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ORIGINAL PAPER

Leaf morphological and genetic differentiation between *Quercus robur* L. and its closest relative, the drought-tolerant *Quercus pedunculiflora* K. Koch

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Abstract
· ***Introduction*** The study of phenotypic and genetic differentiation between incipient species or species that have recently diverged provides insights into the evolutionary history of species complexes and may contribute to our understanding of how species will evolve in contrasting environmental conditions.
· ***Objective*** Here, we characterise the differences in leaf morphology and we estimate the genetic differentiation between *Quercus robur* and its closest relative, the drought-tolerant *Quercus pedunculiflora*. We have examined whether these two ecologically divergent taxa have different genetic structures using both nuclear and chloroplast markers.
· ***Results*** By analysing 844 individual trees from seven *Q. robur* and seven *Q. pedunculiflora* populations and one mixed forest containing both taxa, we found that abaxial laminar pubescence is the most discriminating leaf descriptor between *Q. robur* and *Q. pedunculiflora*. The analysis of seven enzyme-coding gene loci revealed no taxon-specific alleles with a frequency>0.03. The DNA chloroplast haplotypes observed in *Q. pedunculiflora* have been found in our *Q. robur* sample or have been previously reported in *Q. robur*.
· ***Conclusions*** The very low level of nuclear divergence revealed by the isozyme markers and the incomplete sorting of *Q. robur* and *Q. pedunculiflora* populations according to their physical appearance suggests that *Q. pedunculiflora* is an incipient oak species and that the process of ecological speciation is not completed.

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1 Introduction

In species-rich tree genera, such as *Quercus* (oaks), there is often a debate regarding the exact number of species (Rushton 1993). This happens because the species delineation is sometimes a very difficult task (De Queiroz 2007). Traditionally, morphological and ecological criteria are used to delimit one species from another. However, in the last years, genetic data are increasingly used in addressing the species status, as for example in the case of oaks (Muir et al. 2000). When species have only recently diverged or the speciation is ongoing, the phenotypic and genetic differentiation between species is very low. The morphological divergence may be limited to several flower and leaf characters (e.g. in oaks, Schwarz 1993), while the genetic differences may be restricted to a few genomic regions under selection (Turner and Hahn 2007).

In contrast to *Quercus robur* L, the most widespread oak species in Europe, *Quercus pedunculiflora* K. Koch has a more restricted distribution in south-eastern Europe, from Balkan Peninsula across Crimea to the Caucasus and northern part of Anatolia (Schwarz 1993; Menitsky 2005). Both taxa are members of the section *Robur,* series *Pedunculatae* Schwarz. *Q. pedunculiflora* is phenotypically very similar to *Q. robur*, the pedunculate oak (Schwarz 1937; Georgescu and Cretzoiu 1941). There are small morphological differences such as the length of the cupula peduncle, which is usually longer in *Q. pedunculiflora*, or the pubescence on the abaxial surface of the leaf (Schwarz 1993). While *Q. robur* prefers wetter soils which can be subject to flooding for short periods of time, *Q. pedunculiflora* grows under dry conditions being more drought

tolerant (Stanescu et al. 1997; Donita et al. 2004). Its taxonomic position either as distinct species or as subspecies (ecotype) of *Q. robur* sensu lato is a subject of debate among botanists (Georgescu and Morariu 1948; Ciocarlan 2000; Donita et al. 2004; Broshtilov 2006).

Because of its adaptations to a more arid climate, *Q. pedunculiflora* may become a very important tree species for forestry, particularly in view of global climate change. Rising temperatures will result in vegetation shifts to higher altitudes and northern latitudes (Kremer 2007). *Q. pedunculiflora* is considered a keystone species for the forest ecosystems of the wood-steppe zone, the survival of many other plant and animal species depending on it (Donita et al. 2005). Its natural occurrence has been drastically reduced by land conversion to agriculture, human activities and poor forest management over the last centuries. At present, in some geographical areas (e.g. Northern Balkans) its distribution range is very fragmented and comprises only few isolated populations (Stanescu et al. 1997).

Due to its great ecological, economic and social importance, *Q. robur* has been intensively studied across Europe in the last two decades. There is a large body of literature on morphological and genetic variation, population differentiation and divergence of *Q. robur* from other closely related oak species (e.g. Fortini et al. 2009; Lepais et al. 2009; Viscosi et al. 2009). However, until present there is no genetic study which addresses the difference between the two sister species or very closely related taxa, *Q. robur* and *Q. pedunculiflora*, or which investigates the genetic variation in *Q. pedunculiflora*, except for one test on half-sib progenies (Enescu 1993).

