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# Genetic Differentiation between *Quercus frainetto* Ten. and *Q. pubescens* Willd. in Romania

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**Abstract**

Little is known about genetic differences among *Quercus frainetto* and *Q. pubescens*, two species of section *Dascia* Kotschy (subgenus *Lepidobalanus*, white oaks) that reach in Romania the margins of their natural distribution range. A set of genomic SSRs (simple sequence repeats) and EST (expressed sequence tags)-SSRs was used to estimate the genetic differentiation among four natural populations of the two species. *Q. pubescens* had higher values of genetic diversity than *Q. frainetto*, although the differences were not significant ($P>0.05$). Two out of seven marker-loci, QrZAG112 and QpZAG110, displayed very high $F_{ST}$ values. Averaged across loci, the genetic differentiation was high and significant ($F_{ST} = 0.067$, $P<0.05$). Genetic distances were much higher among species than among populations within species. A Bayesian analysis indicated that two is the most appropriate number of genetic clusters. Using a blind procedure (i.e. based on multilocus genotypes only) the vast majority of sampled individuals (90%) could be assigned to the cluster corresponding to their phenotypes. When information about sampling localities was introduced in the assignment test, all individual trees were correctly classified. The higher degree of admixture in *Q. frainetto* as compared to *Q. pubescens* may be explained by different rates of introgressive hybridization.

***Keywords:*** expressed sequence tags (EST), genetic differentiation, oaks, *Quercus frainetto, Quercus pubescens*, simple sequence repeats (SSRs)

## Introduction

The issue of genetic differentiation among closely related species is a central research theme because it provides insights into the evolutionary processes responsible for species divergence. Genus *Quercus* is a long-standing model for studying population divergence, species delimitation and natural hybridization (e.g. Curtu *et al.*, 2009; Gailing *et al.*, 2007; Peñaloza-Ramírez *et al.*, 2010; Zeng *et al.*, 2010). In Europe, most of the studies have focused on genetic differentiation and identification of highly discriminating genomic regions between the most common oak species: *Q. robur* and *Q. petraea* (Jensen *et al.*, 2009; Muir and Schlötterer, 2005; Neophytou *et al.*, 2010; Scotti-Saintagne *et al.*, 2004). By contrast, little is known about genetic differences between the two oak species from Section *Dascia* Kotschy (subgenus *Lepidobalanus* or white oaks), *Q. frainetto* and *Q. pubescens*, although they have a high ecological and economic value in the south-eastern part of the European continent (Abrudan *et al.*, 2009; Ioras *et al.*, 2009).

The actual distribution of *Q. frainetto* in Romania comprises approximately 130 000 ha, 2% of the total forest cover, as much as *Q. robur* (Sofletea and Curtu, 2007). *Q. frainetto* and *Q. pubescens* are elements of the (sub-)Mediterranean flora that reach in Romania the northern limit of their distribution range. *Q. frainetto* is a meso-xerophilous species which can grow on heavy clay soils. *Q. pubescens* is better adapted to xeric conditions, being found on dry sites, such as on limestones and sunny slopes (e.g. in Transylvania) or in the wood steppe in southern Romania. Both species can be easily identified using leaf and fruit characters (Schwarz, 1993).

Few studies have investigated genetic differences between *Q. frainetto* and *Q. pubescens*, or between them and other oak species. Chloroplast DNA markers were found to be polymorphic, but could not discriminate among species (Moldovan *et al.*, 2010; Petit *et al.*, 2002). Highly polymorphic nuclear markers, such as microsatellites (SSRs –simple sequence repeats), are more promising for differentiating among oak species (Curtu *et al.*, 2007; Fortini *et al.*, 2009). Very recently, microsatellite markers have been developed from expressed sequence tags (EST) or sequences of messenger RNA starting from a *Q. robur/Q. petraea* library (Durand *et al.*, 2010). In contrast to genomic SSRs, EST-SSRs have the advantage of being located within the expressed portion of the genome, and thus reflect differences at gene level.

The aim of this study was to estimate the genetic differentiation between the two oak species of section *Dascia* that occur in Romania. It was tested whether genotypic information from a set of seven microsatellite markers is

sufficient to identify the species at population and individual level.

