Turkish Journal of Agriculture and Forestry

Volume 37 | Number 5 Article 14

1-1-2013

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TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2013) 37: 632-641

doi:10.3906/tar-1210-28

Is Quercus virgiliana a distinct morphological and genetic entity among European white oaks?

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Received: 10.10.2012 • **Accepted:** 30.01.2013 • **Published Online:** 28.08.2013 • **Printed:** 25.09.2013

Abstract: The existence of *Quercus virgiliana* Ten. is a subject of debate among botanists and silviculturists. It was considered a distinct species, sometimes as an intraspecific taxonomic unit of *Q. pubescens* L., or was not even recognized as a taxon or infrataxon. This disagreement with regard to the taxonomic classification is explained by the morphological similarities between *Q. virgiliana* and *Q. pubescens*, with a small overlap between particular leaf and fruit traits. The main objective of this study was to evaluate the macromorphological fruit and leaf descriptors in Romanian populations of pubescent oaks. We wanted to find which traits discriminate between the 2 taxa. By using 7 microsatellite markers, we checked whether the 2 taxa have different genetic structures. A total of 918 individuals were sampled for morphological analyses from 20 stands across Romania. The length of cupula peduncle showed the highest discriminating power, but also leaf characters such as abaxial laminar pubescence, lamina length, sinus width, and length of lamina at largest width helped to discriminate between the 2 taxa. The analysis performed at 7 microsatellite loci revealed very little genetic differentiation between *Q. pubescens* and *Q. virgiliana*. Different genetic assignment tests provided no support for 2 genetic entities in our sample. This could be explained by the restricted number of loci used. However, the morphological differences found in this study suggest that *Q. virgiliana* is an intraspecific taxonomic unit of *Q. pubescens* rather than a distinct species.

Key words: Fruit morphology, leaf morphology, microsatellites, *Quercus pubescens*, *Quercus virgiliana*

1. Introduction

Quercus virgiliana has been taxonomically classified in many ways. According to different taxonomic classifications, it is sometimes treated as a distinct species (Schwarz 1993; Şofletea and Curtu 2007; Doniță 2008), sometimes as an intraspecific taxonomic unit of *Q. pubescens,* or sometimes it is not recognized or nominated as a taxon or infrataxon (Camus 1936–1954; Nixon 1993; Bussotti and Grossini 1997; Menitsky, 2005). This disagreement regarding the taxonomic classification of the 2 taxa is derived from their morphological similarities, with small overlaps between particular leaf traits (lamina length, petiole length, number of lobes) and fruit descriptors (peduncle length, cup scales shape), according to various descriptions (Schwarz 1993; Doniță 2008). Among these morphological descriptors, the length of the cupula peduncle, which is usually longer in *Q. virgiliana*, is considered essential for differentiating the 2 oak taxa from the series *Lanuginosae* (Bartha 2001; Trinajstić 2007). Most analyses of leaf morphological characters in oaks from the series *Lanuginosae* carried out in Europe could not differentiate the 2 taxa (Bussotti and Grossoni 1997; Jerše and Batič 2007). By contrast, other macromorphological (Trinajstić 2007) or micromorphological (Fortini et al. 2009) studies (acorn and cup dimensions and the type of hairs on the leaf) revealed some differences between them.

Even if the present distribution range of both taxa is small, in view of climate warming their ecological and social importance will increase. Consequently, it is expected that the area occupied by the 2 taxa will increase in Romania and in the rest of Europe. This prediction is based on scenarios that indicate successional changes or latitudinal and altitudinal migrations of species (Kardol et al. 2010). Marginal forest tree populations will have the strongest response to climate change (He et al. 2005).

Due to their great morphological, ecological, and genetic variability, the greatest problems of taxonomic separation encountered among the European oaks seem to be within the subgenus *Lepidobalanus* (*Quercus*) (Schwarz 1993). Leaf descriptors are the most commonly used morphological variables for distinguishing between oak species. Leaf morphology of European white oak

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species has been studied extensively using both traditional morphometric methods (Kremer et al. 2002; Gugerli et al. 2007; Curtu et al. 2011b) and geometric morphometric methods (Viscosi et al. 2009). In the last 2 decades, different types of genetic markers have been used for species delimitations (Petit and Excoffier 2009). Among them, nuclear microsatellite markers in combination with Bayesian assignment methods proved to be a good distinguishing tool among closely related oak species (Zeng et al. 2010; Curtu et al. 2011a).

