

## Morphological and genetic variation within and among four *Quercus petraea* and *Q. robur* natural populations

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**Abstract:** Numeric-taxonomical and molecular techniques [nuclear simple sequence repeats (nSSRs), expressed sequence tag (EST) SSRs, and chloroplast SSRs] were used to estimate morphological and genetic differentiation within and among 4 *Quercus petraea* and *Q. robur* natural populations in Turkey. Canonical discriminant analysis (CDA) and principal component analysis (PCA) showed that 3 out of 15 studied leaf morphological characters (clustered hairs, number of intercalary veins, petiole length) mainly accounted for the discrimination between species. In accordance with earlier observations, the mean genetic differentiation between *Q. petraea* and *Q. robur* at 8 microsatellite loci ( $F_{ST} = 0.030$ ) and at chloroplast microsatellites ( $G_{ST} = 0.22$ ) was low, potentially as the result of interspecific gene flow or shared ancestral polymorphisms. Chloroplast microsatellite analyses showed unique chloroplast haplotypes in Turkey, suggesting a glacial refuge in this region. Nuclear microsatellite *ssrQrZAG96*, described in other studies as being potentially under directional selection (outlier locus) due to high interspecific differentiation, showed considerably lower differentiation between species in the present study ( $F_{ST} = 0.028$ ). These different patterns of genetic variation suggest different local adaptations of the species in this putative glacial refugial area in Turkey. It may also be that this marker is not directly associated with morphological or adaptive species differences. Interestingly, the analysed *Q. petraea* and *Q. robur* populations appear to have ecological requirements with regard to water availability that are different than those described for European populations. The analysis of additional populations within the species distribution range in Turkey is necessary to confirm this pattern.

**Key words:** *Quercus*, expressed sequence tag, simple sequence repeats, genetic differentiation, leaf morphology

### 1. Introduction

*Quercus* L. is one of the most important woody genera due to its ecological and economic value and contains about 400 species in several sections distributed across 5 continents (Aldrich & Cavender-Bares, 2011). For example, oaks are important for wood and paper production and are associated with a large diversity of insect and fungal communities (McShea et al., 2007; Oran & Öztürk, 2012). The presence of natural hybrids and introgressive forms among oak taxa (Muir & Schlötterer, 2005) has made the clear definition of species boundaries difficult, challenging the biological species concept (van Valen, 1976). Thus, while most individuals can be assigned to distinct species based on morphological characters, individuals with intermediate morphologies occur, especially in the contact zones between 2 or more species (Curtu et al., 2007a).

In the northern hemisphere, oaks are conspicuous members of the temperate deciduous forests (Borazan & Babaç, 2003). Turkey, with 18 species of oak, is one of

the regions of high diversity for *Quercus* species (Yaltırık, 1984), which have a natural distribution of about 6.4 million ha (OGM, 2006) including many subspecies, varieties, and natural hybrids.

Pedunculate (*Quercus robur* L.) and sessile [*Quercus petraea* (Matt.) Liebl.] oaks are 2 sympatric, widespread species with high economic and ecological importance that cover most of Europe from Spain to Russia and Scotland to Turkey (Bodenes et al., 1997). Both species are supposed to exhibit different ecological requirements; *Q. petraea* is the more drought tolerant, generally occurring on better drained soils, and *Q. robur* is usually found on nutrient rich soils prone to temporary water-logging (Aas, 1998; Breda et al., 1993; Zanetto et al., 1996).

In Turkey, both *Q. robur* and *Q. petraea* occur in similar semihumid to arid climates (Uğurlu et al., 2012), suggesting different local adaptations of both species in Turkey when compared to the European species distribution range. Based on the precipitation effectiveness

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index (Erinç, 1965), in the present study *Q. petraea* populations were located in semihumid and *Q. robur* populations in semiarid to arid climate types. Boundaries between these 2 species are not clearly defined due to the absence of species diagnostic characters, and their taxonomic classification is still debated among botanists (Bacilieri et al., 1995). No single morphological character can unambiguously distinguish *Q. robur* from *Q. petraea*; however, by using multivariate statistics based on leaf morphological characters species assignment of most samples was possible (Kremer et al., 2002). Similar to morphological characters, genetic differentiation between species at nuclear genetic markers such as isozymes (Müller-Starck et al., 1996; Curtu et al., 2007b), nuclear microsatellites (nSSRs; Mariette et al., 2002), AFLPs (Coart et al., 2002; Mariette et al. 2002), candidate gene markers (Gailing et al., 2009; Vornam et al., 2011, Sullivan et al., 2013), and maternally inherited chloroplast DNA markers (Petit et al., 2003) is generally low. Rare outlier loci with higher interspecific genetic differentiation than expected under selective neutrality were detected for example among AFLPs (Coart et al., 2002; Mariette et al., 2002), isozymes (Gömöry, 2000; Finkeldey, 2001), and nuclear simple sequence repeats (nSSRs) (Scotti-Saintagne et al., 2004; Muir & Schlötterer, 2005). The generally low genetic differentiation between species was explained by their propensity to hybridise in regions where both species co-occur (Scotti-Saintagne et al., 2004, Lexer et al., 2006) and by the shared ancestral variation among recently separated species (Muir & Schlötterer, 2005).

