysis were collected from areas in the southern Balkans, or, more precisely, areas reaching as far as the southern parts of Macedonia. In addition, in Croatia, the populations from Istria at northern Adriatic coast to the area of Dubrovnik in far south were covered by the sampling, which was not the case with the study of BORDÁCS et al. (2002).

Materials and Methods

The buds, or in a few cases, the leaves of 444 trees from 110 natural populations of oaks of section *Quercus*, species *Q. robur* L., *Q. petraea* (Matt.) Liebl., *Q. pubescens* Willd. and *Q. frainetto* Ten. were collected (*Table 1*) across the area of Croatia to Macedonia. The basic criteria for the selection of the populations was to attempt to sample native woodlands and to adopt a strategy that provided a fairly even sampling over the study area. In areas of particular interest a more intensive sampling strategy was adopted.

The total DNA was extracted from the samples using the CTAB protocol (DOYLE and DOYLE, 1987). The four fragments of cpDNA used by PETIT et al. (2002 a) were amplified using the primers described in *Table 2*.

The PCR program involved an initial cycle of 93 °C for 4 min, followed by 30 cycles of three steps given in *Table 3*. The final extension was done for 10 min at 72 °C.

Two fragments (AS and TF) were digested with restriction enzyme *HinfI* (DEMESURE et al., 1995; TABERLET et al., 1991) and two (DT and CD) with *TaqI* (DEMESURE et al., 1995). Following digestion, each digest was separated on an 8% polyacrylamide gels. The duration of electrophoresis was different, depending on the size of the restriction fragments, and ranged between 1h 20 min for TF to 2h 45 min for CD and DT. The fragments were scored and haplotypes determined according to the system of PETIT et al. (2002 a).

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Table 1. – Basic data of analysed populations.

State	No	Population	Species	Number of individuals	Coordinates		Elevation (m)
Bosnia and Herzegovina	1	Autovac	*Q. pubescens*	1	43°04'	18°32'	1000
	2	Banovici	*Q. petrea*	5	44°25'	18°34'	396
	3	Bileca	*Q. pubescens*	5	42°54'	18°24'	780
	4	Bocac	*Q. petrea*	5	44°31'	17°09'	300
	5	Celinac	*Q. petrea*	1	44°42'	17°19'	260
	6	Dobro polje	*Q. petrea*	5	43°35'	18°31'	741
	7	Drezanka	*Q. petrea*	5	43°31'	17°41'	230
	8	Glamocko polje	*Q. pubescens*	4	43°58'	16°54'	1050
	9	Gorazde	*Q. petrea*	4	43°38'	18°58'	420
	10	Gornji Vakuf	*Q. petrea*	5	43°59'	17°30'	620
	11	Gromiljak	*Q. petrea* *Q. robur*	5	44°00'	18°01'	600
	12	Kifino selo	*Q. pubescens*	4	43°17'	18°13'	1020
	13	Ostrozac	*Q. petrea*	5	43°41'	17°49'	335
	14	Omarska	*Q. petrea*	4	44°53'	16°54'	200
	15	Popovo polje	*Q. pubescens*	1	42°44'	18°15'	320
	16	Sana	*Q. robur*	10	44°58'	16°47'	
	17	Sapna	*Q. petrea*	5	44°32'	18°57'	522
	18	Sipovo	*Q. petrea* *Q. frainetto*	3	44°18'	17°08'	500
	19	Sokolac	*Q. robur*	4	43°55'	18°48'	917
	20	Tegare	*Q. frainetto*	5	44°07'	19°59'	190
	21	Tjentiste	*Q. petrea* *Q. pubescens*	2 2	43°23'	18°46'	462
	22	Turbe	*Q. petrea*	4	44°13'	17°33'	650
	23	Vogosca	*Q. robur* *Q. pubescens*	4 1	43°53'	18°21'	600
	24	Vrbaska gora	*Q. robur*	5	45°09'	17°03'	
	25	Zepce	*Q. petrea*	5	44°25'	18°04'	232
	26	Zvornik	*Q. petrea*	3	44°24'	19°06'	185
Montenegro	27	Dapsici	*Q. petrea*	2	42°50'	19°57'	880
	28	Jasenovo polje	*Q. pubescens*	5	42°51'	18°56'	845
	29	Krstac	*Q. petrea*	4	42°58'	19°35'	985
	30	Sv. Stefan	*Q. pubescens*	3	42°16'	18°53'	55
	31	Tuzi	*Q. pubescens*	4	42°20'	19°20'	140
Serbia - Kosovo	32	Pec	*Q. petrea*	1	42°40'	20°18'	550
	33	Pristina	*Q. pubescens*	5	42°37'	21°11'	650
	34	Prizren	*Q. frainetto*	2	42°12'	20°45'	450
Croatia	35	Bunic	*Q. pubescens*	5	44°41'	15°39'	693
	36	Crnac polje	*Q. robur*	5	45°52'	18°48'	-
	37	Crno jezero	*Q. robur*	3	44°49'	15°15'	-
	38			2	44°48'	15°14'	-
	39	Cep	*Q. robur*	5	46°21'	16°23'	-
	40	Cepikuce	*Q. pubescens*	5	42°49'	17°49'	350