Recently, increasing attention has been given to the variability of *Quercus pubescens*. Many studies were carried out in Italy (Bruschi et al. 2000; Viscosi et al. 2011), Hungary (Bartha 2001), Turkey (Borazan and Babaç 2003), Slovakia (Jedináková-Schmidtová et al. 2004), Croatia (Škvorc et al. 2005; Trinajstić, 2007), and Slovenia (Jerše and Batič 2007). By contrast, no detailed assessment of leaf and fruit morphology and genetic variation of *Q. pubescens* was done in Romania, where this species is reaching the northeastern border of its distribution range (Stănescu et al. 1997). The present forest area covered by oak forests in Romania is about 18% (Abrudan et al. 2009) and the genus *Quercus* L. is represented by 7 oak species sensu stricto, which belong to different sections and series. *Quercus pubescens* Willd. (pubescent oak) and *Q. virgiliana* Ten. (Italian oak) are 2 closely related oak taxa, which are included in the section *Dascia* Kotschy, series *Lanuginosae* Simk (Şofletea and Curtu, 2007). According to the botanical literature, *Q. virgiliana* can be found in Romania with *Q. pubescens* over nearly its entire distribution range, in the wood steppe areas with high temperatures and dryness (Sanda et al. 2004). In view of global warming, the 2 taxa may become very important tree species for forestry because of their adaptations to a more arid climate. In Romania, the utilization of the 2 taxa is stated in the National Forest Policy and Strategy, as a measure for reconstruction of degraded lands and conservation of the ecosystems in the wood steppe (Abrudan et al. 2009).

The aim of the present study was to find macromorphological leaf and fruit descriptors that discriminate between the 2 taxa and that can be used in field determinations. The oak individuals were initially identified in the field based on leaf, fruit, bark, and twig traits. We checked the presence of *Q. virgiliana* in complex with *Q. pubescens* in the sampled stands because we assume that the area occupied in Romania by *Q. virgiliana* was overestimated in the past. The genetic differentiation between the 2 taxa was estimated by means of highly polymorphic molecular markers, which discriminated very well between other European white oak species (Lepais et al. 2006; Curtu et al. 2011a).

2. Materials and methods

2.1. Plant material

In 2009 and 2010, 20 natural stands were sampled throughout the entire Romanian distribution range. Eight stands are located in the Carpathian Basin, namely Aiud (N46.37344, E23.58969, 556 m a.s.l.), Criş (N46.12240, E24.70857, 541 m a.s.l.), Dumbrăveni (N46.25495, E24.58168, 489 m a.s.l.), Hoia (N46.76920, E23.51099, 513 m a.s.l.), Mirăslău (N46.37350, E23.73154, 331 m a.s.l.), Petiş (N46.03513, E24.23512, 453 m a.s.l.), Săcălaia (N46.96560, E23.92908, 439 m a.s.l.), and Şuncuiuş (N46.95505, E22.52139, 512 m a.s.l.). The rest of the stands are located outside the Carpathian Basin, namely Clisura Dunării (N44.49972, E22.10611, 155 m a.s.l.), Comanca (N44.07776, E24.33258, 130 m a.s.l.), Deveselu (N44.06647, E24.36980, 111 m a.s.l.), Vlădila (N44.00480, E24.37756, 110 m a.s.l.), Berca (N45.26853, E26.73302, 254 m a.s.l.), Jugureni (N45.09205, E26.40553, 407 m a.s.l.), Răducăneni (N46.92676, E28.01289, 166 m a.s.l.), Bădeana (N46.16527, E27.57571, 173 m a.s.l.), Breana Roşcani (N45.91884, E27.99315, 194 m a.s.l.), Gârboavele (N45.56593, E28.01197, 89 m a.s.l.), Măcin (N45.22178, E28.25222, 216 m a.s.l.), and Ciucurova (N45.08277, E28.35129, 240 m a.s.l.). According to the literature (Sanda et al. 2004), in some of these stands (Bădeana, Breana Roşcani, Ciucurova, Clisura Dunării, Comanca, Deveselu, Gârboavele, Jugureni, and Măcin) the 2 taxa coexist. From each stand, 50 adult oak trees were sampled at a minimum distance of 30 m, to avoid relatedness as much as possible, except for Clisura Dunării (14 individuals), Ciucurova (16), and Gârboavele (38) stands.

In total, 918 oaks were mapped with a GPS Garmin 62s. Subsequently, 5 fully developed and undamaged leaves were chosen from every tree for the morphological analysis. Moreover, cupula peduncle collection was done. Five to 7 cupula peduncles for each individual were measured. In total, 4590 leaves and 3826 cupula peduncles were measured.