## Materials and methods

*Sampling strategy*

Two populations for each species, *Q. pubescens* and *Q. frainetto*, were selected at four sites across Romania. Collections were made in 2010 from adult individuals at Măcin-Tulcea (45°13'N, 28°14'E) and Săcălaia-Cluj (46°57'N, 23°56'E) for *Q. pubescens*, and Seaca Optășani-Olt (44°43'N, 24°28'E) and Lugoj-Timiș (45°43'N, 21°59'E) for *Q. frainetto*. The total number of trees sampled was 125: 62 *Q. pubescens* individuals (32 at Măcin-Tulcea and 30 at Săcălaia-Cluj) and 63 *Q. frainetto* individuals (31 at Seaca Optășani-Olt and 32 at Săcălaia-Cluj). Habitat data and GPS mapping data were collected at each site. Species identification was carried out following Sofletea and Curtu (2007).

*Genetic analysis*

DNA was extracted from buds using the Qiagen DNeasy96 Plant Kit following the manufacturer protocol, but without liquid nitrogen for material disruption. The DNA was then kept by -60°C until use. Five genomic SSRs (gSSRs) and two EST-SSRs were amplified using Polymerase Chain Reaction (PCR). Both gSSRs (Kampfer *et al.*, 1998; Steinkellner *et al.*, 1997) and EST-SSRs (Durand *et al.*, 2010) were developed from *Q. robur*/*Q. petraea* libraries. More information about the primer pairs, repeat motif and allele length is given in Tab. 1. The forward primer for each locus was fluorescently labelled with Beckman dyes (D2, D3 and D4). The primers were combined into two PCR multiplexes on the basis of annealing temperature and fluorescent label. The first multiplexing reaction included the five gSSRs and the second one the two EST-SSRs. The reactions were performed in a 20 µl volume containing approximately 10 ng template DNA ; 1x Promega colorless PCR buffer; 2 mM of $MgCl_2$; 0.45 mM of each dNTP (Fermentas); for each primer concentrations see Tab. 1; 1.15 U *Taq* DNA polymerase (Promega). Amplification was carried out in a *Corbett* Thermal Cycler. The PCR profile was as follows: 3 minutes of denaturation at 94°C followed by 31 cycles of 50 s denaturation at 94°C, a 40 s annealing step at 52°C for gSSRs (57°C for EST-SSRs), a 1 min 20 s elongation step at 70 °C and a final extension step at 70°C for 12 min. Amplification products were run on a Beckman Coulter Genetic Analyser using Frag-3 method and Size Standard 400. The products were then analyzed using Fragment Analysis Software using default parameters and PA ver1 dye correction.

*Statistical data analysis*

Microsatellite loci were tested for genotyping errors due to non-amplified alleles, large allele drop-out and scoring of stutter peaks using MICRO-CHECKER 2.2.0.3 (Van Oosterhout *et al.*, 2004). For each microsatellite locus and species, number of alleles, number of species-specific alleles, allele frequencies, observed and expected heterozygosity (gene diversity), unbiased estimates of Nei's genetic distances were calculated using the computer software GenAlEx version 6.4 (Peakall and Smouse, 2006). Using FSTAT version 2.9.3.2 (Goudet, 1995), allelic richness (El Mousadik and Petit, 1996; Petit *et al.*, 1998), a measure of the number of alleles that is independent of the sample size, was calculated. The smallest number of the individuals (n) for a locus (QrZAG39) in a species was set to 43. The differences between species were tested using a Student's *t*-test. 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) using the program ARLEQUIN ver 3.5.1.2 (Excoffier *et al.*, 2005) was employed to examine the partitioning of molecular variance into components: within populations, among populations within species and among species. $F_{ST}$ values were estimated for each locus and across loci. $F_{ST}$ values were tested by permuting individual genotypes among populations and species. P-values were calculated using 10 000 permutations.

The frequency-based assignment test (Paetkau *et al.*, 1995) available in GenAlEx v. 6.4 software was first used to assign individuals to species. For each individual, a log likelihood value was calculated for each species, using the allele frequencies of the respective species. An individual was assigned to the species with the highest log likelihood value.