The frequencies of the haplotypes were used to compute diversity and differentiation measures following PONS and PETIT (1995). The within-population genetic diversity h_s, the total diversity h_t, and the coefficient of genetic differentiation G_{st} were calculated. The programs HAPLODIV and HAPLONST, available at http://www.pierroton.inra.fr/genetics/labo/Software, were used. These parameters were computed for the pooled samples regardless of species as well as independently for each of the three most common species (*Q. robur* L., *Q. petraea* (Matt.) Liebl. and *Q. pubescens* Willd.). The analysis was not done for *Q. frainetto* Ten. and mixed populations, because of the insufficient number of samples. Only the populations represented by at least three individuals were included in the analysis.

Results and Discussion

The following seven haplotypes were found in the study region; 2, 4, 5, 6, 7, 17 and 31. PETIT et al. (2002 a) also described sub-haplotypes and those detected in this study include haplotypes 4a, 5a, 5b, 5c, 17a. It was not possible to detect sub-haplotypes for all populations because the additional bands which determine sub-haplotypes could not be always recorded during gel analysis. The position of bands for each haplotype and sub-haplotype are given in *Table 4*.

The geographical distribution of haplotypes is presented in *Figure 1*. Most of the haplotypes (4, 5, 6, 7 and 31) belong to the phylo-geographical lineage A (PETIT et al., 2002 b). These haplotypes originate mostly from south-

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Table 1. – Continuing.