The purpose of this study is to characterise the differences in leaf morphology and to estimate the genetic differentiation between *Q. robur* and its closest relative, *Q. pedunculiflora*. More specifically, we were interested to find the most discriminating leaf variables between *Q. robur* and *Q. pedunculiflora* and to examine using both nuclear and chloroplast markers whether these two ecologically divergent taxa have different genetic structures.

2 Materials and methods

2.1 Plant material

Seven *Q. robur* and seven *Q. pedunculiflora* populations were sampled across the entire distribution range of the two taxa in Romania (Table 1). In addition, one mixed forest with both taxa, Letea Natural Reserve, was sampled in the Danube Delta. All populations are naturally regenerated and currently managed as gene reserves, with two exceptions (P-SNA and P-PUN—Table 1). Twigs with leaves and buds were collected during 2008 from approximately 50 adult trees per population. The trees were located at least 50 m apart from each other in order to avoid, as much as possible, the sampling of related individuals.

2.2 Morphological analysis

We focused on leaf descriptors because of the difficulty to obtain acorns and cupulas. The distance between two

Table 1 Geographic location and climate conditions of the sampled *Q. robur* and *Q. pedunculiflora* populations and of the mixed forest with both oak taxa

Abbreviations	Population	County	Latitude N	Longitude E	Altitude (m)	Pa	Ps	T	MTH	MTC
Q. robur populations										
R-NOR	Noroieni	Satu Mare	47°52′	22°55′	120	670	410	9.5	19.5	−2.5
R-PAU	Păunoaia	Prahova	44°45′	25°58′	150	588	361	10.6	22.8	−3.1
R-CEN	Cenuşa	Iaşi	47°03′	27°14′	300	550	364	9.1	20.1	−3.8
R-DAC	Dacia	Braşov	45°58′	25°06′	540	690	430	8.4	18.0	−4.0
R-BAZ	Bazoş	Timişoara	45°45′	21°30′	98	625	356	10.9	21.6	−1.2
R-VAM	Vânju Mare	Mehedinţi	44°26′	22°50′	89	570	327	11.5	22.7	−1.5
R-RES	Reşca	Olt	44°10′	24°25′	75	540	330	10.6	23.3	−2.0
Q. pedunculiflora populations										
P-BRC	Braniştea Catârilor	Olt	43°53′	24°14′	65	550	300	11.2	23.3	−2.0
P-CIO	Ciornuleasa	Călăraşi	44°13′	26°45′	60	445	307	11.0	22.0	−3.0
P-URZ	Urziceni	Ialomiţa	44°32′	26°49′	60	470	300	10.8	22.0	−2.5
P-SNA	Snagov	Ilfov	44°37′	26°21′	90	540	330	11.2	22.5	−2.9
P-BAN	Băneasa	Constanţa	44°03′	27°53′	110	470	260	11.3	23.0	−1.4
P-PUN	Punghina	Mehedinţi	44°15′	22°50′	50	570	327	11.5	23.4	−1.5
P-VIS	Viişoara	Brăila	44°52′	27°39′	30	500	285	10.5	22.3	−2.3
Mixed forest of *Q. robur* and *Q. pedunculiflora*										
LT	Letea	Tulcea	45°20′	29°31′	5	380	220	11.4	22.0	−0.5

The climatic data for each population was recorded at the closest meteorological station

Pa annual precipitation (millimetres), *Ps* precipitation during vegetation season (millimetres), *T* mean annual temperature (°C), *MTH* mean annual temperature of the hottest month (°C), *MTC* mean annual temperature of the coldest month (°C)

consecutive mast years can reach up to 10 years or more in both taxa. Three completely developed leaves collected from the part of the crown exposed to sunlight and from each of the 844 sampled individuals were analysed. A set of leaf morphological descriptors, which are commonly employed for oak species identification (Kremer et al. 2002), was used in this study. Leaf morphology was assessed by means of five dimensional variables (lamina length, petiole length, lobe width, sinus width and length of lamina at the largest width), two counted variables (number of lobes and number of intercalary veins); two observed variables (basal shape of the lamina and the abaxial laminar pubescence),which were scored as an index (Kremer et al. 2002) and five transformed characters (lamina shape, petiole ratio, lobe depth ratio, percentage venation and lobe width ratio). The dimensional descriptors were measured on leaf pictures with WinFOLIA software (Regent Instruments 2007). The basal shape of the lamina was scored from 1 to 9 and the pubescence density was ranked from 1 (no pubescence) to 6 (dense hairiness). The pubescence on the abaxial lamina was assessed with a stereomicroscope (×30). Mean values of each leaf descriptor per population and taxon as well as the *P* values for the comparisons (*t* test) between *Q. robur* and *Q. pedunculiflora* were calculated using STATISTICA v8 software (StatSoft 2008). The data set for seven pure populations of *Q. robur* and seven pure populations of *Q. pedunculiflora* was used for the construction of a discriminant function using the same software. This function was then applied for classifying the trees sampled in the mixed forest from the Danube Delta as either *Q. robur* or *Q. pedunculiflora*.