2.2. Morphological analysis

Leaf morphological assessment was done for all 918 oaks according to the protocol used in a study performed in Western and Central Europe to discriminate between *Q. robur* and *Q. petraea* (Kremer et al. 2002). Five dimensional characters (lamina length, petiole length, lobe width, sinus width, and length of lamina at largest width), 2 counted variables (number of lobes and number of intercalary veins), 2 observed variables (abaxial laminar pubescence and basal shape of the lamina), and 5 transformed variables (lamina shape, petiole ratio, lobe depth ratio, percentage venation, and lobe width ratio) were assessed. The abaxial laminar pubescence was evaluated according to the grading system described by Kissling (1977) with a stereomicroscope (×30) and basal shape of the

lamina was scored as an index varying from 1 to 9. The leaf dimensional traits were assessed using WinFOLIA software. In addition, for 754 trees the length of cupula peduncle was also assessed with a digital slide caliper (Würth, model CR 2032) (Table 1).

According to Bartha (2001), a first separation between *Q. pubescens* and *Q. virgiliana* was mainly based on average values for length of the cupula peduncle. However, other characters, which are described in the literature to be specific for the 2 taxa (i.e. lamina length, petiole length, intensity of abaxial laminar pubescence, type of scales for cupula) were also considered. Individuals with the average value for cupula peduncle length less than 0.8 cm in combination with specific leaf characters were classified as *Q. pubescens*, and the oaks with values of cupula length more than 1.5 cm, also in combination with specific leaf characters, were classified as *Q. virgiliana*. These threshold values for cupula length were established according to different dendrological descriptions. The rest of the trees with average values for length of cupula peduncle between 0.8 and 1.5 cm were considered to be specimens with intermediate morphology (Table 1). Mean, minimum, and maximum values of all morphological traits were calculated using STATISTICA v8 software (StatSoft 2008).

Table 1. Sample size in each oak stand.

Stand	Number of individuals…										
	assigned to…				for which the length of peduncle was assessed				used in genetic analysis		
	PUB	INT	VIR	Total	PUB	INT	VIR	Total	PUB	VIR	Total
Aiud	45	4	1	50	45	4	1	50	13	1	14
Bădeana	15	18	17	50	15	18	17	50	2	9	11
Berca	48	2	0	50	48	2	0	50	3	0	3
Breana Roșcani	49	0	1	50	17	0	1	18	2	1	3
Ciucurova	15	1	0	16	15	1	0	16	0	0	0
Clisura Dunării	14	0	0	14	0	0	0	0	0	0	0
Comanca	47	3	0	50	47	3	0	50	3	0	3
Criș	48	2	0	50	42	2	0	44	7	0	7
Deveselu	42	6	2	50	42	6	2	50	2	2	4
Dumbrăveni	45	5	0	50	33	5	0	38	10	0	10
Gârboavele	38	0	0	38	22	0	0	22	3	0	3
Hoia	37	8	5	50	37	8	5	50	1	2	3
Jugureni	50	0	0	50	0	0	0	0	0	0	0
Măcin	48	2	0	50	48	2	0	50	0	0	0
Mirăslău	42	6	2	50	42	6	2	50	4	1	5
Petiș	49	1	0	50	49	1	0	50	3	0	3
Răducăneni	40	7	3	50	40	7	3	50	3	1	4
Săcălaia	40	9	1	50	6	9	1	16	9	1	10
Șuncuiuș	30	11	9	50	30	11	9	50	3	7	10
Vlădila	45	4	1	50	45	4	1	50	2	1	3
Total	787	89	42	918	623	89	42	754	70	26	96

Footnote: PUB = *Q. pubescens*, INT = intermediate morphology, VIR = *Q. virgiliana*.

Statistical analyses were done with the same software. In analysis of variance (ANOVA) all differences were considered significant when P values were below 0.05. Cluster analysis with Ward's clustering method and Manhattan (city-block) distances was carried out. Because of the incomplete data set (no information about length of peduncle for 2 stands, Clisura Dunării and Jugureni), the analysis was performed for the rest of the 18 stands and only positive correlated dimensional traits were taken into account (i.e. leaf dimensional traits and length of cupula peduncle). Principal component analysis (PCA) was performed on a correlation matrix of 754 oak trees, for which the length of cupula peduncle was also assessed. The input files for ANOVA and PCA contained the mean values for each morphological descriptor and for every tree, while for cluster analysis the mean population values for each trait were used.

2.3. Genetic analysis

A subsample of 96 oak individuals out of the 918 individuals (Table 1) was genotyped at 7 microsatellite loci in order to evaluate the genetic differences between the 2 taxa. Among them, according to the morphological stratification presented above, 70 trees were *Q. pubescens*-like individuals and 26 oaks were *Q. virgiliana*-like individuals (Table 1). To identify genetic differences between the 2 taxa, no microsatellite analysis was carried out on individuals with intermediate morphology. Two reference populations of sessile oak and pedunculate oak were used in the assignment analysis to reduce the effect of unbalanced data due to the low number of individuals classified as *Q. virgiliana*.