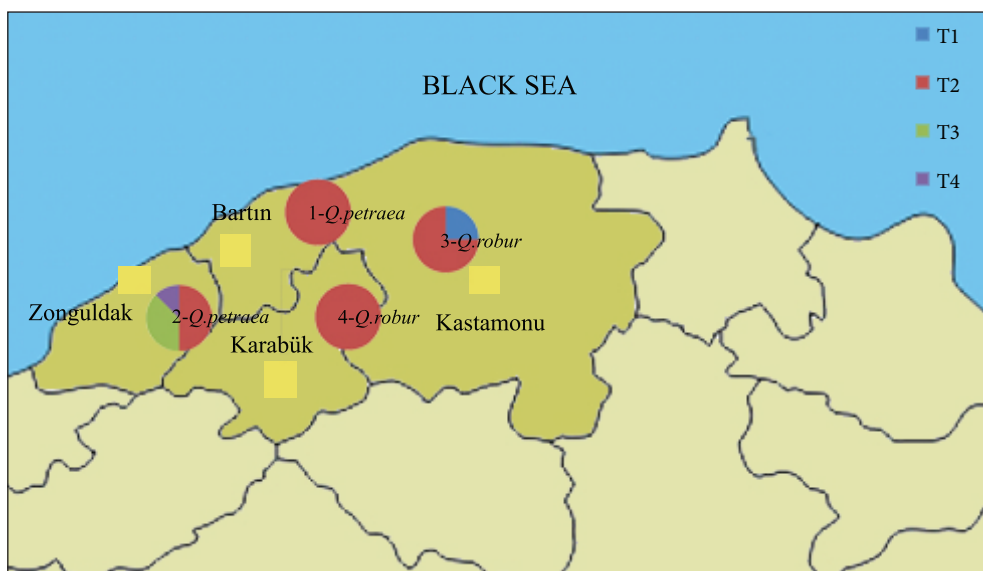
The aims of the present study were to (i) analyse leaf morphological variation within and between 2 *Q. robur* and *Q. petraea* populations from north-western Turkey

that were identified in the field using the taxonomic key by Aas (1993) and to (ii) assess patterns of genetic variation in these populations at 4 nSSRs (Steinkellner et al., 1997; Kampfer et al., 1998), 4 newly developed and annotated expressed sequence tag (EST)-SSR markers with known positions on *Q. robur* genetic linkage maps (Durand et al., 2010), and 2 informative chloroplast SSRs (Deguilloux et al. 2003). In earlier studies, one of these markers, nSSR *ssrQrZAG96*, was identified as an outlier locus since it showed genetic differentiation between species approximately 10-fold higher than other nSSRs due to a high frequency of short repeat alleles and low genetic variation in *Q. robur*, potentially as the result of a selective sweep (Muir & Schlötterer, 2005). Allele frequency differences at outlier loci have most likely evolved in small populations as a result of genetic drift and selection. While outlier loci between *Q. robur* and *Q. petraea* related to different local adaptations of both species have been hypothesised as the result of positive selection in the core distribution range of the species in Europe and the southern Balkan region (Scotti-Saintagne et al., 2004; Neophytou et al., 2010), these genetic analyses are lacking in other potential glacial refugial regions such as northern Turkey (Petit et al., 2002).

## 2. Materials and methods

### 2.1. Plant material

From their natural distribution in Turkey 4 populations separated by a distance of about 50 km were sampled (Figure 1; Table 1). Species were identified in the field using the taxonomic key by Aas (1993). Each population was represented by 50 individuals. A minimum distance of 50 m was kept between trees in order to avoid the



**Figure 1.** Haplotype frequencies (pie charts) in the studied *Quercus petraea* and *Q. robur* populations.

**Table 1.** Sample locations for *Quercus petraea* and *Q. robur* populations.

Population no.	Species	Forest district location	Altitude (m)	Exposure	Latitude (°)	Longitude (°)	Soil	Associated species
1	<i>Q. petraea</i>	Bartın-Abdipaşa	173	NE	41°31'04"	32°32'42"	Loam clay	<i>Cornus mas</i> L., <i>Alnus</i> sp., <i>Carpinus betulus</i> L.
2	<i>Q. petraea</i>	Zonguldak-Devrek	364	NW	41°07'23"	31°59'58"	Clay loam	<i>Arbutus unedo</i> L., <i>Crataegus monogyna</i> Jacq.
3	<i>Q. robur</i>	Kastamonu-Daday	915	SW	41°27'52"	33°20'48"	Sandy loam	<i>Q. cerris</i> L., <i>Q. pubescens</i> Willd., <i>Berberis vulgaris</i> L.
4	<i>Q. robur</i>	Karabük-Eflani	936	SE	41°28'41"	32°51'54"	Sandy loam	<i>Q. cerris</i> L., <i>Q. pubescens</i> Willd., <i>Arbutus unedo</i> L.

sampling of related trees. Adult trees were about 30–70 years old, 15–30 cm in diameter at breast height (DBH), and originated from natural regeneration. Five fresh and fully expanded leaves were collected in the mid-to-upper crown of the trees and from the first flush of the year. Leaf samples were dried with silica gel in zip-lock plastic bags for 2 weeks and stored in paper bags.