Tab. 1. Characteristics of the seven investigated loci

| Type of marker | Locus | Nucleotide motif | Linkage group (LG) | Beckman Dye | Primer concentration (µM) | Allele size (bp) |
|---|---|---|---|---|---|---|
| Genomic SSRs (gSSRs) | QrZAG11 | di | 10 | D3 | 0.34 | 242-289 |
| | QrZAG39 | di | 5 | D2 | 0.28 | 105-169 |
| | QrZAG96 | di | 10 | D3 | 0.25 | 140-180 |
| | QpZAG110 | di | 8 | D4 | 0.18 | 205-243 |
| | QrZAG112 | di | 12 | D4 | 0.09 | 82-112 |
| EST-SSRs | GOT004 | di | 2 | D4 | 0.20 | 266-306 |
| | PIE040 | tri | - | D3 | 0.33 | 171-190 |

The Bayesian clustering method implemented in STRUCTURE software version 2.3.3 (Pritchard *et al.*, 2000) was further used to determine the genetic structure of the sampled populations. Two model approaches have been used. The first approach was a *blind procedure* that did not use any prior information about species and geographic location. The second one took into consideration the sampling location (with LocPrior model). This model is recommended when molecular data is not very informative to help the detection of population structure (Hubisz *et al.*, 2009). 20 independent runs were done for K, number of clusters, ranging from 1 to 5. The program was run with correlated allele frequency (Falush *et al.*, 2003). Each run consisted in 50 000 burn-in steps followed by $10^6$ iterations. To estimate the number of clusters (K), an *ad hoc* measure, $\Delta K$, which is based on the rate of change in the 'log probability of data' (L($K$)) between successive K values, was calculated (Evanno *et al.*, 2005). The software STRUCTURE HARVESTER (Earl, 2011) was used for $\Delta K$ estimation.

**Results and discussion**

The seven genomic SSR and EST-SSR markers have revealed high levels of polymorphism in both species (Tab. 2). However, a strong reduction in variability was observed at two genomic dinucleotide microsatellite loci, QrZAG112 and QpZAG110, in *Q. frainetto*. The two oak species share the most frequent alleles at the investigated loci. Nevertheless, numerous species-specific alleles (i.e. alleles that are found only in each population of one species) were also observed, but the vast majority of them are rare (relative frequency<0.05). Most of the rare alleles may simply be species-specific because of the limited sample size. Allelic richness is highly sensitive to sampling errors, especially at highly polymorphic microsatellite markers (Litt and Luty, 1989). Only five *Q. pubescens* and two *Q. frainetto* specific alleles with higher frequency (>0.05) were detected in the four oak populations. Allele 92bp (Fig. 1a) at locus QrZAG112 (frequency=0.17) and allele 245bp at locus QrZAG11 (frequency=0.10) are among the five specific allele for *Q. pubescens*. By contrast, *Q. frainetto* specific alleles have been detected in the EST regions: allele 184bp at locus PIE040 (frequency=0.10) and allele 304bp (frequency=0.06) at locus GOT004.

No evidence for scoring errors due to stuttering and large allele drop-out was found in the microsatellite data set. However, Micro-checker software indicated that null (non-amplified) may be present at two marker-loci: QrZAG39 and GOT004. Interestingly, null alleles were also reported at locus QrZAG39 in *Q. robur* and *Q. petraea* (Neophytou *et al.*, 2010). The high number of alleles detected at locus GOT004 (see Tab. 2) which differs only 1 base pairs (bp) in length, although the repeat motif is dinucleotide $(TG)_n$, supports the assumption of high mutation rates in the flanking regions of the microsatellite sequence including the primer's binding site.

Mean values of genetic diversity measures were higher in *Q. pubescens* than in *Q. frainetto*, but the differences are not significant (P>0.05) (Tab. 2). Higher allelic richness and gene diversity in *Q. pubescens* might be explained by the greater propensity of this species to hybridize with other white oak species (*Q. petraea*, *Q. robur*) as compared to *Q. frainetto*. Indeed, in a four-oak-stand (Bejan-Deva) in west-central Romania, there was evidence for more hybrids between *Q. pubescens* and the three other species than hybrids that have *Q. frainetto* as parental species (Curtu *et al.*, 2007). Some hybrid combinations that involve *Q. frainetto*, such as *Q. frainetto* x *Q. robur*, were extremely rare in the same mixed stand. Since *Q. pubescens* and *Q. frainetto* have very similar proportions in the Bejan Forest, the differences in the hybridization rates were not influenced by their relative abundance (Lepais *et al.*, 2009). Therefore, the reproductive barriers seemed to be stronger in *Q. frainetto* than in *Q. pubescens*. Moreover, *Q. pubescens* hybridizes extensively with *Q. petraea* in other parts of the natural range (e.g. in Italy, Salvini *et al.*, 2008).