DNA was extracted from winter buds using the Qiagen DNeasy 96 Plant Kit following the manufacturer's protocol, but without liquid nitrogen (Toader et al. 2009). Then the DNA was kept at –60 °C until use. Seven genomic SSRs (gSSRs) were amplified using polymerase chain reaction (PCR). The primers were combined into 2 PCR multiplexes on the basis of annealing temperature and fluorescent label. The first multiplexing reaction included 4 gSSRs (ssrQpZAG112, ssrQpZAG96, ssrQpZAG11, and ssrQpZAG110) and the second one only 3 (ssrQpZAG87, ssrQpZAG20, and ssrQpZAG7). More information about the 7 microsatellite loci is given in Table 2. The reactions were performed in a 10-µL volume containing 1 µL of template DNA (1:40), 2 µL of 5X PCR buffer, 0.90 µL of $MgCl_2$ (25 mM), 1 µL of dNTPs (2 mM), and 0.10 µL of Promega *Taq* DNA polymerase (5 U/µL). For primers' concentrations, see Table 2.

Amplification was carried out in an Eppendorf Master Cycler. The PCR profile was as follows: 3 min of denaturation at 94 °C, followed by 30 cycles of 45 s denaturation at 94 °C, a 35 s annealing step at 51 °C, a 1 min 50 s elongation step at 69 °C, and a final extension step at 69 °C for 15 min. The correct amplification of loci was checked by using 2 µL of PCR products mixed with 3 µL of dye and migrated on 1.5% agarose gels for 25 min at 100 V. Amplification products were run on a Beckman Coulter Genetic Analyzer using Frag-3 method and Size Standard 400. The products were then analyzed using Fragment Analysis Software using default parameters and PA ver. 1 dye correction (WellRED dye-labeled primers were used).

2.4. Genetic statistical data analysis

All 7 microsatellite loci were tested for genotyping errors due to nonamplified alleles, large allele dropout, and scoring of stutter peaks using MICRO-CHECKER 2.2.0.3 (Van Oosterhout et al. 2004). Genetic diversity was evaluated by calculating the number of alleles, and observed and expected heterozygosity (gene diversity) with GenALEx, version 6.4 software (Peakall and Smouse 2006). F_{ST} values were calculated for each locus and across loci with the same program. A hierarchical analysis of molecular variance (AMOVA) was done with the same program in order to determine the partitioning of molecular variance among the 2 analyzed taxa. In addition, allelic richness (Petit et

Table 2. Characteristics of the 7 microsatellite loci.

Locus	Nucleotide motif	Beckman dye	Primer concentration (uM)	Allele size (bp)
ssrQpZAG112	di	D4	0.20	82–112
ssrQpZAG96	di	D3	0.80	140–180
ssrQpZAG11	di	D3	0.60	242–289
ssrQpZAG110	di	D4	0.90	205–243
ssrQpZAG87	di	D3	0.55	103–183
ssrQpZAG7	di	D4	0.65	116–157
ssrQpZAG20	di	D3	0.80	159–213

al. 1998), which is a measure independent of the sample size, was calculated using FSTAT version 2.9.3.2 (Goudet 2002). GenePop software version 4.1 (Rousset 2008) was also used for estimating the number of null alleles, the number of common alleles, and the number of private alleles for each taxon.

The assignments of individuals to species were performed using the frequency based assignment test (Paetkau et al. 1995), available in GenALEx software and Bayesian clustering method implemented in STRUCTURE software, version 2.3.3 (Pritchard et al. 2000). Within STRUCTURE, 2 model approaches were used. The first one took into consideration the sampling location (LocPrior model), while the second one did not use any prior information about the taxa (the blind procedure). In addition, in order to obtain a better separation among the 2 pubescent oak taxa by using the STRUCTURE software and to diminish the effects that may occur as a result of unbalanced number of trees analyzed per taxon, 43 pedunculate oaks (*Q. robur*) sampled from Podul Iloaiei stand (Iaşi County) and 49 sessile oak (*Q. petraea*) individuals sampled from Cristian stand (Braşov County) were also used. Ten independent runs were performed for K (number of clusters) ranging from 1 to 5, when only the 2 pubescent oak taxa were taken into consideration (96 individuals). The maximum value of K was 7, when all 4 oak taxa were analyzed (188 individuals). In both cases, the program was run with correlated allele frequencies. The burn in was set to 100,000 steps followed by 200,000 iterations. The number of clusters (K) was estimated based on ΔK values, which is considered more precise than the output provided by the software STRUCTURE (Evanno et al. 2005). ΔK was estimated with the STRUCTURE HARVESTER program (Earl and Von Holdt 2011).