Precipitation effectiveness indexes were 30.7 for Bartın, 37.3 for Zonguldak, 16.2 for Kastamonu, and 13.8 for Karabük (Erinç, 1965). According to these precipitation effectiveness indices, *Q. petraea* populations in Bartın and Zonguldak are located in semihumid and *Q. robur* populations in Kastamonu and Karabük in semiarid and arid climates. To determine these indices, 41 annual (1970–2011) climate records were used.

## 2.2. Assessment of leaf morphological characters

Five leaves from each of the 200 trees were measured for biometrical verification in every population at 15 leaf characters. The variables comprised 5 directly measured, 5 observed and counted, and 5 synthetic characters according to the methods used previously in Kremer et al. (2002) and Aas (1993). The following characters were assessed: 1) lamina length (LL), 2) petiole length (PL), 3) lobe width at the tip of the widest lobe (LW), 4) sinus width (SW), 5) length of lamina at largest width (WP), 6) number of lobes (NL), 7) number of intercalary veins (NV), 8) basal shape of the lamina (BS), 9) clustered hairs (CH), 10) lobe tip shape (LT), 11) lamina shape or obversity (OB), 12) petiole ratio (PR), 13) lobe depth ratio (LDR), 14) percentage venation (PV), and 15) lobe width ratio (LWR). The character, clustered hairs, was assessed with a stereomicroscope ( $\times 30$ ) on a scale from 0 (no hairs) to 2 (densely clustered hairs), counting both stellate and simple hairs. The character lobe tip shape was scored from 0 (not pointed), 1 (slightly pointed), or 2 (pointed). All leaves were measured in the same orientation, and all measurements were taken from the lower right sides of each leaf.

Principal component analysis (PCA) was used as an ordination technique to classify population samples into

distinguishable groups and to reduce the total number of morphological characters. The proportion of variation accounted for by each principal component was expressed as the eigenvalue divided by the sum of eigenvalues.

Canonical discriminant analysis (CDA), a dimension-reduction technique, was instrumental for identifying the morphological characters with high discriminatory power (Teshome et al., 1997). Stepwise discriminant analysis using the principal component clusters was carried out to determine the most discriminatory variables to enter into the discriminant functions. The discriminant functions were then used to estimate the percentage of specimen classifications into selected groups using a probabilistic approach to classify individuals in handling cases known to be intermediate between groups. Individual samples were classified into either species when both methods (PCA, CDA) yielded corresponding results or were considered unclassified in the case of contradictory results.

In addition, means, standard deviations, and ranges of characters were calculated separately for populations. To quantify the amount of variation distributed among populations and species for leaf morphological characters one-way ANOVAs were performed. In order to assess the effect of leaf size variation on leaf characters, correlations between LL and the other leaf characters were evaluated using Pearson's correlation coefficients (Kremer et al., 2002). SPSS version 20 (SPSS Inc., 2002) was used for all analyses of leaf morphological characters.

## 2.3. Genetic marker analyses

Total genomic DNA was extracted from dried leaves using the DNeasy96 Plant Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA content was checked on 0.8% agarose gels after staining with ethidium bromide. DNA concentration was adjusted for further applications.

Four nSSR markers: *ssrQpZAG1/5*, *ssrQpZAG36*, and *ssrQpZAG104* developed for *Q. petraea* (Steinkellner et al., 1997) and *ssrQrZAG96* developed for *Q. robur* (Kampfer et al., 1998) and 4 genic (EST)-SSR markers: *FIR004*, *FIR053*, *GOT004*, and *POR016* developed for *Q. petraea* and *Q.*

*robur* (Durand et al., 2010) were selected for genotyping the 200 individual trees. The 10- $\mu$ L PCR reactions were composed of 2  $\mu$ L of 5  $\times$  HOT FIREPol Blended Master Mix with 10 mM of MgCl<sub>2</sub> (0.6 U of Taq polymerase, 2 mM of dNTPs) (Solis BioDyne, Estonia), 1.3  $\mu$ L of forward (synthesised with fluorescent dye by Applied Biosystems, Foster City, CA, USA) (Table 2) and reverse (synthesised by Sigma Aldrich, St Louis, MO, USA) primers (5  $\mu$ M of each), and 1.5  $\mu$ L of DNA template (~2 ng). The samples were amplified in a Peltier thermal cycler (GeneAmp<sup>®</sup> PCR system 2700, Applied Biosystems, Foster City, CA, USA) with the following program: (1) initial denaturation at 95 °C for 15 min; (2) 35 cycles at 94 °C for 45 s, annealing at 55 °C (57 °C for loci FIR004, FIR053, and GOT004) for 45 s, elongation at 72 °C for 45 s; and (3) a final extension at 72 °C for 20 min. In addition, informative chloroplast microsatellites specifically developed for oaks (Deguilloux et al., 2003; Table 2), *ucd4* and *udt4*, were analysed in 8 samples per population, in addition to a reference sample with a known haplotype, following the protocol described in Gailing et al. (2007). The PCR products were separated on an ABI Prism<sup>®</sup> Genetic Analyzer 3730 and scored using GeneMapper<sup>®</sup> v.4.0 (Applied Biosystems, Foster City, CA, USA).

