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GENETIC DIVERSITY AND DIFFERENTIATION AMONG FOUR *QUERCUS ROBUR* AND *Q. PETRAEA* NATURAL POPULATIONS IN ROMANIA

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Abstract: *A set of non-genic SSRs (simple sequence repeats) and EST (expressed sequence tags)-SSRs was used to estimate the genetic diversity and differentiation among four natural populations of two native oak species: Quercus robur and Q. petraea. Similar values of genetic diversity were found in both species. Two out of ten loci, QrZAG112 and QpZAG110, displayed very high F_{ST} values. Averaged across loci, the genetic differentiation was high and significant (F_{ST} = 0.045, $p<0.05$). Genetic distances were much higher between species than between populations within species. A Bayesian analysis indicated that a few sampled trees may be the result of introgressive hybridization.*

Key words: *Quercus robur, Quercus petraea, expressed sequence tags (EST), microsatellites (SSRs), genetic differentiation, hybridization.*

1. Introduction

The genus *Quercus* has approximately 500 species [1], widely distributed across the Northern Hemisphere. However, compared with Central America and Southeast Asia, the diversity of species is lower in Western Palaearctic (Europe, North Africa and Western Asia). Among the 29 accepted species [19] in this ecozone, pedunculate (*Quercus robur* L.) and sessile oak [*Q. petraea* (Matt.) Liebl.] are the most widespread and important oak species, both economically and ecologically. Quite often these species are sympatric, due to widely overlapping distribution range. Recent genetic studies have shown that natural hybridization can occur in mixed oak stand (e.g. [7], [22], [26]), with evidence of unidirectional mating from male *Q. petraea* to female *Q. robur* [36]. This pattern can be due to a strong reproductive isolating mechanism between these two oak species [27] and it was proposed to explain the colonization of climax forest species *Q. petraea* via pollen flow into the range occupied by the postpioneer species *Q. robur* [36]. In spite of interspecific gene flow, the two taxa exhibit large morphological differences in acorn peduncle length and leaf shape [2], [24]. In addition, they grow under a wide range of climatic and edaphic conditions and show significant eco-physiological differences. Thus, *Q. robur* is more tolerant to light, water-logging and

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hypoxia [16], [33] and prefer nutrient rich soils with higher pH [20]. On the other hand, *Q. petraea* is more drought tolerant due to a better water use efficiency [37], generally occurring on better drained and more acidic soils [39]. This adaptation to different ecological niches even in sympatric areas was interpreted as evidence in maintaing species integrity [3].

Although at European level *Q. robur* and *Q. petraea* are among the most studied forest species using genetic markers, in Romania only few studies focused on them (e.g. [5], [6], [28]). However, no study in Romania investigated the genetic diversity and differentiation within and among these closely related species in multiple populations. Thus, the aim of this study was to fill the gap, using nuclear microsatellites as genetic markers.

2. Material and Methods

2.1. Plant Material

Two populations for each species, *Q. robur* and *Q. petraea*, were selected from their natural distribution at four sites across Romania. The sampling was made from adult individuals at Prejmer (Braşov county; 45°44′N, 25°44′E) and Podu Iloaiei (Iaşi county; 47°12′N, 27°16′E) for *Q. robur*, and Cristian (Braşov county; 45°38′N, 25°33′E) and Ronişoara (Maramureş county; 47°54′N, 23°55′E) for *Q. petraea*. A total number of 192 individuals were sampled: 94 *Q. robur* trees (48 and 46 at Prejmer and Podu Iloaiei, respectivelly) and 98 *Q. petraea* trees (51 and 47 at Cristian and Ronişoara, respectivelly). Species identification was carried out following [40] and [43].