3. Results

3.1. Morphological and taxonomic assessment

Among the 754 individuals, only 42 trees (5.6%) showed a cupula peduncle longer than 1.5 cm and 623 trees (82.6%) showed one shorter than 0.8 cm (Table 1). These oak trees were classified as *Q. virgiliana* and *Q. pubescens*-like individuals, respectively. However, in addition to the most discriminating character (length of cupula), other particular leaf traits for one or another taxon were also considered for the classification. The remaining 89 trees (11.8%), for which the length of cupula peduncle ranged between 0.8 and 1.5 cm, were considered as individuals with intermediate morphology. The mean and standard deviation values for all sampled trees and separated for each taxon are given in Table 3.

Table 3. Mean and standard deviations of the 15 fruit and leaf descriptors for all sampled trees and separated for each taxon.

Descriptor	*Q. pubescens* (623 oaks)	*Q. virgiliana* (42 oaks)	All (918 oaks)
Abaxial laminal pubescence	4.2 ± 0.9	3.7 ± 1.1	4.2 ± 0.86
Basal shape of the lamina	4.0 ± 0.7	3.9 ± 0.7	4.0 ± 0.71
Number of lobes	9.7 ± 1.4	9.3 ± 1.6	9.5 ± 1.47
Number of intercalary veins	2.2 ± 1.3	2.2 ± 1.3	2.2 ± 1.3
Lamina length (mm)	82.1 ± 13.7	96.8 ± 21.2	83.5 ± 15.1
Petiole length (mm)	14.3 ± 3.4	14.9 ± 4.1	14.4 ± 3.58
Lobe width (mm)	29.2 ± 5.1	30.8 ± 5.7	29.2 ± 5.31
Sinus width (mm)	12.2 ± 3.9	15.1 ± 5.4	12.5 ± 4.18
Length of lamina at largest width (mm)	43.5 ± 8.4	51.6 ± 11.3	44.3 ± 9.15
Lamina shape	52.9 ± 5	53.5 ± 4	53.0 ± 5.06
Petiole ratio	14.8 ± 2.9	13.5 ± 3.1	14.8 ± 3.02
Lobe depth ratio	57.5 ± 12.2	50.8 ± 13.4	56.6 ± 12.27
Percentage venation	23.5 ± 14.1	23.9 ± 13.5	23.4 ± 13.95
Lobe width ration	35.7 ± 4.4	32.3 ± 4.3	35.3 ± 4.4
Length of cupula peduncle (mm)	4.0 ± 2.1	22.9 ± 8	5.8 ± 5.44

ANOVA revealed high phenotypic variability for the 2 oak taxa. Most of the variables differed significantly among taxa, stands, and trees within stands, respectively. In addition to the length of cupula peduncle, by using Levene's test for homogeneity of variances, it was found that "taxon" effect is statistically significant ($P < 0.05$) for another 4 variables (abaxial laminar pubescence, lamina length, sinus width, and length of lamina at largest width). The same variables and 2 more (i.e. lobe width and percentage venation) presented significant differences between stands for the F-test.

Two main groups were revealed in the cluster diagram (Figure 1). The first one is represented by stands (Breana Roşcani, Criş, Măcin, Berca, and Petiş) in which the oak trees have mainly smaller leaves, shorter petioles, and sessile or shorter cupula peduncles. The second is divided in 2 subgroups and the one from the right part of the diagram comprises stands in which individuals have longer leaves, petioles, and cupula peduncles (data not shown).

In PCA, the first 2 factors accounted for 41.99% of the total variance when all 15 variables were taken into account. Lamina length (19%), length of lamina at the largest width (19%), sinus width (18%), and lobe width (9%) were variables that gave the highest contribution to the first factor. No clear separation between the 2 taxa was revealed. The morphological groups corresponding to the 2 taxa were overlapping (Figure 2). Therefore, another PCA was performed with the variables that gave significant differences among taxa in ANOVA (i.e. abaxial laminar pubescence, lamina length, sinus width, length of lamina at largest width, and length of cupula peduncle). In this case, the first 2 principal components explained 69.71% of the whole variability. It should also be noted that *Q. virgiliana*-like individuals tended to separate themselves and to form a distinct group (Figure 3).