For each locus and population, observed number of alleles (A), expected heterozygosity  $H_e$  (gene diversity; Nei, 1987), and observed heterozygosity ( $H_o$ ) were calculated using the GENALEX 6.41 computer program (Peakall & Smouse, 2006). The inbreeding coefficient was calculated as  $F_{IS} = (H_e - H_o)/H_e$ . Possible divergence from the Hardy–Weinberg equilibrium (HWE) was also determined by the exact test method based on Markov chain analysis according to Guo and Thompson (1992) in GENEPOP version 3.4 (Raymond & Rousset, 1995). Pairwise differentiation between populations was calculated as  $F_{ST}$  and tested for significance using a 2-sided test (10,000 permutations) implemented in GENEPOP. Genetic differentiation among populations based on chloroplast haplotype frequencies ( $G_{ST}$ ) was calculated with the RAREFAC program (Petit et al., 1998).

### 3. Results

#### 3.1. Leaf morphological characters

Most characters showed significant positive and negative correlations with lamina length (Table 3). The mean of leaf characters was mostly higher in *Q. petraea* than in *Q. robur* populations (Table 4). Significant differences

**Table 2.** Characteristics of primers for *Quercus petraea* and *Q. robur*.

Locus	Primer sequences (5'–3')	Repeat motif	Size (bp)	Lg	T <sub>a</sub> (°C)	Citation	Putative function
ssrQpZAG1/5	PET- GCTTGAGAGTTGAGATTTGT GCAACACCCCTTAACTACCA	(GT) <sub>5</sub> (GA) <sub>9</sub>	159–183	7	55	Steinkellner et al., 1997	N.A.
ssrQpZAG36	VIC- ATCAAAATTTGGAAATTTAGAGAG ACTGTGGTGGTGGAGTCTAACATGTAG	(AG) <sub>19</sub>	201–229	2	55	Steinkellner et al., 1997	N.A.
ssrQrZAG96	VIC- CCCAGTCACATCCACTACTGTCC GGTTGGGAAAAGGAGATCAGA	(TC) <sub>20</sub>	139–178	10	55	Kampfer et al., 1998	N.A.
ssrQpZAG104	FAM- CACCACTACCGCTGTGAGATAG GATGGTACAGTAGCAACATTC	(AG) <sub>16</sub> AT(GA) <sub>3</sub>	183–241	2	55	Steinkellner et al., 1997	N.A.
FIR004	FAM- TCTCTCTCAGGGCAGCTTCT AACCAAACCTCAGATCCAGATTCA	(CT) <sub>18</sub>	123–179	3	57	Durand et al., 2010	Unknown
FIR053	PET- AGTTTCCCCACATTTGTTGC TACCATGCACCAAGCAATTC	(GTG) <sub>7</sub>	133–176	5	57	Durand et al., 2010	Glutaredoxin
GOT004	FAM- GGGCATATTGATCGCTTAGG TGAGCATTACATATCCATGAT	(TG) <sub>12</sub>	273–315	7	57	Durand et al., 2010	Aquaporins, MIP family, TIP family (drought stress) Alexandersson et al., 2010
POR016	NED- GCAACAGCAGAGCCAAAAT CAGCGGCTTTGAGGTAATTC	(GGT) <sub>6</sub>	120–149	6	55	Durand et al., 2010	Heat shock protein 70 (stress) Rizhsky et al., 2004
<i>ucd4</i> *	FAM- TTATTTGTTTTTGGTTTCACC TTTCCCATAGAGAGTCTGTAT	(T) <sub>12</sub>	93–96	-	Touch down	Deguilloux et al., 2003	
<i>udt4</i> *	FAM- GATAATATAAAGAGTCAAAT CCGAAAGGTCCTATACCTCG	(A) <sub>9</sub>	144–145	-	Touch down	Deguilloux et al., 2003	

\*: chloroplast microsatellites, Lg: linkage group.

**Table 3.** Correlation between leaf variables and lamina length (LL).

Variable	Coefficient of correlation	P values
PL	0.33	0.000
LW	0.76	0.000
SW	0.40	0.000
WP	0.54	0.000
NL	0.56	0.000
NV	0.35	0.000
BS	0.03	0.337
LT	-0.29	0.000
CH	-0.63	0.000
OB	-0.02	0.558
PR	-0.07	0.038
LDR	0.11	0.000
LWR	-0.27	0.000
PV	-0.38	0.000

among populations and between species were observed for the majority of the characters at 0.001 and 0.01 levels. Differentiation between species based on morphological characters was relatively high ( $\sigma_s^2 = 24\%$ ), and CH contributed a significant portion to this differentiation ( $F_{CH} = 2743$ ;  $\sigma_s^2, CH = 83\%$ ;  $P < 0.001$ ).

PCA and CDA revealed a bimodal distribution of synthetic variables with partial overlap. For most samples both methods yielded similar results, and leaf samples were classified as either *Q. robur* or *Q. petraea*. When PCA and CDA resulted in a different morphological assignment, leaves were considered unclassified (2.9% of the samples; Figure 2). The numbers of unclassified leaves (5 leaves per tree, 250 leaves per population) in the Abdipasa, Devrek, Daday, and Eflani populations were 10, 3, 13, and 3, respectively. The numbers of trees with variable leaf

morphology (not all 5 leaves were unambiguously assigned to the same species) were 5 for Abdipasa, 2 for Devrek, 5 for Daday, and 3 for Eflani. Exclusion of these trees had only very minor effects on genetic variation within and among populations (data not shown).