State	No	Population	Species	Number of individuals	Coordinates		Elevation (m)
Croatia	41	Dilj	*Q. pubescens*	3	45°17'	17°55'	-
	42	Dragov lug	*Q. robur*	5	46°20'	16°48'	-
	43	Dreznicko polje	*Q. robur*	5	45°09'	15°05'	-
	44	Dubica	*Q. robur*	5	45°17'	16°44'	-
	45	Djurdjenovac	*Q. robur*	5	45°34'	18°08'	-
	46	Goranska Dobra	*Q. robur*	5	45°26'	14°59'	-
	47		*Q. petraea*	5	45°24'	15°03'	-
	48	Grobnik	*Q. pubescens*	3	45°23'	14°32'	-
	49	Gunja	*Q. robur*	5	44°57'	18°49'	-
	50	Gusevac	*Q. robur*	5	45°13'	18°29'	-
	51	Haljevo	*Q. robur*	5	45°43'	18°37'	-
	52	Imotski	*Q. pubescens*	6	43°27'	17°09'	-
	53	Imotski (Lug)	*Q. frainetto*	5	43°27'	17°09'	-
	54	Jablanac	*Q. pubescens*	3	44°43'	14°54'	-
	55	Karadza	*Q. pubescens*	2	43°34'	16°49'	425
	56	Karin	*Q. pubescens*	3	44°07'	15°35'	230
	57	Kljucevi	*Q. robur*	5	45°11'	17°21'	-
	58	Konavle	*Q. pubescens*	4	42°33'	18°17'	160
	59	Krizancija	*Q. robur*	4	46°15'	16°44'	-
	60	Lanisce	*Q. pubescens*	3	45°25'	14°08'	-
	61	Laudonov gaj	*Q. robur*	5	44°38'	15°39'	635
	62	Lipovljani	*Q. robur*	5	45°26'	16°49'	-
	63	Liznjan	*Q. pubescens*	4	44°49'	13°58'	49
	64	lug Drobna	*Q. robur*	5	45°59'	16°42'	-
	65			5	45°57'	16°35'	-
	66	Medvidja	*Q. pubescens*	3	44°06'	15°46'	503
	67	Motovun forest	*Q. robur*	5	45°20'	13°50'	-
	68	Novska	*Q. robur*	5	45°21'	16°55'	-
	69	Okucani	*Q. robur*	5	45°11'	17°10'	-
	70	Orlovac	*Q. robur*	5	45°33'	15°44'	-
	71	Pakostane	*Q. pubescens*	3	43°54'	15°31'	58
	72	Papuk	*Q. pubescens*	3	45°30'	17°38'	-
	73	Peljesac	*Q. pubescens*	4	42°51'	17°39'	236
	74	Pula	*Q. pubescens*	3	44°55'	13°51'	-
	75	Repaš	*Q. robur*	5	46°09'	17°10'	-
	76	Roski slap	*Q. pubescens*	3	43°52'	15°59'	-
	77	Visovacka brina	*Q. pubescens*	3	45°48'	15°37'	67
	78	Samobor	*Q. robur*	3	45°50'	15°58'	-
	79	Sarkanj	*Q. robur*	5	45°52'	18°48'	-

east Europe. Haplotype 5 is the most common member of lineage A in the study region and the distribution of its three sub-haplotypes is shown in *Figure 2*. The records of haplotype 5 in the populations of Albania and Macedonia represent the most south-easterly locations yet discovered for this haplotype.

Haplotype 31 occurred in Croatia and these populations represent the most southerly locations yet discovered for this haplotype. Prior to this sampling the most southerly location was in Romania (BORDÁCS et al., 2002). Two sub-haplotypes of haplotype 31 are present along the Neretva river valley. Furthermore, the sea level of the Adriatic during the glaciation was much lower than today. All this implies a possible refugium at the ancient mouth of the Neretva river, probably along the stream that existed southeast of the present island of Vis, in the Adriatic, during the glacial period.

The populations containing haplotype 4 in Montenegro are also by far the southernmost populations of this haplotype yet discovered in Europe. Interestingly, it was not found either along the coast or in the *Q. robur* populations in Croatia, but was present throughout central-eastern Bosnia and Herzegovina and Hungary (BORDÁCS et al., 2002) along the edge of the Pannonian plain and further to the north-west and north-east. Its presence in Romania indicates the spread of this haplotype along the eastern edge of Pannonia, but unfortunately there is a lack of data for Serbia.

Haplotype 6 was found in eastern Croatia (Slavonia) and in one Bosnian population, but it always occurred in mixed populations, and north of haplotype 4. Haplotype 6 was previously found in Romania (BORDÁCS et al., 2002), both in pure and mixed populations. All populations found since then seem to be situated north of the

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Table 1. – Continuing.