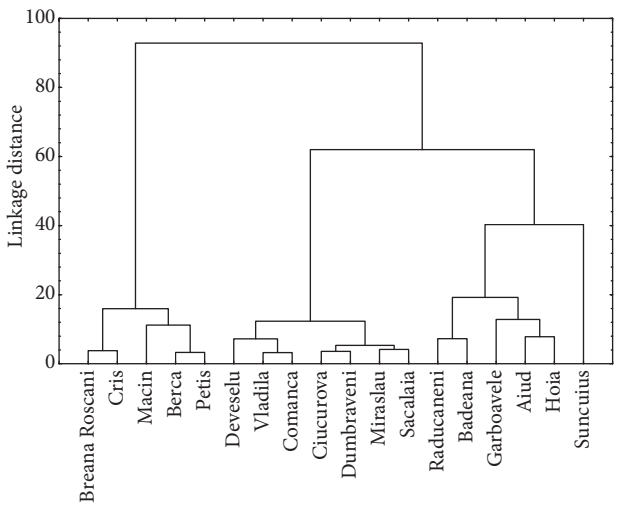

Figure 1. Cluster diagram.

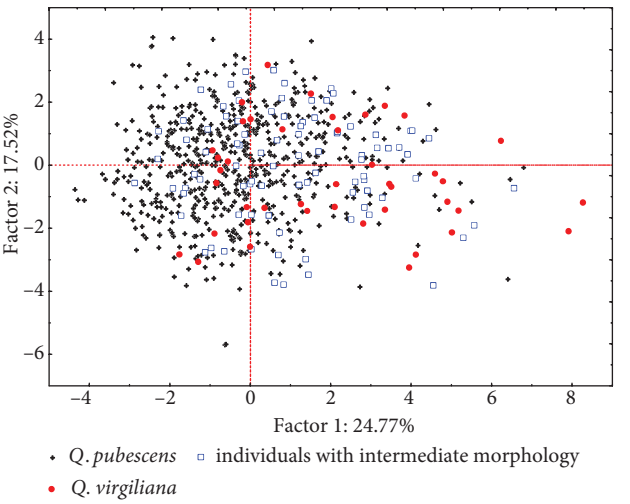

Figure 2. PCA diagram (15 variables).

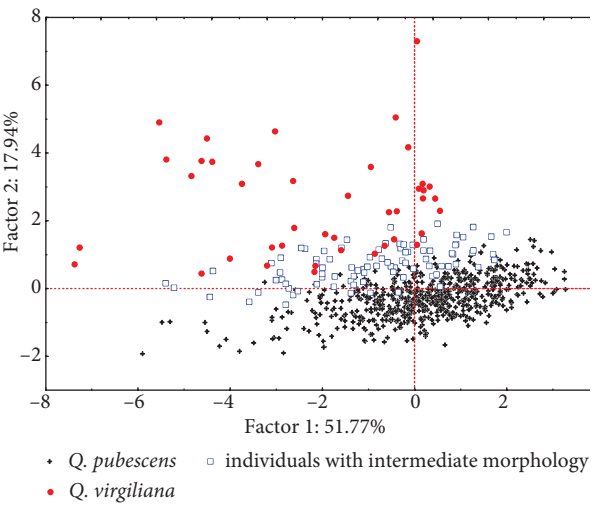

Figure 3. PCA diagram (5 variables: abaxial laminar pubescence, lamina length, sinus width, length of lamina at largest width, and length of cupula peduncle).

3.2. Microsatellite analysis

All microsatellite loci were highly polymorphic in both taxa (Table 4). A total of 157 alleles were observed over the 7 analyzed SSR loci. The mean number of alleles per locus was 22 and ranged from 14 (locus ssrQpZAG87) to 40 (locus ssrQpZAG11). Among them, there were alleles that seemed to be specific to *Q. pubescens* and *Q. virgiliana*, but only at a low frequency (<0.05). The highest number of private alleles was recorded at locus ssrQpZAG11, with 19 private alleles for *Q. pubescens* and 5 for *Q. virgiliana*. Overall, GenePop software indicated that the mean frequency of private alleles was only 0.018.

MICRO-CHECKER found no evidence for large allele dropout or scoring errors due to stuttering. Instead, the

Table 4. Genetic parameters estimated at the 7 microsatellite loci.