Two out of seven loci displayed very high $F_{ST}$ values (Tab. 3). The two loci are genomic SSRs markers. However, one of the EST-SSR loci, PIE004, also shows a relatively high $F_{ST}$ value. Interestingly, locus QrZAG112 (Fig. 1) discriminated also very well between *Q. petraea* and *Q. robur* (Scotti-Saintagne *et al.*, 2004). Not the same situation was found for another outlier locus between *Q. pe-*

Tab. 2. Diversity measures of the seven microsatellite loci investigated in *Q. pubescens* (PUB) and *Q. frainetto* (FRA)

| Locus | $N_a$ | | A | | $H_o$ | | $H_e$ | |
|---|---|---|---|---|---|---|---|---|
| | PUB | FRA | PUB | FRA | PUB | FRA | PUB | FRA |
| QrZAG11 | 18 | 10 | 17.1 | 9.0 | 0.755 | 0.545 | 0.844 | 0.610 |
| QrZAG39 | 21 | 21 | 20.1 | 21.0 | 0.796 | 0.744 | 0.929 | 0.922 |
| QrZAG96 | 20 | 17 | 19.6 | 16.2 | 0.880 | 0.855 | 0.929 | 0.903 |
| QpZAG110 | 15 | 12 | 14.4 | 11.3 | 0.788 | 0.474 | 0.853 | 0.462 |
| QrZAG112 | 11 | 5 | 10.7 | 4.6 | 0.810 | 0.194 | 0.819 | 0.181 |
| GOT004 | 24 | 25 | 23.8 | 23.4 | 0.844 | 0.857 | 0.937 | 0.940 |
| PIE040 | 10 | 12 | 9.9 | 10.9 | 0.739 | 0.732 | 0.701 | 0.686 |
| Average | 17.0 | 14.6 | 16.5 | 13.8 | 0.802 | 0.629 | 0.859 | 0.672 |

Note: Na –number of alleles, A – allelic richness after rarefaction (n=43 individuals), Ho – observed heterozygosity, He – expected heterozygosity.