State	No.	Population	Species	Number of individuals	Coordinates		Elevation (m)
	80	Sibenik	*Q. pubescens*	3	43°49'	15°58'	-
	81	Skakavac	*Q. robur*	5	45°29'	15°42'	-
	82	Slatina	*Q. robur*	5	45°42'	17°48'	-
		Jasenovaca		5	45°40'	17°48'	-
	83	Slivno-Raba	*Q. pubescens*	4	42°58'	17°31'	-
	84	Smrdljivac-Kom	*Q. pubescens*	3	44°12'	16°03'	160
	85	Staza	*Q. pubescens*	4	43°15'	17°06'	420
	86	Strahinscica	*Q. robur* *Q. petraea*	5	46°01'	15°56'	912
	87	Sveti Ilija	*Q. pubescens*	5	43°00'	17°09'	-
	88	Svibovica	*Q. robur*	5	46°02'	17°14'	830
Croatia	89	Trilj	*Q. pubescens*	1	43°37'	16°45'	-
	90	Trnovacke Bare	*Q. robur*	5	44°30'	15°17'	350
	91	Var. Podravske sume	*Q. robur*	5	46°21'	16°19'	-
	92	Velika Gorica	*Q. robur*	5	45°40'	16°10'	-
	93	Vidova Gora	*Q. pubescens*	2	43°19'	16°36'	-
	94	Vrbanja	*Q. robur*	5	45°01'	18°59'	580
	95	Vrlika	*Q. pubescens*	5	43°52'	16°29'	-
	96	Zelendvor	*Q. robur*	5	46°20'	16°11'	460
	97	Zagreb	*Q. robur*	1	45°50'	15°58'	-
	98	Zdenacki gaj	*Q. robur*	5	45°42'	17°48'	-
Serbia	99	Morovic	*Q. robur*	5	45°02'	19°11'	-
	100	Kicevo	*Q. pubescens* *Q. frainetto*	2 1	41°29'	20°54'	690
Macedonia	101	Kratovo	*Q. pubescens*	3	42°09'	22°3,6'	-
	102	Pelagonija	*Q. robur*	1	41°09'	21°22'	595
	103	Resen (Bigla)	*Q. frainetto* *Q. petrea*	3 3	41°4,1'	21°4,3'	627
	104	Gziq	*Q. frainetto*	1	41°50'	19°45'	1007
	105	Katerlis	*Q. frainetto*	2	42°05'	20°05'	450
	106	Liqe I kuq	*Q. pubescens*	3	42°10'	20°15'	650
Albania	107	Kerpice	*Q. pubescens*	3	40°40'	20°10'	350
	108	Bishnice	*Q. pubescens*	2	40°54'	20°25'	600
	109	Faqja prroit Melleze	*Q. petrea* *Q. frainetto*	3	41°05'	19°55'	1050
	110	Shpati kalimash	*Q. frainetto*	1	42°05'	20°17'	800

Table 2. – Pairs of primers used for amplification.

Fragment	Primers	
	Name	Sequence
AS	*psa*A [PSI (P700 apo-protein A1)]	5'-ACTTCTGGTTCCGGCGAACGAA-3'
	*trn*S [tRNA-Ser(GGA)]	5'-AACCACTCGGCCATCTCTCCTA-3'
DT	*trn*D [tRNA-Asp(GUC)]	5'-ACCAATTGAACTACAATCC-3'
	*trn*T [tRNA-Thr(GGU)]	5'-CTACCACTG AGTTAAAAGGG
CD	*trn*C [tRNA-Cys(GCA)]	5'-CCAGGTCAAATCTGGGTGTC-3'
	*trn*D [tRNA-Asp(GUC)]	5'-GGGATTGTAGTTCAATTGGT-3'
TF	*trn*T [tRNA-Thr(UGU)]	5'-CATTACAAATGCGATGCTCT-3'
	*trn*F [tRNA-Phe(GAA)]	5'-ATTGAACTGGTGACACGAG-3'

Table 3. – Duration of amplification and temperature steps for each primer pair.

Fragment	Denaturation		Hybridisation		Elongation	
	Temperature	time	Temperature	time	Temperature	time
AS	93 °C	45 s	57,5 °C	45 s	72 °C	4 min
DT	93 °C	45 s	54,5 °C	45 s	72 °C	2 min
CD	93 °C	45 s	58,0 °C	45 s	72 °C	4 min
TF	93 °C	45 s	57,5 °C	45 s	72 °C	2 min

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Table 4. – Position of bands for each haplotype and sub-haplotype found in the region.