Locus	Taxon	N_a	N_e	A	H_o	H_e
ssrQpZAG112	*Q. pubescens*	14	5.8	11.18	0.857	0.828
	Q. virgiliana	12	6.6	11.67	0.808	0.849
ssrQpZAG96	*Q. pubescens*	22	14.8	17.44	0.899	0.932
	Q. virgiliana	15	11.2	14.87	0.84	0.91
ssrQpZAG110	*Q. pubescens*	22	6	14.88	0.825	0.833
	Q. virgiliana	12	3.9	12	0.583	0.741
ssrQpZAG11	*Q. pubescens*	35	12.6	21.52	0.809	0.921
	Q. virgiliana	21	15.2	20.71	0.64	0.934
ssrQpZAG87	*Q. pubescens*	13	3.9	9.02	0.729	0.744
	Q. virgiliana	7	2.7	7	0.708	0.631
ssrQpZAG7	*Q. pubescens*	21	12.7	16.49	0.871	0.921
	Q. virgiliana	16	11	15.79	0.88	0.909
ssrQpZAG20	*Q. pubescens*	18	7.1	13.32	0.87	0.859
	Q. virgiliana	15	6.5	15	0.917	0.846
	Mean *Q. pubescens*	21	9	14.79	0.837	0.862
	Mean *Q. virgiliana*	14	8	13.86	0.768	0.832
	Overall mean	17,3	8.5	14.32	0.802	0.847

Footnote: N_a = number of alleles, N_e = effective number of alleles, A = allelic richness, H_o = observed heterozygosity, H_e = expected heterozygosity.

software reported that null alleles could be present at locus ssrQpZAG11. The frequencies of these nonamplified (null) alleles ranged from 5.5% in the case of *Q. pubescens,* to 17.8% for *Q. virgiliana*. The latter value could be explained by the limited sample size, and so we decided not to exclude locus ssrQpZAG11 from the analysis.

Allelic richness, which is independent of population size, was slightly higher in *Q. pubescens* than in *Q. virgiliana* (Table 4). The other genetic parameters showed the same trend, but all parameters might be influenced by the lower sample size in *Q. virgiliana*, especially when using microsatellite markers that have large numbers of alleles per locus. All loci showed very small F_{ST} values. Only locus ssrQpZAG20 showed a relatively high F_{ST} value (4.5%). The overall value for F_{ST} between the 2 taxa was only 0.01, a value that indicates almost no genetic differentiation between the studied oak taxa. Moreover, this very low level of divergence was also confirmed by AMOVA, which revealed that the genetic differentiation between the 2 taxa was only 0.02 ($P < 0.05$).

By using the genetic assignment procedure implemented in the software GenALEx ver. 6.4, no separation between the 96 analyzed pubescent oaks resulted. This was further supported by the Bayesian clustering analysis implemented in the software STRUCTURE, which did not allow a clear delineation of the 2 taxa, with or without a priori grouping of individuals to taxa. In the case of the 188 individuals, the STRUCTURE HARVESTER program determined that 3 genetic clusters (K) best fit the data. This is in agreement with the existence of 3 morphological groups, each of them corresponding to pedunculate oak, sessile oak, and pubescent oak-Italian oak individuals, respectively.

4. Discussion

In general, the leaf morphological survey indicated that there are small differences between the sampled stands from Romania and others from the rest of Europe. With respect to lamina length, it can be observed that the total mean value for all 918 trees (83.5 mm) is higher than the values reported in recent studies done on *Q. pubescens* in Italy (Bruschi et al. 2000), Croatia (Škvorc et al. 2005; Franjić et al. 2006), and Slovenia (Jerše and Batič 2007). In this context, the slightly larger size of leaves in some stands from Romania could be due to the presence of the 42 Italian oaks and 89 intermediate individuals between the 2 taxa in the sampled stands, or by the specific habitat conditions at the northern natural distribution range of pubescent oak. Kleinschmit (1993) reported the existence of adaptive reactions in European oaks, which are driven by environmental conditions. It was also reported that the transition from glabrous to pubescent leaves is due to the environmental conditions related mainly to soil water content (Drake and Muller-Bombois 1993). Thus, more densely pubescent oak individuals are generally located in drier sites. This was also observed during our field exploration. Smaller but hairier leaves were sampled in drier sites.

Compared to the values from other studies (Dupouey and Badeau 1993; Bruschi et al. 2000), the mean number of lobes is smaller in our study. In contrast, the average value that we obtained for petiole length is higher than those reported in Croatia (Škvork et al. 2005; Franjić et al. 2006) and Slovenia (Jerše and Batič 2007). According to Dupouey and Badeau (1993), the pilosity and number of intercalary veins are very important discriminant morphological descriptors between *Q. robur*, *Q. petraea*, and *Q. pubescens*. In our study, the mean value for all stands for number of intercalary veins is 2 times higher than that in France (Dupouey and Badeau 1993), indicating a high level of leaf irrigation at the northern natural distribution range of *Q. pubescens*. Instead, the values found for the length of cupula peduncle are smaller than those reported for *Q. pubescens* from similar studies in France (Dupouey and Badeau 1993) and Slovenia (Jerše and Batič 2007).