2.3 Isozyme analysis

Enzymes were extracted from meristematic tissues after removing the bud scales. Separation of enzymes from crude homogenates was done by horizontal starch–gel electrophoresis following standard procedures (Müller-Starck et al. 1996; Zanetto et al. 1996). Seven enzyme systems were analysed (Enzyme Commission number and controlling locus are given in parenthesis): aspartate aminotransferase (2.6.1.1; *Aat-B*), isocitrate dehydrogenase (1.1.1.42, *Idh-B*), menadione-reductase (1.6.99.2; *Mnr-A*), 6-phosphogluconate-dehydrogenase (5.3.1.9; *6-Pgdh-B*), phosphoglucose-isomerase (5.3.1.9; *Pgi-B*), phosphoglucomutase (2.7.5.1; *Pgm-A*) and shikimic acid dehydrogenase (1.1.1.25; *Skdh-A*). The seven enzyme-coding gene loci showed Mendelian inheritance in controlled crosses (Müller-Starck et al. 1996; Zanetto et al. 1996). Alleles were labelled in accordance with the relative migration rate of the corresponding band as related to the most common one. At every locus, 100 was assigned to the most frequent alleles.

2.4 Chloroplast DNA analysis

DNA was extracted from buds and leaves using the Qiagen DNeasy96 Plant Kit following the manufacturer protocol, but without using liquid nitrogen for disruption. The sample size for the chloroplast DNA analysis comprises five randomly selected trees per population. Four large regions of the chloroplast DNA were amplified, and each one was cut with restriction enzymes: *psa*A–*trn*S (AS) with *Hinf*I, *trn*D–*trn*T (DT) with *Taq*I, *trn*C–*trn*D (CD) with *Taq*I and *trn*T–*trn*F (TF) with *Hinf*I. The methods used are described in details elsewhere (Toader et al. 2009). The restriction fragments were then run on 8% polyacrylamide gels and stained with SYBR Gold (Molecular Probes). Nomenclature of the chloroplast haplotypes follows (Petit et al. 2002a). Chloroplast haplotypes, which did not fit exactly the restriction patterns, were named after the most similar ones and by adding prime (e.g. haplotype 15′).

2.5 Statistical data analysis

For each enzyme-coding gene locus and population, percentage of polymorphic loci, observed and effective number of alleles, number of private alleles (i.e. alleles found only in one taxon), allele frequencies, observed and expected heterozygosity, fixation index, divergence from Hardy-Weinberg equilibrium, Nei's genetic distances was calculated using the computer software GenAlEx version 6.3 (Peakall and Smouse 2006). An unweighted pair group method arithmetic average (UPGMA) dendrogram of the oak populations based on Nei's genetic distance, was constructed using MEGA version 4 (Tamura et al. 2007). A hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN software version 3.5.1.2 (Excoffier et al. 2005). The significance of the *F* statistics was tested by permuting individuals between the populations. The number of permutations was set to 10,000. The frequency of the chloroplast DNA haplotypes and the population statistics for chloroplast data were calculated using the HAPLODIV software (Pons and Petit 1995).

3 Results

3.1 Leaf morphology

Only one out of five dimensional variables showed significant differences between *Q. robur* and *Q. pedunculiflora* populations (Table 2). The petiole length was on average approximately 2 mm longer in *Q. pedunculiflora*. The morphological differences were more substantial at the observed variables. While *Q. robur* leaves show almost no trichomes on the abaxial surface of the leaf, *Q. pedunculi-*

Table 2 Mean and standard deviation values of 14 leaf descriptors in seven populations of *Q. robur* and seven populations of *Q. pedunculiflora*, respectively

Leaf descriptor	*Q. robur*		*Q. pedunculiflora*		*P* value
	Mean	SD	Mean	SD	
Lamina length (mm)	116.91	±9.72	123.29	±11.20	0.28
Petiole length (mm)	6.03	±0.84	8.33	±0.67	**0.00**
Lobe width (mm)	37.73	±3.50	40.69	±3.86	0.16
Sinus width (mm)	14.29	±1.52	13.14	±1.68	0.20
Length of lamina at largest width (mm)	69.80	±5.16	72.03	±7.77	0.54
Basal shape of the lamina	8.26	±0.33	7.65	±0.35	**0.00**
Abaxial laminar pubescence	1.01	±0.03	4.62	±0.09	**0.00**
Number of lobes	10.06	±0.60	9.95	±0.66	0.72
Number of intercalary veins	4.17	±0.69	4.28	±0.24	0.70
Lamina shape	59.69	±2.23	58.25	±1.48	0.18
Petiole ratio	4.99	±0.82	6.41	±0.69	**0.00**
Lobe depth ratio	61.35	±2.25	67.15	±2.61	**0.00**
Percentage venation	42.15	±6.32	43.97	±2.33	0.48
Lobe width ratio	32.36	±1.69	33.13	±0.81	0.29