Gel	DT	CD	AS	TF	haplotype
Bands positions	91211	19221	142923	2020211	2
	11111	11231	162223	2020212	4a
	11211	11231	162223	2020212	5a
	11211	11231	162223	2030213	5b
	11211	11231	162223	2010211	5c
	21211	11131	162223	2020212	6
	11291	11231	162224	2020212	7
	11311	11231	142223	2020212	17a
	21211	11231	162223	2020212	31

refugial area (with the exception of one population in southern Italy which is of dubious origin) (PETIT et al., 2002 b). The refugium of origin for these haplotypes is supposed to be located in the Carpathian basin (BORDÁCS et al., 2002).

Haplotypes 4, 5, 6 and 31, are genetically closely related and may originate from the same refugial area in the southern Balkans, even though FINESCHI et al. (2002) and PETIT et al. (2002 b) reported that haplotypes 5 and 6 originated from Italy and south Balkan. Our evidence suggests that these haplotypes may have probably survived in refugia in the Balkans.

Haplotype 7, within the study area, is only found in the Northwest of Croatia with the exception of one mixed population in central Bosnia (Gornji Vakuf) and two in Croatia (Sarkanj and Gusevac). At least one of these mixed populations (Sarkanj) is known to have been artificially established through plantation. In previous studies (PETIT et al., 2002 b), it was found in the Pyrenees and through the Alps to north-east Europe. This haplotype is phylogenetically distant from other haplotypes of lineage A found in this study (PETIT et al., 2002 a). Its geographical distribution clearly indicates that it does not originate from the Balkan refugia, but most probably from the south-east Pyrenees, with probable secondary refugia south of the Alps, as reported by PETIT et al. (2002 b).

Haplotypes 2 and 17 belong to different lineages (C and E, PETIT et al., 2002 a), and both are thought to be of Apennine origin (PETIT et al., 2002 b). Haplotype 2 colonised south-west and central Croatia across the Northern Adriatic, which was a mainland during the last glacial period. The fact that haplotype 2 in our samples does not occur further south-east than the southern Croatia supports this theory. Haplotype 17 tends to have a more eastern distribution and its populations are dispersed through most of the studied area. Haplotypes 2 and 17, therefore follow the colonisation routes across Italy and Central Balkans to the north, north-east and east (*Figure 3*).

There is a clear geographical structure in the distribution of haplotypes. The individual distribution of each haplotype in Europe is shown in *Figure 3* with additional data from PETIT et al. (2002 a, b).