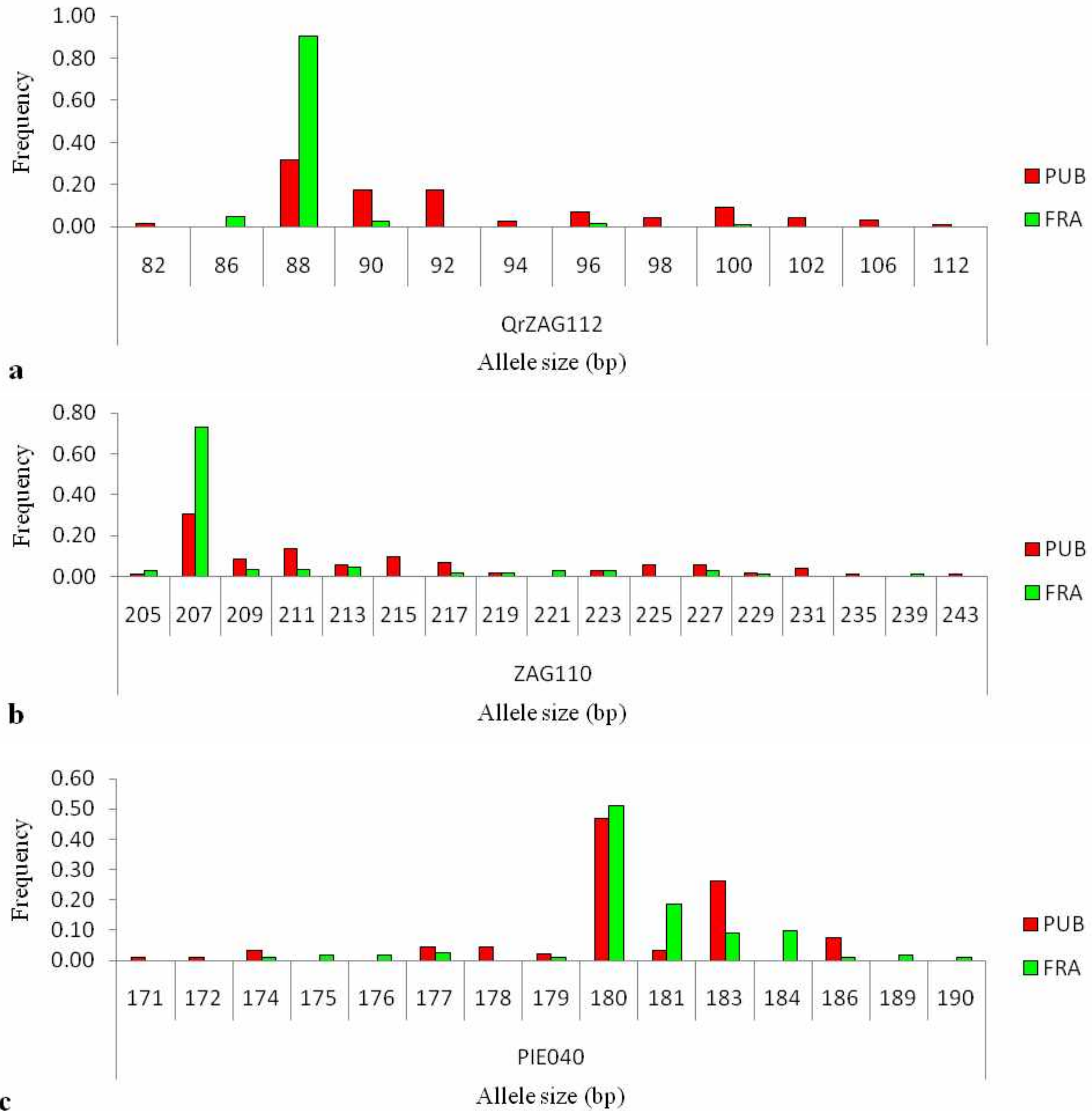


Fig. 1. Allele frequency spectra at loci with high FST values: genomic SSRs (a and b) and EST-SSRs (c). Abbreviations: PUB – *Q. pubescens,* FRA – *Q. frainetto*, bp – base pairs

Tab. 3. Pairwise $F_{ST}$ values between *Q. pubescens* and *Q. frainetto* for the seven microsatellite loci

| Locus | $F_{ST}$ value | P value (10 000 replicates) |
|---|---|---|
| QrZAG112 | 0.290 | 0.00 |
| QpZAG110 | 0.130 | 0.00 |
| QrZAG11 | 0.049 | 0.00 |
| PIE040 | 0.041 | 0.00 |
| GOT004 | 0.010 | 0.01 |
| QrZAG96 | 0.009 | 0.02 |
| QrZAG39 | 0.002 | 0.43 |
| All | 0.067 | 0.00 |

*traea* and *Q. robur* (QrZAG96) that shows an extremely low value for $F_{ST}$ in the present investigation (Tab. 3). Loci with high $F_{ST}$ values are very likely situated in genomic regions under selection (Lexer *et al.*, 2006; Neophytou *et al.*, 2010; Scotti-Saintagne *et al.*, 2004). Genetic differentiation between both species was high and significant ($F_{ST}$=0.067; P<0.05), when all loci where considered jointly. Significant differences between *Q. frainetto* and *Q. pubescens* were also reported for a set of five genomic SSRs in Italy (Fortini *et al.*, 2009).

The four populations cluster according to species rather than geographic origin (Fig. 2). The genetic distance

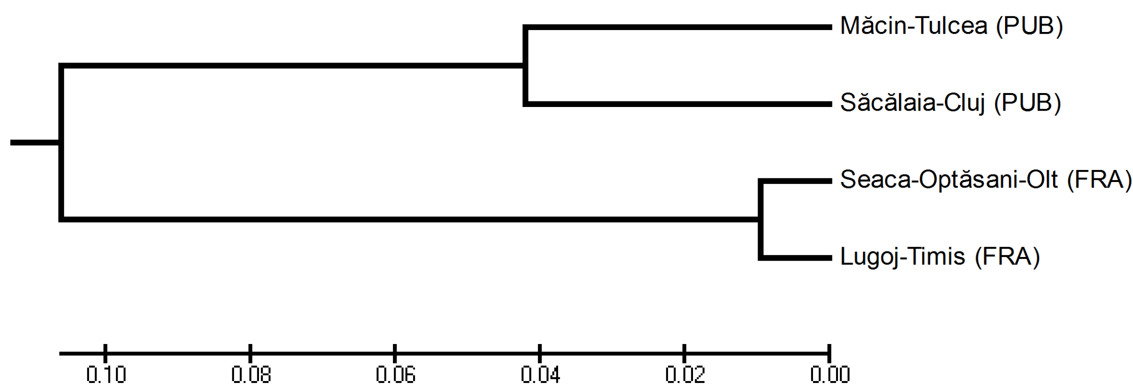


Fig. 2. UPGMA dendrogram based on unbiased estimates of Nei's genetic distances between *Q. pubescens* (PUB) and *Q. frainetto* (FRA) populations at seven microsatellite loci