2.2. Genetic Analysis

DNA was isolated from buds using the DNeasy96 Plant Kit (Qiagen) following the manufacturer's instructions and the protocol modified by [45]. DNA content was checked on NanoDrop 8000 spectrophotometer (Thermo Scientific) and the DNA concentration was adjusted for further applications. Eight potentially selectively neutral non-genic microsatellites (nuclear simple sequence repeats or nSSRs; ZAG type in this study) and two genic microsatellites (expressed sequence tag or EST-SSRs; PIE type in this study) were used in two multiplexing PCRs reactions. One set, named kit1, comprises the five following loci: ZAG9, ZAG11, ZAG96, ZAG110 and ZAG112 and the second one, named kit2, five others: ZAG7, ZAG20, ZAG87, PIE215 and PIE223 [10], [23], [42]. The following PCR profile for multiplex reactions was set up: an initial denaturation step at 94 ºC lasting 3 minutes followed by 30 cycles of 50 s denaturation at 95 ºC, a 1 min annealing step at 55 ºC, a 2 min elongation step at 72 ºC and a final extension step at 72 ºC for 15 min. PCR reactions were performed in a Peltier thermocycler (Corbett) and after that, PCR products were run in a 8800 Genetic Analyser (Beckman Coulter) capillary sequencer, using Frag-3 method and Size Standard 400. All genotypes were visually scored by analyzing the raw data, using default parameters and PA ver1 dye correction.

2.3. Statistical Data Analysis

The presence of null alleles, scorring errors due to stuttering and large allele dropout was checked using the Micro-Checker 2.2.3 [47] with 1000 randomizations and a 95% confidence interval. For each microsatellite locus, population and species, the number of alleles, number of species-specific alleles, allele frequencies, observed and expected heterozygosity (gene diversity), Wright's inbreeding coefficient (F_{IS}) were calculated using GenAlEx 6.5 [34]. Allelic richness, a

diversity measure that is not influenced by the sample size [12], [35] was calculated using the program FSTAT 2.9.3.2 [18], with a rarefaction of n = 37 individuals (the minimum population size found in Ronişoara at ZAG96 locus). Nei's minimum genetic distance [32] between each pair of populations was computed with the Populations software [25], using 200 replications for the bootstrap resampling. The resulting distance matrix was used to build a UPGMA dendrogram with MEGA 6 [44]. For genetic differentiation among populations and species, pairwise FST's were computed using the software ARLEQUIN 3.5.2.2 [15], with the significance test based on 10000 permutations. With the same software, a hierarchical Analysis of Molecular Variance (AMOVA, [14]) was employed to test how genetic variation is partitioned into the following components: among groups of populations (species in our case), among populations within groups and within populations.

A Bayesian clustering method implemented in the program STRUCTURE 2.3.3 [17], [38] was used to determine the genetic structure of the sampled populations. The program was set to run five independent runs of a burn-in period of 200000 iterations and 10^6 Markov chain Monte Carlo repetitions after burn-in for each value of K (K=1 to 6), with admixture and correlated allele models. We used both a blind procedure that did not use any prior information about species and geographic location and the LocPrior model that took into consideration the sampling location. To determine the optimal number of groups (K), the highest posterior probabilities of data and the calculation of an ad hoc statistic, ΔK, was used according to [38] and [13], respectivelly. The online platform Structure Harvester [11] was used for ΔK estimation. Trees assigned to pure species were defined as having a proportion of ancestry above 0.9 in one cluster ($Q \geq 0.900$) while individuals with intermediated admixture coefficient ($0.100 \geq Q \geq 0.900$) were assigned as hybrids sensu lato.

3. Results and Discussions

No evidence for null alleles, nor scoring errors due to stuttering and large allele drop-out was found in the microsatellite data set. These findings are in accordance with genetic studies that used subsets of the same microsatellites [4], [8], [9], [21], [30]. Within populations, levels of genetic variation differed markedly among loci (Table 1). High levels of polymorphism in both species were found at all loci. A total number of 235 alleles in 192 multilocus genotypes were identified. However, the EST-SSRs had fewer alleles compared with non-genic microsatellites. Thus, the former had between 11 and 12 alleles and the latter showed between 16 and 47 alleles. The two species shared only 51.5% of total alleles, the remainining been private to one of the species. However, only six private alleles had frequencies >0.05. The vast majority of shared alleles had similar allelic frequencies in both species. Nevertheless, there are few alleles that showed high interspecific difference in allelic frequencies. Thus, 10 alleles from seven loci (ZAG7, ZAG11, ZAG20, ZAG96, ZAG112, PIE215 and PIE223) and two alleles from two loci (ZAG96 and ZAG112) had relative frequencies differences between 0.10 and 0.30 and above 0.60, respectivelly. These observations can be related to the fact that closely related oak species, which have recently diverged, share ancestral polymorphism [29].