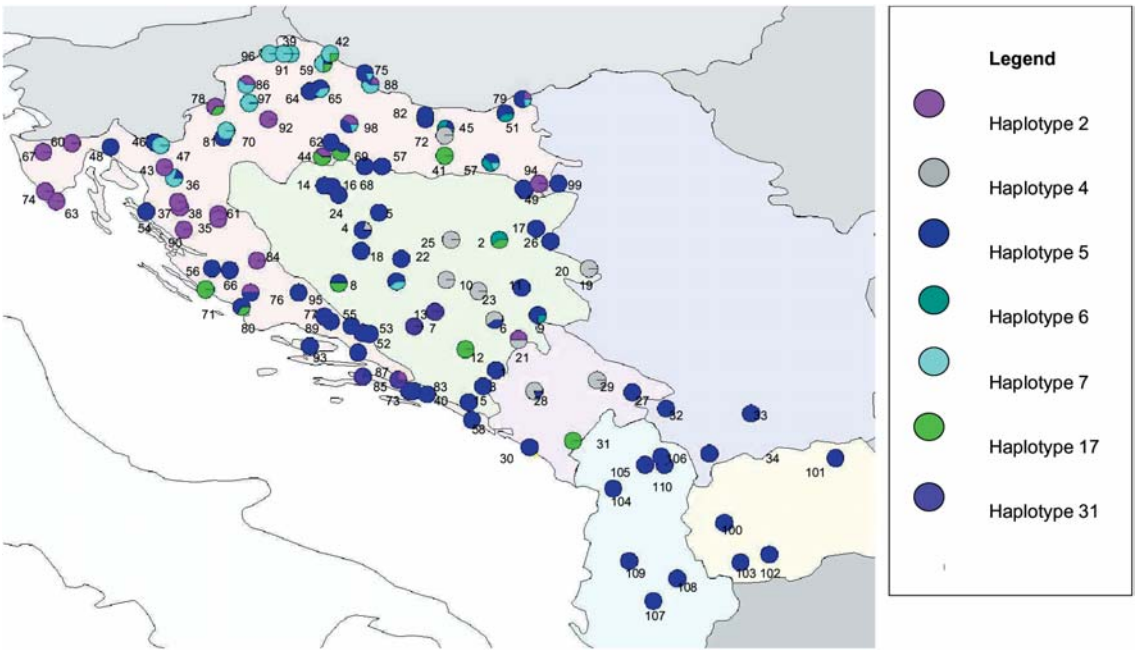

Figure 1. – Distribution of haplotypes of oak within Croatia, Bosnia and Herzegovina, Montenegro, Kosovo, Serbia, Macedonia and Albania, based on the data contained in *Table 1* (1–110 populations).

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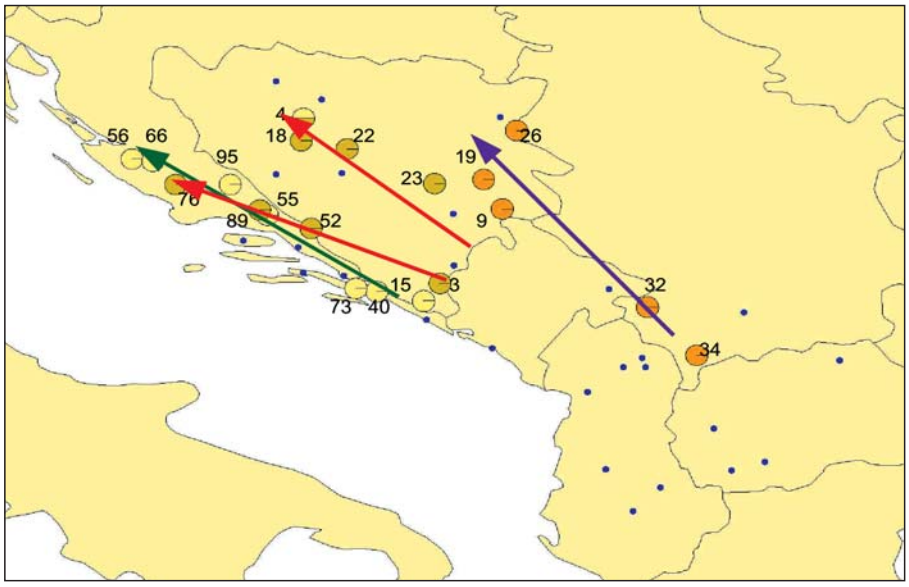

Figure 2. – Distribution of sub-haplotypes of haplotype 5. Populations for which sub-haplotype was not detected are marked with blue dots. Arrows show most probable colonisation routes for each sub-haplotype.