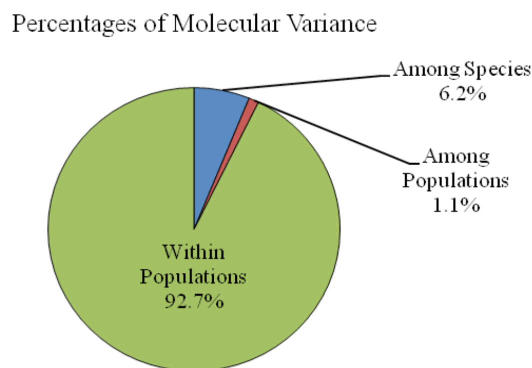


Fig. 3. Results of Analysis of Molecular Variance (AMOVA)

among *Q. frainetto* populations is much smaller than that among *Q. pubescens* populations, which is consistent with the geographic distances among populations. Moreover, in accordance with AMOVA, the genetic variation among *Q. pubescens* and *Q. frainetto* is nearly six fold higher than the variation among populations within species (Fig. 3).

Using a genetic assignment procedure implemented in software GenAlEx ver. 6.4., the taxonomic status of the sampled individuals, that had either a *Q. pubescens* or *Q. frainetto* phenotype, was determined. In 96% of the cases, the molecular data indicated the correct status (Fig. 4). Only five individuals, four *Q. pubescens* and one *Q. frainetto*, were not correctly assigned. In the Bayesian analysis, the uppermost level of structure corresponds to two clusters (Fig. 5). Each species was represented by one cluster

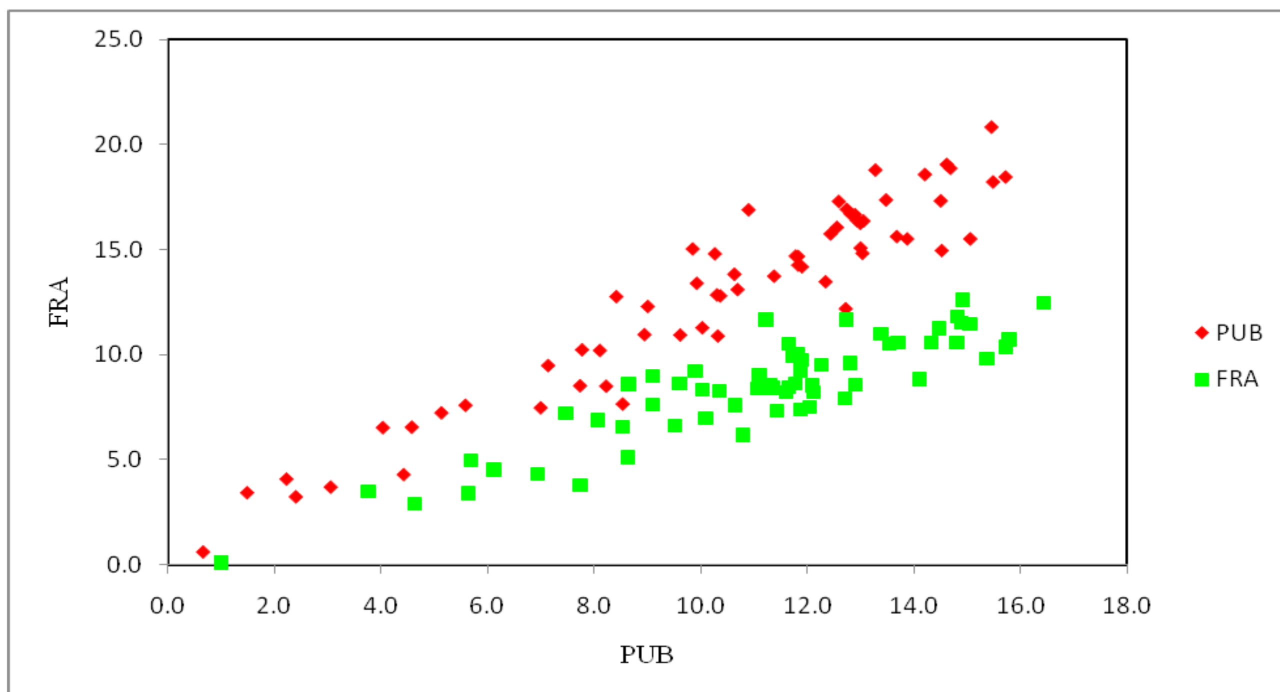


Fig. 4. Genetic assignment of *Q. pubescens* (PUB ) and *Q. frainetto* (FRA) samples by using the frequency-based test implemented in the software GenAlEx ver. 6.4