P values for the comparisons between taxa are given. Statistical significant values ($P<0.05$) are given in bold

flora leaves are densely pubescent (Table 2). Basal shape had smaller ear-like lobes (i.e. auricles) in *Q. pedunculiflora* populations. Two composed variable showed also significant differences ($P<0.05$). One descriptor is petiole ratio, which is positively correlated with the petiole length. The second descriptor, lobe depth ratio, exhibited greater values in *Q. pedunculiflora* populations, which means that the leaves of this taxon have deeper sinuses compared to *Q. robur*.

The discriminant analysis, which was performed using the two groups of pure populations, showed that the pubescence on the abaxial surface of the leaf (PU) has the lowest value of Partial Wilks' Lambda (0.07), followed by petiole length, lobe width, basal shape and lamina shape, but with much higher values (0.98–099). The Partial Wilks' Lambda indicates that variable 'pubescence' contributes most to the overall discrimination. The first two discriminating variables were retained for the construction of the following discriminant function between *Q. robur* and *Q. pedunculiflora*: ID=686−(228×PU)−(5.8×PL). This function gave positive values for *Q. robur* and negative values for *Q. pedunculiflora* (Fig. 1). Based on this discriminant function, the individuals sampled in the mixed oak forest from the Danube Delta were then classified as either *Q. robur* (47 samples) or *Q. pedunculiflora* (52 samples).

3.2 Isozyme analysis

The seven enzyme-coding gene loci were polymorphic in all oak populations. A total of 41 alleles were observed in our sample (Table 3). The two oak taxa shared the common alleles at all loci but each harboured rare alleles (frequency≤0.03) which were not found in the other taxon: four alleles in *Q. robur* and five alleles in *Q. pedunculiflora*. The private allele with the highest frequency, *Pgi-B-129* (Table 3), was detected in all *Q. robur* pure populations. The other three *Q. robur* private alleles were observed in two up to five populations. Most of the alleles unique to *Q. pedunculiflora* were observed in two but not the same populations, only the private allele, *Idh-B-82*, occurred in one population (P-BAN).

The mean number of alleles per locus varied between 3.00 and 3.86 in *Q. robur* populations and between 2.86 and 3.86 in *Q. pedunculiflora* populations. When averaged across populations, this parameter was slightly higher in *Q. robur* than in *Q. pedunculiflora* (Table 4). The same trend was observed for the effective number of alleles (A_e). However, the mean value of observed heterozygosity (H_o) was the same in both taxa. Only three out of the 56 tests for

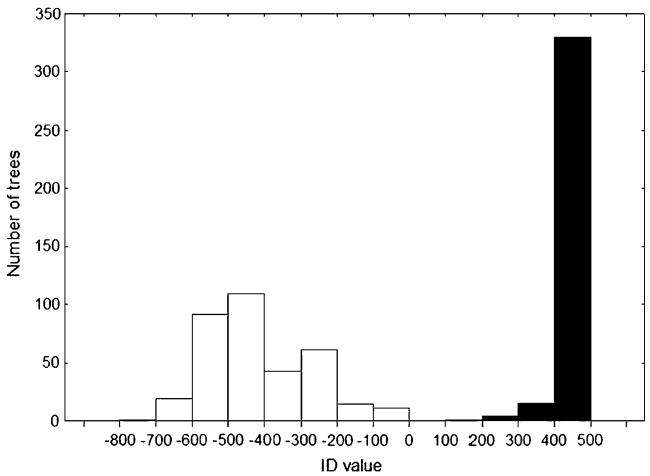

Fig. 1 Distribution of the discriminant function values according to the oak taxon: *Q. pedunculiflora* (*unshaded*) and *Q. robur* (*shaded*)

Table 3 Mean allele frequencies at each enzyme-coding gene locus in *Q. robur* and *Q. pedunculiflora*