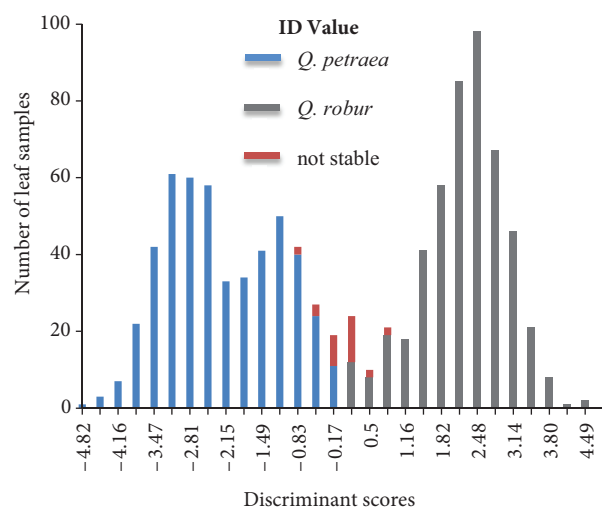
According to CDA, 97.4% of the trees from *Q. petraea* populations and 96.8% of the trees from *Q. robur* populations were classified correctly by the discriminant function. The first discriminant function, which differentiated species, was determined mostly by CH and the number of NV. PCA showed that there were 7 components to be extracted for these leaf characters, which explained 86.9% of the total variance. The first synthetic function explained 26.3% of the variance, and 6 dominant characters (LL, NL, CH, LW, WP, and PV) showed a high loading on the first component. The other studied characters, apart from LT, showed high loading on the different components separating the populations. Some leaf characters (CH, SW, BS, and PV) showed high negative loadings on different components (1, 2, and 7).

### 3.2. Nuclear genetic variation within populations

Within populations, levels of genetic variation differed markedly among loci (Table 5). The number of alleles per population varied from 6 (POR016) to 22 (FIR004), with an average of 14.1 alleles across all populations and loci. Between 18 and 22 alleles per population were detected at FIR004, the most polymorphic locus in the present study. Overall, similar levels of genetic variation were observed at nSSR and EST-SSR markers. The observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) within populations ranged from 0.262 for POR016 to 0.959 for *ssrQpZAG1/5* and from 0.546 for POR016 to 0.931 for FIR004, respectively (Table 5). Mean  $H_o$  and  $H_e$  for *Q. robur* (0.799, 0.863) were slightly higher than for *Q. petraea* (0.727, 0.810). Levels of genetic variation ( $H_o$ ,  $H_e$ )

**Table 4.** Mean values, standard deviations, and phenotypic ranges for metric (in millimetres), counted, and observed leaf characters in *Quercus* populations.

Characters	1 - <i>Q. petraea</i>		2 - <i>Q. petraea</i>		3 - <i>Q. robur</i>		4 - <i>Q. robur</i>	
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
LL	107.6 $\pm$ 1.6	78–168	106.5 $\pm$ 1.4	64–152	84.9 $\pm$ 1.0	65–117	78.9 $\pm$ 1.1	57–117
PL	13.4 $\pm$ 0.3	17–25	14.5 $\pm$ 1.1	13–20	10.6 $\pm$ 0.3	5–13	9.4 $\pm$ 0.3	4–11
LW	32.9 $\pm$ 0.7	20–61	32.3 $\pm$ 0.5	17–49	26.9 $\pm$ 0.4	18–46	25.7 $\pm$ 0.4	15–42
SW	13.6 $\pm$ 0.4	4–33	11.3 $\pm$ 0.3	5–21	10.9 $\pm$ 0.3	5–20	9.9 $\pm$ 0.3	1–19
WP	56.1 $\pm$ 1.5	26–95	59.5 $\pm$ 2.6	20–102	45.1 $\pm$ 1.0	18–71	43.9 $\pm$ 1.0	19–80
NL	12.3 $\pm$ 1.8	7–17	14.1 $\pm$ 2.0	9–20	10.7 $\pm$ 1.7	6–15	10.3 $\pm$ 1.8	4–15
NV	1.5 $\pm$ 1.4	0–6	0.8 $\pm$ 1.1	0–5	3.1 $\pm$ 1.6	0–9	3.5 $\pm$ 1.7	0–9
BS	4.4 $\pm$ 2.6	1–8	4.1 $\pm$ 2.3	1–8	4.3 $\pm$ 1.8	1–9	3.9 $\pm$ 1.7	1–9
CH	1.8 $\pm$ 0.4	1–2	1.9 $\pm$ 0.3	1–2	0.7 $\pm$ 0.5	0–1	0.2 $\pm$ 0.4	0–1
LT	0.3 $\pm$ 0.5	0–1	0.3 $\pm$ 0.5	0–1	0.9 $\pm$ 0.7	0–2	0.8 $\pm$ 0.9	0–2



**Figure 2.** Distribution of discriminant function values according to *Quercus petraea* and *Q. robur*.

were similar for *Q. robur* and *Q. petraea* populations at *ssrQrZAG96*, which showed reduced genetic variation in *Q. robur* in earlier studies (see discussion below).

The number of markers showing significant heterozygote deficits varied from 2 (*Q. robur*-3) to 5 (*Q. robur*-4) per population (Table 5). Highly significant and positive  $F_{IS}$  values across most populations, as an indicator for null alleles, were observed for FIR053 and POR016. *Q. robur* populations revealed significant and positive  $F_{IS}$  values at *ssrQrZAG96*, but no significant departure from HWE was observed for *Q. petraea* populations.