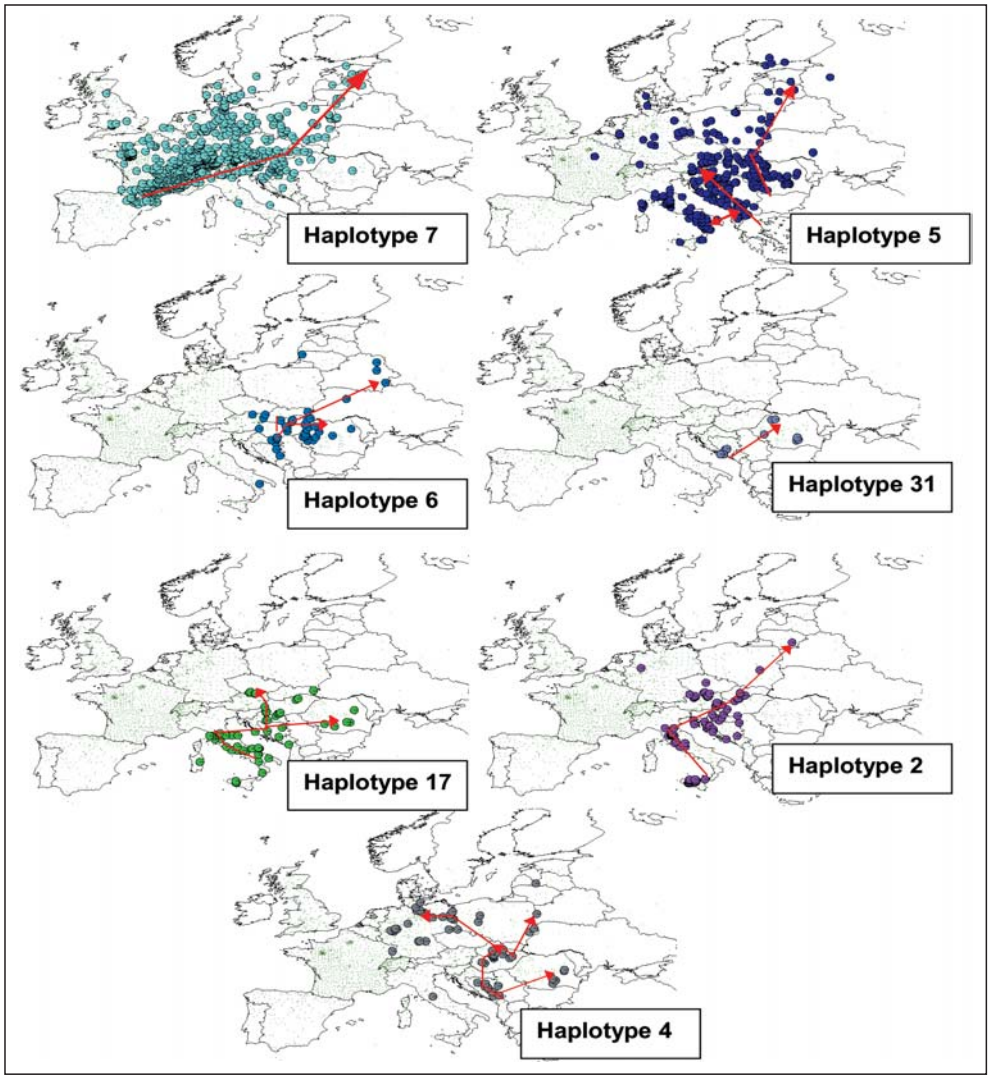

Figure 3. – Distribution of haplotypes of oaks found in the central Balkans and in the rest of Europe with additions from PETIT et al., 2002 (a, b). Arrows show the most probable colonisation routes.

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Table 5. – Levels of diversity and differentiation by species.

Species	No. of populations (≥3 individuals)	Harmonic mean of individuals per population	Number of haplotypes	h_s (standard error)	h_t (standard error)	G_{st} (standard error)
Q. robur	41	4.50	6	0.200 (0.0462)	0.707 (0.0362)	0.717 (0.0628)
Q. petraea	16	4.20	7	0.198 (0.0683)	0.718 (0.0868)	0.724 (0.0943)
Q. pubescens	37	3.42	6	0.103 (0.0364)	0.602 (0.0755)	0.830 (0.0578)
All populations	98	4.01	8	0.160 (0.0272)	0.700 (0.0370)	0.772 (0.0380)

– haplotype 31 is present to the south of haplotype 4, while closely related haplotype 6 is present only to the north, with a probable parallel colonisation route with haplotype 4 to eastern Hungary (*Figures 1* and *3*).