Enzyme-coding gene locus	Allele	*Q. robur*	*Q. pedunculiflora*
Idh-B	139	0.000	**0.006**
	118	0.408	0.522
	105	0.000	**0.005**
	100	0.587	0.458
	82	0.000	**0.001**
	74	0.005	0.007
Pgm-A	130	**0.005**	0.000
	115	0.458	0.298
	100	0.488	0.682
	83	0.018	0.008
	75	0.030	0.012
6-Pgdh-B	124	0.009	0.002
	110	0.034	0.025
	100	0.941	0.945
	88	0.014	0.002
	52	0.001	0.025
Skdh-A	113	0.017	0.013
	105	0.008	0.011
	100	0.956	0.933
	95	0.000	**0.008**
	93	0.018	0.032
	88	0.000	**0.003**
Mnr-A	144	0.016	0.002
	122	0.009	0.023
	100	0.913	0.952
	78	0.040	0.016
	63	0.004	0.004
	22	0.019	0.004
Pgi-B	160	0.006	0.005
	129	**0.030**	0.000
	123	0.002	0.015
	117	0.001	0.006
	100	0.954	0.952
	71	**0.002**	0.000
	37	0.005	0.023
Aat-B	120	**0.008**	0.000
	108	0.006	0.023
	100	0.929	0.899
	90	0.017	0.020
	85	0.032	0.041
	79	0.008	0.016

The private alleles are in bold characters

each taxon showed significant deviations ($P<0.05$) from genotypic frequencies expected under Hardy–Weinberg equilibrium. *Q. robur* as well as *Q. pedunculiflora* showed on average a very low heterozygote deficit, but this measure varied from one population to another (Table 4).

The hierarchical AMOVA showed that most of the total genetic variation is within populations and only a small fraction ($F_{ST}=0.039$) is among the 16 populations (Table 5). The variation between taxa, averaged over all loci, was more than double in comparison to the variation among populations within taxa. The most discriminating enzyme-coding gene loci between taxa were *Pgm-A* and *Idh-B* (Table 5). Four other loci (*6-Pgdh-B*, *Skdh-A*, *Mnr-A* and *Aat-B*) differentiated better within populations of *Q. robur* and *Q. pedunculiflora*, respectively, and not between the two taxa.

The dendrogram constructed using Nei's genetic distances between pairs of populations revealed two clusters which comprise mostly populations of a single taxon (Fig. 2). Two *Q. pedunculiflora* populations situated in south-western Romania (P-PUN and P-BRC) were grouped together with other *Q. robur* populations from the same geographical region. One *Q. pedunculiflora* population (P-LT), situated in the Danube Delta, was grouped together with the single *Q. pedunculiflora* population (P-BAN) located between the Danube and the Black Sea (Fig. 2). The *Q. robur* population from the Danube Delta (R-LT) was included in one cluster with *Q. pedunculiflora* populations from the same geographical area, and not with the other *Q. robur* populations.

3.3 Chloroplast DNA analysis

A total of eight chloroplast haplotypes were detected in the 16 oak populations. Most of the observed haplotypes belong to lineage A (Petit et al. 2002b) of the European oaks (haplotypes 4a, 4b′, 5a, 5c and 6), two haplotypes belong to lineage E (haplotype 13 and haplotype 15′) and one haplotype belong very likely to lineage F (haplotype 9′). Twelve out of the 16 oak populations were fixed for one or another haplotype (Fig. 3). The most common chloroplast haplotypes irrespective of taxon were haplotype 4a (frequency=0.325) and haplotype 5a (frequency=0.263). Three chloroplast haplotypes (haplotypes 4a, 5a and 9′) are shared by *Q. robur* and *Q. pedunculiflora* (Fig. 3). Four chloroplast haplotypes were apparently specific for *Q. robur* (haplotype 6, 4b′, 5c and 15′) and one (haplotype 13) for *Q. pedunculiflora*. As expected, *Q. robur*, which covers a larger area in this study, has a higher level of total genetic diversity compared to *Q. pedunculiflora*, which is confined to south-eastern Romania ($h_T=0.891$ for *Q. robur* versus $h_T=0.721$ for *Q. pedunculiflora*). On the contrary, the coefficient of genetic differentiation, G_{ST}, has a higher value in *Q. pedunculiflora* ($G_{ST}=0.930$ versus $G_{ST}=0.761$ for *Q. robur*), which shows the very low level of within population diversity, as it has been described for maternally inherited markers and measured in many tree species.

Table 4 Summary of genetic diversity over seven enzyme-coding gene loci for each population and taxon

Population	*N*	N_a	N_e	H_o	*F*
R-NOR	56	3.000	1.418	0.247	−0.023
R-PAU	55	3.286	1.420	0.210	−0.014
R-CEN	54	3.429	1.378	0.206	0.003
R-DAC	53	3.571	1.440	0.240	0.014
R-BAZ	52	3.143	1.368	0.209	0.008
R-VAM	55	3.857	1.395	0.200	0.030
R-RES	55	3.429	1.414	0.244	−0.033
R-LT	47	3.571	1.344	0.185	0.046
Mean for *Q. robur*	53.4	3.411	1.397	0.218	0.004
SE	0.363	0.144	0.058	0.023	0.012
P-BRC	55	3.286	1.426	0.249	−0.008
P-CIO	52	3.429	1.389	0.236	−0.048
P-URZ	51	3.286	1.338	0.216	0.010
P-SNA	50	3.286	1.357	0.229	−0.068
P-BAN	52	3.857	1.376	0.247	−0.021
P-PUN	54	3.571	1.403	0.198	0.075
P-VIS	51	2.857	1.280	0.171	0.009
P-LT	52	3.286	1.359	0.198	0.067
Mean for *Q. pedunculiflora*	52.1	3.357	1.366	0.218	0.002
SE	0.207	0.115	0.051	0.023	0.016