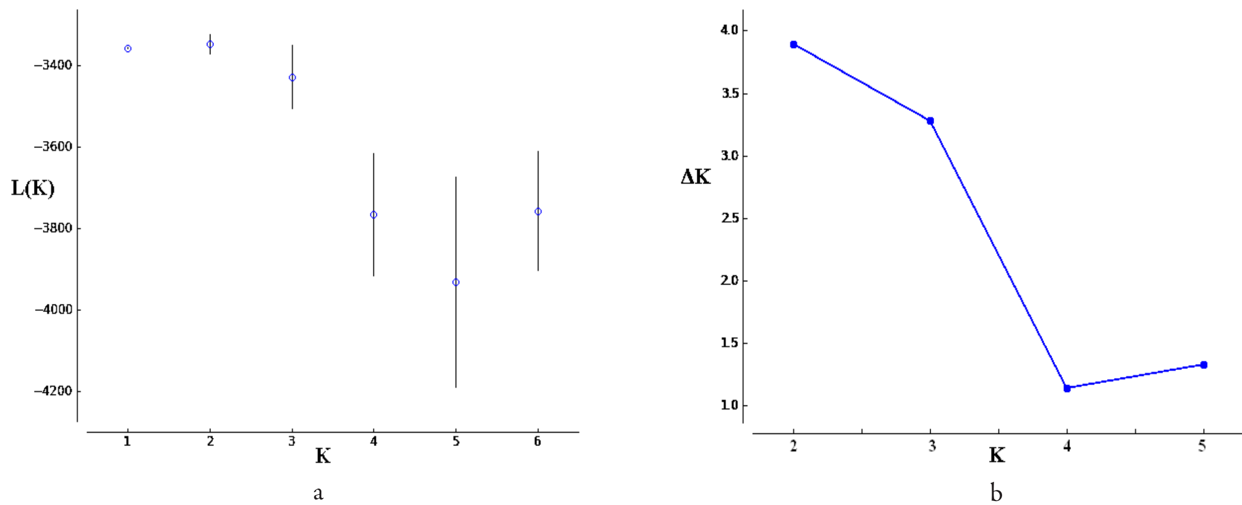


Fig. 5. a - Mean L(K) (±SD) over 20 runs for each K value; b - ΔK calculated as $\Delta K = m|L''(K)|/s[L(K)]$. The modal value of this distribution is the true K or the uppermost level of structure, here two clusters