– haplotype 17 is not present in southwest Croatia, while haplotype 2 is quite rare in northeast (*Figures 1* and *3*).

– sub-haplotypes of haplotype 5 are also geographically structured. Sub-haplotype 5c was found only to the north of haplotype 4, sub-haplotype 5b is common in central Bosnia, and haplotype 5a in south-west Croatia (Dalmatia), between the areas colonised by haplotypes 31 to the west, and 4 to the northeast. Sub-haplotypes 5a and 5b are equally present in the rest of Dalmatia (*Figures 1*, *2* and *3*).

The estimates of genetic diversity and differentiation are given in *Table 5* for the whole data set as well as separately for each species. The overall mean coefficient of differentiation G_{st} for all species combined is 0.772, for populations which had three or more individuals, which means that the majority of cpDNA diversity (77.2%) is distributed among rather than within populations. Similar results were obtained for the four species when studied separately. This value of G_{st} is a lower value than has previously been reported for other regions of Europe (PETIT et al., 2002a). Diversity within stands (h_s) is lowest for *Q. pubescens* and highest for *Q. robur*, while genetic differentiation (G_{st}) is highest for *Q. pubescens* populations and lowest for *Q. robur*. Such ranking of species is a general trend which has also been found elsewhere in Europe (DUMOLIN-LAPÈGUE et al., 1999, BORDÁCS et al., 2002, PETIT et al., 2002 a, b). These results can be explained by the fact that *Q. robur* is under the strongest anthropogenic influence in the central Balkans as well as elsewhere in Europe. Its acorns were often transferred between stands. Its seedlings were intensively cultivated in nurseries and distributed to different areas. Thus, as a consequence of this artificial gene flow, often between very distant populations the level of intra-population diversity has increased while the G_{st} decreased. This anthropogenic influence is less pronounced for *Q. petraea* populations, while completely absent in *Q. pubescens*, because this species is economically much less valuable.

In this study, 22.8% of the studied populations were mixed haplotypes. This variability is less than in other parts of Europe (PETIT et al., 2002 a). In addition to mixing haplotypes within populations, it can be a consequence of a large number of populations with haplotype 5, which decreases diversity between populations.

For a precise location of the refugia it is necessary to collect more fossil pollen data for the oak species, covering the period between 15.000 to 10.000 B.P., especially in the Neretva river valley, the Skadar Lake and in south Bosnia.

Conclusion

The seven haplotypes found across the broad region covering Croatia, Bosnia and Herzegovina, Montenegro, Kosovo, Serbia, Macedonia and Albania consisted of haplotypes 2, 4, 5, 6, 7, 17, 31, as well as the subtypes of haplotypes 4 (a), 5 (a, b, c) and 17 (a).

The highest haplotype variability was found in the Neretva river valley and in the south of Bosnia. This could be a consequence of localization of the glacial refugia in the mouth of the Neretva river (haplotype 31) and probably along the banks of the Skadar Lake (haplotype 4).

Haplotype 5 was the most abundant, with three subhaplotypes. The haplotype 2 is present in the west, but haplotype 17 is found in Montenegro, Bosnia and Herzegovina and Croatia. The haplotype 4 has an interesting distribution from Zeta and Komarnica river to the valley of the Bosna river. Haplotype 7 was found in just one population (Gornji Vakuf). Haplotype 6 is distributed just to the north of haplotype 4.

Of the four haplotypes originating from the Balkans refugia, one is present in the entire study area, while the distribution of the others is more geographically structured.

Out of the seven observed haplotypes, four belong to the Balkan refugium (haplotypes 4, 5, 6 and 31), two are of Apennine origin (haplotypes 2 and 17), taking into consideration the possibility that haplotype 5 can be either of Balkan or Apennine origin, and one is clearly not from either of these two refugial areas (haplotype 7). It most probably belongs to different glacial refugia, most likely the Pyrenees or the Southern Alps.

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