N sample size, N_a number of alleles per locus, N_e effective number of alleles, H_o observed heterozygosity, *F* heterozygote deficit, *SE* standard error

4 Discussion

The morphological characterization of the pure populations of *Q. robur* and *Q. pedunculiflora* showed that one leaf descriptor, abaxial laminar pubescence, contributes most to the differentiation between the two taxa. At the population level, three more leaf variables (petiole length, basal shape and lobe depth ratio) confirmed other minor leaf morphological differences described in the literature (Schwarz 1993; Stanescu et al. 1997). However, based solely on these last three leaf variables, one could not distinguish between individuals of *Q. robur* and *Q. pedunculiflora*. In dendrological manuals (e.g. Stanescu et al. 1997), it is also mentioned a rare variety (var. *virescens*) of *Q. pedunculiflora* which exhibits a very low density of hairs on the abaxial surface of the leaf. However, this variety was not detected in our populations of *Q. pedunculiflora*, although we covered very well the actual natural occurrence of this taxon in Romania by including in this survey the most representative populations, the majority of them selected as forest genetic resources.

The high density of fasciculate hairs observed on the lower surface of *Q. pedunculiflora* leaves is undoubtedly an adaptation for survival on drier sites, which is also particular to *Quercus pubescens*. Other leaf morphological traits of *Q. pedunculiflora* also show similarities to *Q. pubescens*: longer petiole (8.33±0.67) and deeper sinuses compared to *Q. robur*. These morphological similarities may support the hypothesis of introgression of genetic material from *Q. pubescens*, a species which has the petiole length ranging from 8 to 15 mm (20 mm), and in many instances deep sinuses (Bussotti 1998). The values of the discriminant function indicate a larger variance in *Q. pedunculiflora* than in *Q. robur* (see Fig. 1). This higher

Table 5 Locus by locus and global AMOVA

Enzyme-coding gene locus	F_{ST}	*P* value	F_{SC}	*P* value	F_{CT}	*P* value
Idh-B	0.030	0.00	0.003	0.22	0.027	0.00
Pgm-A	0.075	0.00	0.018	0.00	0.058	0.00
6-Pgdh-B	0.016	0.00	0.015	0.00	0.001	0.29
Skdh-A	0.014	0.00	0.013	0.00	0.001	0.23
Mnr-A	0.021	0.00	0.014	0.00	0.007	0.05
Pgi-B	0.007	0.03	0.001	0.29	0.006	0.00
Aat-B	0.012	0.00	0.010	0.00	0.002	0.17
Weighted average over all loci	0.039	0.00	0.011	0.00	0.028	0.00

Fixation indices: F_{ST} among populations, F_{SC} among populations within taxa, F_{CT} among populations between taxa

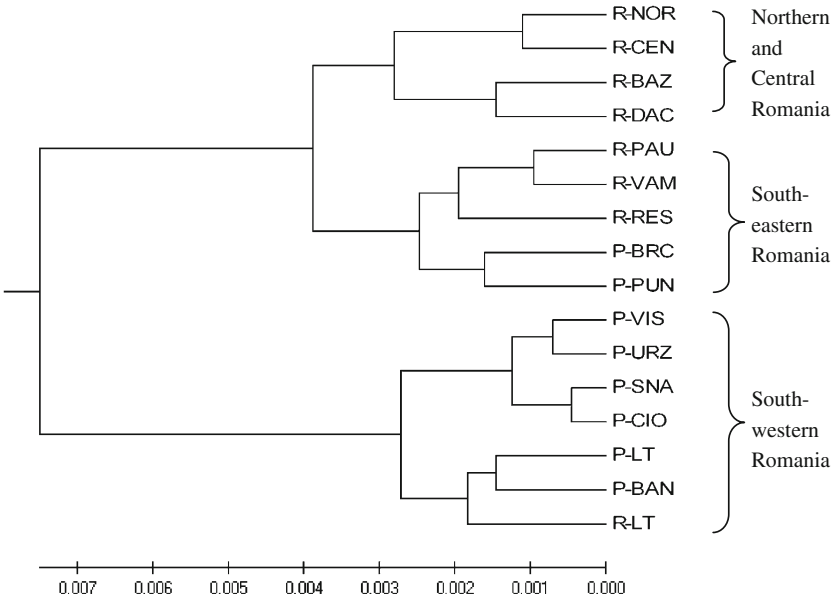

Fig. 2 UPGMA cluster analysis using Nei's distances obtained from seven isozyme markers for 16 oak populations