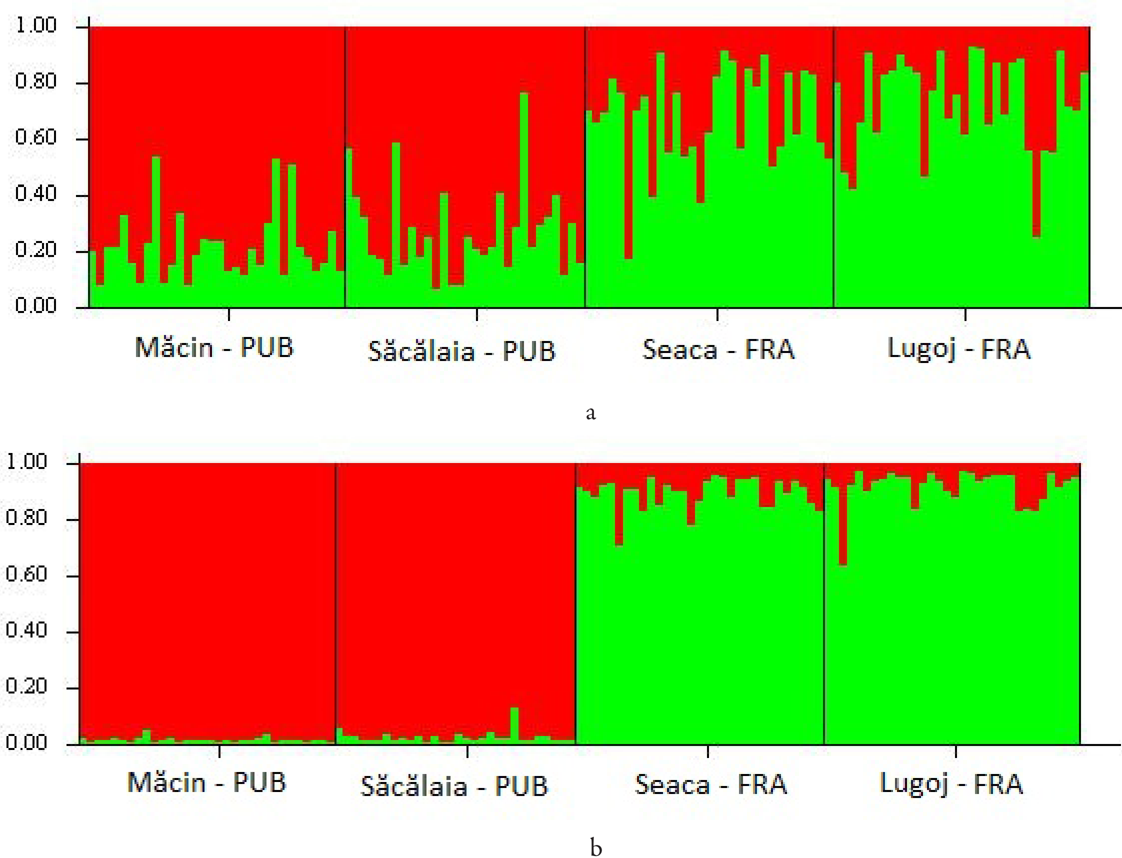


Fig. 6. Structure clustering results obtained for K=2 clusters with: a – no prior information, b – LocPrior model. Each individual is represented by a thin vertical bar partitioned into two color segments proportional to its membership in each genetic cluster. The four populations of *Q. pubescens* (PUB) and *Q. frainetto* (FRA) are separated by black lines and identified at the bottom

and the admixture coefficient (Q), corresponding to the assignment probability of each individual to each cluster, was used to infer the species status. There was a clear correspondence between the genetic cluster and the species designation (Fig. 6). When no prior information was used in the assignment procedure, 90% of the individuals had the highest admixture coefficient (Q>0.50) for the genetic group corresponding to their phenotype (Fig. 6a). The percentage reaches 100% if information about the sampling localities is considered in the admixture model (Fig. 6b).

Among the individuals that were not correctly classified (10%) using the *blind procedure*, no one had a probability larger than 0.85 for the wrong cluster. Most of them had coefficients of admixture between 0.50 and 0.60, a proportion which usually correspond to hybrids, although only pure stands for both species have been sampled. Two hypotheses may explain the wrong assignment of the 13 individuals: (1) the molecular data was not informative or (2) these individuals are natural hybrids. The first hypothesis is supported by the absence (at Săcălaia-Cluj) and low-density (at Măcin-Tulcea) of *Q. frainetto* individuals in the vicinity of the two *Q. pubescens* populations. Moreover, closely related oak species, which have recently diverged, share ancestral polymorphism (Muir and Schlötterer, 2005). On the other hand, hybridization events are more likely in the two *Q. frainetto* stands since *Q. pubescens* populations are mentioned to occur in their proximity (Sofletea and Curtu, 2007). Indeed, the degree of admixture was larger for *Q. frainetto* populations than for *Q. pubescens* populations (0.30 versus 0.25), which suggests more introgression in *Q. frainetto*. However, hybridization by long distance pollen dispersal may explain the occurrence of hybrids between the two species in apparently isolated *Q. pubescens* populations. The same hypothesis was invoked for the presence of hybrids with *Q. pyrenaica* and *Q. pubescens* in a mixed stand consisting only of *Q. robur* and *Q. petraea* trees, although the nearest *Q. pyrenaica* and *Q. pubescens* populations are localised tens of kilometres away (Lepais *et al.*, 2009).

**Conclusions**

Higher values of genetic diversity measures were observed in *Q. pubescens* than in *Q. frainetto*. Two out of seven microsatellite loci discriminated very well between the two species. The molecular analysis demonstrates that *Q. pubescens* and *Q. frainetto* can be unambiguously designated at population level. Using seven microsatellite markers the species of an individual can be easily identified when sampling location is known. In the absence of any information about geographical location, the species can still be determined with a high probability.

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