### 3.3. Nuclear genetic differentiation between species

The majority of analysed microsatellite loci showed a significant ( $P < 0.05$ ) differentiation among species pairs (Table 6). Only EST-SSRs FIR053, GOT004, and POR016 showed low and nonsignificant interspecific differentiation. UPGMA using the 8 microsatellite loci grouped populations according to species with 100% bootstrap support. According to  $F_{ST}$ , the highest differentiation between species was observed at loci *ssrQpZAG104* and *ssrQpZAG36* (Table 6). In particular, the frequencies of short repeat alleles differed between species. Thus, allele 183 bp at *ssrQpZAG104* showed higher frequencies in *Q. robur* populations, and allele 201 bp at *ssrQpZAG36* was more frequent in *Q. petraea* populations (data not shown). The genetic differentiation between species at *ssrQrZAG96* was relatively low ( $F_{ST} = 0.028$ , Table 6). The short repeat allele 139 bp showed the most pronounced allele frequency differences between species and was absent in *Q. petraea* (data not shown).

### 3.4. Chloroplast haplotypes

Chloroplast haplotype T2 was predominant in all populations. The other haplotypes were present only in

the 2 - *Q. petraea* and 3 - *Q. robur* populations (Figure 1). Haplotypes were shared among species ( $G_{ST} = 0.22$ ). Haplotypes T1, T2, and T3 were different from haplotypes characteristic for refugial regions in the Balkan (2, 4, 5, 7–26, 17) and Iberian peninsulas (10, 11, 12) (Table 7). Only haplotype T4 in the easternmost population showed the same fragment sizes as haplotype 1 from the refugial area in southern Italy (Table 7).

## 4. Discussion

### 4.1. Leaf morphological characters

In the present study, leaf morphological analyses using 2 multivariate statistical techniques clearly demonstrated that the 4 oak populations consisted of 2 species, *Q. robur* and *Q. petraea*, as revealed by the bimodal distribution of leaf synthetic variables. Other studies using multivariate analyses to distinguish between *Q. petraea* and *Q. robur* in different parts of Europe (Iestwaart & Feij, 1989; Aas, 1993; Dupouey & Badeau, 1993; Borovics, 1999; Bakker et al., 2001; Kremer et al., 2002; Ponton et al., 2004) also showed a bimodal distribution of synthetic leaf variables, although various sampling methods were used. In accordance with these earlier studies, in the present study both species showed wide phenotypic variation with respect to leaf morphological characters and an absence of single morphological characters that clearly differentiate between species (Aas, 1993; Dupouey & Badeau, 1993; Kremer et al., 2002; Curtu et al., 2007b).

In accordance with Aas (1993), the present study showed that CH was highly discriminative between the species, producing both the highest contribution in the canonical discriminant function (0.76) and contributing a significant portion to this differentiation ( $F_{CH} = 2743$ ;  $\sigma_s^2$ , CH = 83%;  $P < 0.001$ ). In addition, NV and petiole length discriminated between both species ( $P < 0.001$ ), as shown in earlier studies (Saintagne et al., 2004).

Some leaf samples (2.9%) could not be classified unequivocally (i.e. by PCA or CDA) into one species or another due to their intermediate characteristics. This percentage was slightly inferior to the percentages observed by other authors (Aas, 1993; Bacilieri et al., 1995; Kremer et al., 2002). Interestingly, a larger proportion of unclassified leaves were found in populations 1 - *Q. petraea* and 3 - *Q. robur* than in the other populations. On the other hand, Aas (1993) reported that a larger proportion of intermediate oaks were present within populations of *Q. petraea*.

Large leaf morphological variation and the absence of species diagnostic leaf characters as observed in the present and earlier studies is likely caused by nongenetic variation (phenotypic plasticity), within species genetic variation for leaf characters, and hybridisation between species resulting in new gene combinations. The analyses of more