leaf morphological variation in *Q. pedunculiflora* may also represent an evidence of introgression from *Q. pubescens*, the only oak species that co-occurs with *Q. pedunculiflora* in the wood steppe of south-eastern Romania (Stanescu et al. 1997). Hybridization of *Q. robur* with other *Quercus* species, which show adaptations to drought, such as *Q. pubescens*, may have played a role in the formation of *Q. pedunculiflora*. Subsequent introgression following hybridization may be responsible for the transfer of few adaptations (e.g. pilosity) to *Q. robur* populations situated on the ecological margin of species distribution. Evidence for adaptive introgression between species has been reported in other plant species: sunflower and Louisiana Iris (see Arnold and Martin 2009 and references therein). For testing the hypothesis of introgressive hybridization, future investigations should include all oak species present in the region, which are potentially linked by gene flow. The importance of considering all species when studying hybridization was very well illustrated in a recent study on oak species in France (Lepais et al. 2009).

To our knowledge, this is the first investigation which aims at genetically differentiating the most widespread

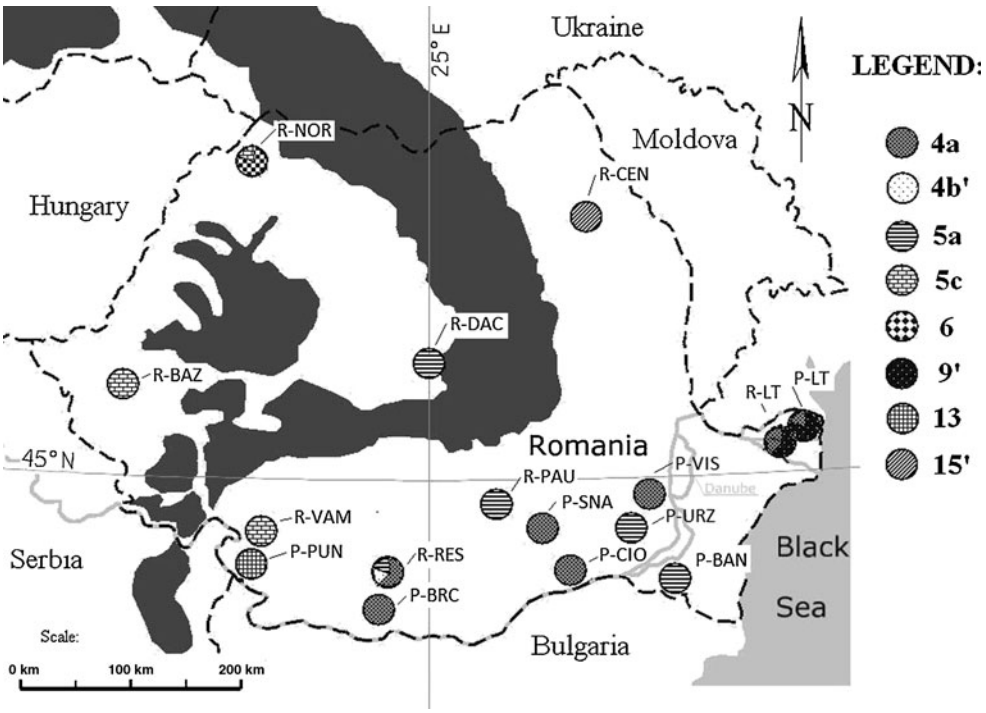

Fig. 3 Geographical distribution of the chloroplast DNA haplotypes. The *shadow area* represents the Carpathian Mountains (elevation over 500 m)

European white oak species, *Q. robur*, from *Q. pedunculiflora*, a less known taxon, which occurs in the wood steppe of south-eastern Europe. The presence of nine private alleles, most of them with very low frequency, may be mainly due to the small sample size for each population. However, the rare alleles are more susceptible to the loss through genetic drift than the more frequent ones (Nei and Chakraborty 1975). In case of private allele *Pgi-B-129*, which occurs in seven *Q. robur* populations, and shows frequencies≥0.05 in three populations, it might be that there was not enough time to spread across *Q. pedunculiflora* populations since it appeared through a mutation. On average, *Q. robur* showed a higher allelic richness and gene diversity than *Q. pedunculiflora*, although the differences are not significant ($P>0.05$). The greater fragmentation of natural distribution and smaller population size may lead to stronger effects of genetic drift in *Q. pedunculiflora* as compared to *Q. robur*.