In a similar study on leaf morphology of the *Q. robur*–*Q. petraea* complex (Ponton et al. 2004), it was shown that leaf characters are not independent from each other as shown by a positive correlation between them. In addition, our findings suggest that there is also a positive correlation between leaf dimensional characters and length of cupula peduncle. However, taxonomic determinations, which use a combination of characters (lamina length, lobe width, petiole length, length of cupula peduncle) proved to be informative, but other relevant characteristics should be taken into account.

With regard to PCA, the lack of separation of *Q. virgiliana* from *Q. pubescens* was also reported in Turkey (Borazan and Babaç 2003). However, using only 5 descriptors, a better separation of the 2 taxonomic groups represented by pubescent oak and Italian oak individuals was achieved. Between them were interposed most of the individuals with intermediate characters for specific descriptors that differentiate the 2 taxa. These individuals with intermediate morphology (11.8%) could be putative hybrids. Therefore, the mixed stands with a high number of individuals displaying intermediate morphology (e.g., Şuncuiuş, Bădeana, Gârboavele, Aiud, and Hoia) can be considered valuable stands under the assumption that the high level of morphological variability corresponds to an increased genetic diversity. However, this was not shown by our small set of SSR markers. Morphological stratification in the 3 groups revealed that *Q. virgiliana* leaves are on average less hairy than those of *Q. pubescens*, but the intermediate group is very close to *Q. pubescens*. Since the density of hairs is associated with drought resistance (Stănescu et al. 1997; Bruschi et al. 2000) and the Italian oak is less xerophyllous than the pubescent oak (Bartha 2001; Doniță 2008), there is a correlation between morphological criteria used in the present study to separate the 2 taxa and their ecological requirements.

Therefore, based on macromorphological descriptors, *Q. virgiliana*-like individuals could not be unambiguously distinguished from the *Q. pubescens* individuals mainly because of the existence of many intermediate morphological forms between them.

The higher values of genetic diversity measures observed in *Q. pubescens* than in *Q. virgiliana* can be explained by the smaller sample size of the latter. *Q. pubescens* and *Q. virgiliana*-like individuals share the most frequent alleles at the 7 loci. The results based on our set of genetic markers do not support the existence of 2 species, but rather suggest that *Q. virgiliana* is an intraspecific taxonomic unit of pubescent oak. This hypothesis was also confirmed by the very low level of divergence in AMOVA (0.02), which could also mean that they cannot be distinguished. Compared to our results, by using a similar number of microsatellite markers for other related oak species, higher genetic differentiation among the 2 studied species was obtained for *Q. petraea*–*Q. pubescens*, 0.059 (Bruschi et al. 2000), or for *Q. pubescens*–*Q. frainetto*, 0.062 (Curtu et al. 2011a). This high differentiation is mainly due to a few outlier loci, which are potentially under divergent selection. However, until markers located in genomic regions under divergent selection are applied, genetic differences between *Q. pubescens* and *Q. virgiliana* individuals are not excluded.

Moreover, the genetic assignment tests, based on the allele's frequencies or on Bayesian method, revealed only 1 genetic cluster corresponding to the 2 taxa. The results obtained for the observed heterozygosity are consistent

with those reported for *Q. pubescens* in France (Lepais et al. 2006) and with those reported for other white oak species, such as sessile oak or pedunculate oak in stands from Greece, Bulgaria, and Germany (Neophytou et al. 2010).

In conclusion, our morphological survey supports the hypothesis that *Q. virgiliana* is not a separate species, but rather an intraspecific taxonomic unit of *Q. pubescens*. However, a very low molecular divergence between the 2 taxa was found. This result has to be treated with caution because our investigations were confined to only one region of occurrence of *Q. virgiliana* individuals. The analysis of additional markers located in differentiation hot spots of the oak genome is necessary to test whether *Q. pubescens* and *Q. virgiliana* are separate species.

Acknowledgments

This study was supported by the Sectoral Operational Program Human Resources Development (SOP HRD), financed from the European Social Fund, and by the Romanian Government under the contract number POSDRU/88/1.5/S/59321 and by CNCS-TE-73-2010 project. We are grateful to Andras Tothpal and numerous friends and colleagues from the Forest Districts for supporting us during the fieldwork. We also thank Viorel Roşca, park director, and Bogdan Băjenariu, biologist at the National Park Munţii Măcinului, for allowing us to collect samples of pubescent oak. The authors would like also to thank the 2 anonymous reviewers for their constructive comments, which helped to improve the manuscript.

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