**Table 5.** Summary of genetic variation within species at 8 microsatellite loci.

Population	Species	Locus	A	H <sub>o</sub>	H <sub>e</sub>	F <sub>IS</sub>
1	<i>Quercus petraea</i>	ssrQpZAG1/5	11	0.771	0.860	0.103
		ssrQpZAG36	10	0.659	0.572	-0.153
		ssrQrZAG96	13	0.841	0.885	0.050
		ssrQpZAG104	16	0.750	0.840	0.108*
		<b>Mean</b>	<b>12.5</b>	<b>0.755</b>	<b>0.789</b>	<b>0.027</b>
		FIR004	22	0.929	0.931	0.003
		FIR053	11	0.600	0.839	0.285***
		GOT004	11	0.952	0.848	-0.123
		POR016	6	0.262	0.546	0.520***
		<b>Mean</b>	<b>12.5</b>	<b>0.686</b>	<b>0.791</b>	<b>0.171</b>
		<b>Overall mean</b>	<b>12.5</b>	<b>0.721</b>	<b>0.790</b>	<b>0.099</b>
2	<i>Quercus petraea</i>	ssrQpZAG1/5	11	0.826	0.819	-0.009
		ssrQpZAG36	12	0.792	0.692	-0.143
		ssrQrZAG96	14	0.917	0.877	-0.045
		ssrQpZAG104	18	0.771	0.880	0.124*
		<b>Mean</b>	<b>13.8</b>	<b>0.827</b>	<b>0.817</b>	<b>-0.018</b>
		FIR004	20	0.955	0.908	-0.052
		FIR053	13	0.500	0.856	0.416***
		GOT004	13	0.682	0.886	0.231***
		POR016	9	0.409	0.721	0.433**
		<b>Mean</b>	<b>13.8</b>	<b>0.637</b>	<b>0.843</b>	<b>0.257</b>
		<b>Overall mean</b>	<b>13.8</b>	<b>0.732</b>	<b>0.830</b>	<b>0.119</b>
3	<i>Quercus robur</i>	ssrQpZAG1/5	12	0.830	0.848	0.021
		ssrQpZAG36	15	0.870	0.878	0.009
		ssrQrZAG96	19	0.826	0.926	0.108**
		ssrQpZAG104	17	0.739	0.883	0.163**
		<b>Mean</b>	<b>15.8</b>	<b>0.816</b>	<b>0.884</b>	<b>0.075</b>
		FIR004	20	0.938	0.929	-0.009
		FIR053	15	0.762	0.851	0.105
		GOT004	16	0.896	0.907	0.012
		POR016	10	0.500	0.701	0.287
		<b>Mean</b>	<b>15.3</b>	<b>0.774</b>	<b>0.847</b>	<b>0.099</b>
		<b>Overall mean</b>	<b>15.5</b>	<b>0.795</b>	<b>0.865</b>	<b>0.087</b>
4	<i>Quercus robur</i>	ssrQpZAG1/5	12	0.959	0.862	-0.113
		ssrQpZAG36	15	0.822	0.901	0.088*
		ssrQrZAG96	18	0.867	0.927	0.065**
		ssrQpZAG104	17	0.911	0.876	-0.041
		<b>Mean</b>	<b>15.5</b>	<b>0.890</b>	<b>0.892</b>	<b>-0.000</b>
		FIR004	18	0.900	0.919	0.021*
		FIR053	14	0.660	0.844	0.218***
		GOT004	15	0.880	0.890	0.011
		POR016	8	0.420	0.662	0.366**
		<b>Mean</b>	<b>13.8</b>	<b>0.715</b>	<b>0.829</b>	<b>0.154</b>
		<b>Overall mean</b>	<b>14.6</b>	<b>0.802</b>	<b>0.860</b>	<b>0.077</b>
<b>General mean</b>	<b>14.1</b>	<b>0.763</b>	<b>0.836</b>	<b>0.096</b>		

Abbreviations: A: number of alleles; H<sub>e</sub>: expected heterozygosity; H<sub>o</sub>: observed heterozygosity; F<sub>IS</sub>: inbreeding coefficient; \*: deviation from Hardy-Weinberg equilibrium (P < 0.05; \*\*: P < 0.01; \*\*\*: P < 0.001).

**Table 6.** Interspecific  $F_{ST}$  values at the 8 microsatellite loci.

Locus	$F_{ST}$	P values (10,000 replicates)
ssrQpZAG1/5	0.020	0.0396
ssrQpZAG36	0.083	<0.0001
ssrQrZAG96	0.028	<0.0001
ssrQpZAG104	0.061	<0.0001
FIR004	0.023	<0.0001
FIR053	0.007	0.2599
GOT004	0.013	0.1139
POR016	0.005	0.3860
ssrQpZAG1/5*	0.032	<0.0001
ssrQpZAG36*	0.040	<0.0001
ssrQrZAG96*	0.232	<0.0001
ssrQpZAG1/5**	0.040	<0.0001
ssrQpZAG36**	0.072	<0.0001
ssrQrZAG96**	0.308	<0.0001
ssrQpZAG104**	0.062	<0.0001

\*:  $F_{ST}$  values and significances according to Muir and Schlötterer (2005); \*\*:  $F_{ST}$  values and significances according to Curtu et al. (2007b).

and highly discriminating microsatellite or alternative molecular markers and leaf morphological characters in a common garden are necessary to better distinguish between these possible causes for leaf morphological variation.

#### 4.2. Nuclear genetic variation within species

At both nSSR and EST-SSR markers genetic variation was slightly higher in *Q. robur* than in *Q. petraea* due to the relatively low variation observed in population 1 - *Q. petraea*. In a mixed stand in Romania (Curtu et al., 2007b) and in other mixed stands in Europe (Mariette et al., 2002), slightly higher genetic variation at nSSRs was observed in *Q. petraea* than in *Q. robur* populations. Likewise, higher genetic variation in *Q. petraea* was found at other DNA markers such as AFLPs (Mariette et al., 2002) and at isozyme markers (Müller-Starck et al., 1996; Zanetto et al., 1996). Interestingly, locus ssrQrZAG96, which showed a lower variability in *Q. robur* than in *Q. petraea* in other studies (Muir & Schlötterer, 2005), showed slightly higher genetic variation in both *Q. robur* populations than in the *Q. petraea* populations in the present study (see discussion below). The analysis of additional populations in Turkey is necessary to draw conclusions regarding a general trend in species variation.