The amount of nuclear differentiation between the two taxa (0.028) is slightly lower than the value estimated throughout Europe with the same category of genetic markers for the differentiation between *Q. robur* and *Quercus petraea* (Zanetto et al. 1994). However, the isozyme diversity between *Q. robur* and *Q. petraea* across regions in Central and Eastern Europe (0.020) was lower compared to our study when one outlier locus, *Gludh-A*, is excluded (Gömöry et al. 2001). Higher values for isozyme differentiation among *Q. robur*, *Q. petraea* and *Q. pubescens* are reported in a series of other studies (Finkeldey 2001; Belletti et al. 2005; Curtu et al. 2007). The values of Nei's distances estimated in our study are also lower than those reported in other isozyme studies on European oak species (Zanetto et al. 1994).

The level of genetic differentiation between *Q. robur* and *Q. pedunculiflora* varied across the enzyme-coding gene loci. Two loci, *Idh-B* and *Pgm-A*, have better discriminated between taxa than among populations within taxa. Interestingly, isocitrate dehydrogenase (*Idh*) is one of the few enzyme systems at which selection effects have been detected (see Gömöry et al. 2010 and references therein). Moreover, *Idh-B* and *Pgm-A* loci showed high F_{ST} values in a recent study within the white oak complex in Slovakia (Gömöry and Schmidtova 2007) or between Asian *Quercus* species (Potenko et al. 2007). Such markers that experience high levels of gene flows within species but little introgression (interspecific gene flow) are useful for species delimitation (Petit and Excoffier 2009).

If *Q. robur* and *Q. pedunculiflora* were distinct species, then the populations should have clustered according to physical appearance rather than geographic location. However, unlike other studies (Zanetto et al. 1994; Muir et al. 2000; Potenko et al. 2007) not all the populations of the same taxon are grouped together. For instance, the samples of *Q. robur* from the Danube Delta are grouped together with two *Q. pedunculiflora* populations from the same geographical area rather than with other *Q. robur* populations. These results support the hypothesis that *Q. pedunculiflora* is an incipient species which has recently evolved under the dry conditions of the wood steppe in south-eastern Europe. The ecological specialisation of *Q. robur* populations to a more arid climate has conducted to the formation of a sister or minor species, *Q. pedunculiflora*, a taxon which exhibits several adaptations to drought such as hairiness on the abaxial surface of the leaf or thicker bark compared to *Q. robur*. As in other oak species, the gene flow between *Q. robur* and *Q. pedunculiflora*, especially in areas of sympatry, like the mixed forest in the Danube Delta, is still very intense. Flowering phenology does not seem to represent a reproductive barrier between *Q. robur* and *Q. pedunculiflora* since the two taxa flowered simultaneously in a mixed stand in Eastern Romania (Chesnoiu et al. 2009). Recent studies have found substantial gene flow during the process of speciation (Hey 2006). Ecological divergence in the presence of gene flow may be explained by the fact that natural selection can prevent gene flow at some genes (e.g. the genes responsible for adaptation to dry conditions) and it can enable other genes to pass between populations (Hey 2006).

The two taxa share the most frequent chloroplast haplotypes, but also one rare haplotype of presumably Caucasian origin, which was observed for the first time in the Danube Delta. The sharing of both common and rare chloroplast haplotypes supports also the existence of gene flow between oak species in the recent past (Lexer et al. 2006). Haplotype 13, which in our study seems to be present only in one *Q. pedunculiflora* population from south-western Romania, was previously reported in *Q. robur* populations from central Romania (Bordács et al. 2002). So, there are no specific haplotypes for *Q. pedunculiflora*, all of them being detected in *Q. robur* either in this study or in previous surveys on material from the same regions (Bordács et al. 2002). On the other hand, haplotype 6, which was observed only in one *Q. robur* population in north-western Romania, is very common in the Carpathian Basin, where no *Q. pedunculiflora* trees are mentioned to occur, at least in Romania. The haplotypes detected in this study match very well those previously found in the northern Balkans and are likely to have migrated from the Balkan and Italian Peninsulas, but also from the eastern part of the Black Sea (Petit et al. 2002a).

The very low level of nuclear differentiation revealed by the isozyme markers as well as the lack of differentiation at the chloroplast DNA level between *Q. robur* and *Q. pedunculiflora* might be explained by ongoing or recent divergence of the two taxa. Even though *Q. robur* and *Q.*

pedunculiflora exhibit different morphology and ecological preferences, the genetic differences between them are quite small and are very probably restricted to a few genomic regions under selection. Whether the formation of *Q. pedunculiflora* is mainly due to the direct adaptation of *Q. robur* to drier habitats or to introgressive hybridization with other xerophilous oak species (e.g. *Q. pubescens*) remains to be tested. Future research should use more marker loci distributed throughout the whole oak genome and/or adaptive markers, which will hopefully be soon available, to detect possible 'genomic islands' of incipient speciation.

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