#### 4.3. Nuclear genetic differentiation between species

With the exception of locus ssrQrZAG96, interspecific  $F_{ST}$  values showed values similar to those observed in other *Q. robur* and *Q. petraea* population pairs in Europe (see

**Table 7.** Characterisation of chloroplast haplotypes with microsatellites in *Quercus petraea* and *Q. robur*.

Haplotype	<i>ucd4</i>	<i>udt4</i>
T1	94	142
T2	95	142
T3	96	142
T4	95	145
1*	95	145
2*	93	145
4*	95	144
5*	94	144
7-26*	94	144
10-11-12*	95	143
17*	96	145

\*: haplotypes described for European *Q. robur* and *Q. petraea* populations (Petit et al., 2002; Gailing et al., 2007). Fragment sizes are given in base pairs.

Tables 6 and 7 in Curtu et al., 2007b; Table 3 in Muir & Schlötterer, 2005). Locus ssrQpZAG104 showed a relatively high frequency of the short repeat allele 183 bp in *Q. robur* populations and a very low frequency in both *Q. petraea* populations. Allele frequency differences between species observed at allele 183 bp were very similar to those observed in a mixed *Q. petraea* and *Q. robur* stand in Romania (Curtu et al., 2007b). Likewise, between species similar allele frequency differences of the short repeat allele 201 bp at ssrQpZAG36 were observed in the present study (Figure 1) and in the Romanian populations (Curtu et al., 2007b). Similar allele frequency differences between species within the species distribution range at these short repeat alleles with supposedly low mutation rates suggest limited gene flow between *Q. robur* and *Q. petraea*.

The average genetic differentiation between *Q. petraea* and *Q. robur* at 8 microsatellite loci was very low ( $F_{ST} = 0.030$ ), which is similar to the results described in Curtu et al. (2007b) ( $G_{ST} \approx F_{ST} = 0.026$ , when outlier locus ssrQrZAG96 was excluded) and the mean value for 7 mixed stands of *Q. robur* and *Q. petraea* in central and western Europe ( $F_{ST} \approx G_{ST} = 0.015$ ; Mariette et al., 2002). The overall low level of genetic differentiation between species might be explained by shared ancestral characters within species (Muir and Schlötterer, 2005) and/or by interspecific gene flow (Lexer et al., 2006). Among the 8 loci analysed, ssrQrZAG96 was described as an outlier locus in earlier studies, due to higher than expected interspecific differentiation under selective neutrality (Scotti-Saintagne et al., 2004; Muir & Schlötterer, 2005). Thus, the short repeat allele (139 bp in the present study) was nearly fixed in *Q. robur* (Muir & Schlötterer, 2005;



Curtu et al., 2007b), resulting in low genetic variation at this gene locus across the distribution range of *Q. robur* in Europe, which indicates a selective sweep in this species (Muir & Schlötterer, 2005). Interestingly, the signatures of this selective sweep were not observed in Turkish *Q. robur* populations at *ssrQrZAG96*. While the 139 bp allele showed the highest frequency difference between species at this locus, frequency differences were much lower than those observed in earlier studies of European populations (Muir & Schlötterer, 2005; Curtu et al., 2007b). The short repeat allele 139 bp had a 21% frequency in *Q. robur* and was absent in the 2 *Q. petraea* populations in the present study. The less pronounced allele frequency differences at *ssrQrZAG96* resulted in considerably lower genetic differentiation between species ( $F_{ST} = 0.028$ ) than was observed in earlier studies in western, central, and southern Europe (Curtu et al., 2007b; Muir & Schlötterer, 2005; Neophytou et al., 2010). For example, the interspecific differentiation at this locus was 30.8% ( $G_{ST} = 0.308$ ) in a mixed stand in Romania (Curtu et al., 2007b) and 23.2% ( $F_{ST} = 0.232$ ) across species pairs from 5 European countries (Muir & Schlötterer, 2005). Locus *ssrQrZAG96* also mapped to a quantitative trait locus (QTL) associated with PL ratio [PL/(PL + LL)], a character that differentiates between *Q. robur* and *Q. petraea* (Saintagne et al., 2004).

Compared with previous studies conducted in European population pairs, lower genetic differentiation between species for outlier locus *ssrQrZAG96* was found. This indicates that different evolutionary factors (selection, drift) acted on populations within the species distribution range and/or that this marker is not directly linked to genomic regions associated with morphological or adaptive species differences.

It should also be noted that Turkish populations are (1) located close to putative refugial regions with likely large and stable historical population sizes along the western coast of the Black Sea (Petit et al., 2002) and (2) species appear to show different ecological requirements with regard to water availability. The observation of 3 unique chloroplast haplotypes in the Turkish populations

and the relatively large number of 4 haplotypes in this geographically restricted region is in accordance with the presence of glacial refugia in the Black Sea region of Turkey. These 2 factors may be related to the different patterns of interspecific differentiation observed in the present study at *ssrQrZAG96*. Additional analyses of *Q. robur* and *Q. petraea* populations in Turkey could reveal whether signatures of a selective sweep at gene locus *ssrQrZAG96* are related to geographic location and environmental conditions.

## 5. Conclusions

Multivariate statistical analyses allowed the assignment of most individual samples to species; only 2.9% of the samples could not be classified unequivocally to either species. Similar levels of genetic variation within and among species were observed at nuclear genetic markers in other studies conducted in the European distribution range of the species. Only outlier locus *ssrQrZAG96*, which showed high interspecific differentiation in European population pairs interpreted as the result of a selective sweep, revealed low differentiation between species in the present study. The analysis of additional populations in Turkey at functional gene markers is necessary in order to better understand these different patterns of species differentiation.

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