The observed heterozygosity (Ho) and expected heterozygosity (He) within populations ranged from 0.156 for ZAG112 to 0.960 for ZAG7 and ZAG20 and from 0.145 for ZAG112 to 0.935 for ZAG11, respectively (Table 1).

Summary of genetic variation within population and species Table 1

Population	Species	Locus	N	Na	Ar	Ho	He	F_{IS}
Prejmer	*Q. robur*	ZAG7	48	14	13.4	0.896	0.888	-0.01
		ZAG9	47	13	12.3	0.957	0.859	-0.11
		ZAG11	44	21	19.5	0.909	0.914	0.01
		ZAG20	48	12	11.0	0.792	0.822	0.04
		ZAG87	48	20	18.0	0.896	0.878	-0.02
		ZAG96	48	6	5.4	0.292	0.309	0.05
		ZAG110	44	15	13.9	0.750	0.790	0.05
		ZAG112	48	15	13.9	0.771	0.807	0.04
		PIE215	48	5	4.8	0.646	0.694	0.07
		PIE223	48	6	6.0	0.771	0.766	-0.01
		ZAG7	48	14	13.4	0.896	0.888	-0.01
Podu Iloaiei	*Q. robur*	ZAG7	42	17	16.5	0.929	0.887	-0.05
		ZAG9	44	14	13.3	0.864	0.878	0.02
		ZAG11	43	25	23.8	0.860	0.934	0.08
		ZAG20	42	14	13.3	0.881	0.861	-0.02
		ZAG87	42	20	19.0	0.905	0.858	-0.05
		ZAG96	43	12	11.1	0.535	0.526	-0.02
		ZAG110	44	17	15.8	0.750	0.785	0.04
		ZAG112	44	23	21.5	0.864	0.891	0.03
		PIE215	42	7	7.0	0.690	0.755	0.09
		PIE223	42	7	6.9	0.714	0.782	0.09
Total	***Q. robur***	**Mean**	**89.9**	**18.1**	**13.3**	**0.782**	**0.800**	**0.02**
Cristian	*Q. petraea*	ZAG7	50	19	17.7	0.960	0.922	-0.04
		ZAG9	50	12	11.6	0.860	0.867	0.01
		ZAG11	49	21	18.6	0.857	0.844	-0.02
		ZAG20	50	17	16.1	0.960	0.913	-0.05
		ZAG87	49	18	16.6	0.857	0.862	0.01
		ZAG96	49	19	17.9	0.898	0.908	0.01
		ZAG110	49	21	19.2	0.837	0.855	0.02
		ZAG112	51	4	3.5	0.314	0.284	-0.11
		PIE215	50	11	10.3	0.740	0.786	0.06
		PIE223	49	9	8.4	0.592	0.665	0.11
Ronisoara	*Q. petraea*	ZAG7	45	19	17.8	0.822	0.906	0.09
		ZAG9	46	13	12.6	0.848	0.880	0.04
		ZAG11	39	19	18.5	0.872	0.830	-0.05
		ZAG20	43	16	15.5	0.930	0.895	-0.04
		ZAG87	45	18	17.1	0.867	0.866	0.00
		ZAG96	37	21	21.0	0.865	0.919	0.06
		ZAG110	40	15	14.8	0.825	0.853	0.03
		ZAG112	45	3	2.8	0.156	0.145	-0.07
		PIE215	45	9	8.9	0.822	0.761	-0.08
		PIE223	45	9	8.5	0.778	0.779	0.00
Total	***Q. petraea***	**Mean**	**92.6**	**17.5**	**13.9**	**0.784**	**0.793**	**0.01**

Abbreviations: N – number of individuals, Na – number of alleles, Ar – allelic richness after rarefaction (n=37 genotypes), Ho – observed heterozygosity, He – expected heterozygosity, F_{IS} – fixation index.

However, mean Ho and He for *Q. robur* and for *Q. petraea* were almost similar. On the other hand, the mean allelic richness (Ar) for *Q. petraea* was slightly higher than for *Q. robur*. These results are consistent with studies from close regions [30].

Genetic differentiation between both species was high and significant (F_{ST}=0.045; P<0.05), when all loci where considered jointly. With the exception of locus ZAG9, all loci showed significant interspecific F_{ST} values. Two non-genic SSRs markers displayed high interspecific F_{ST} values (0.172 and 0.157 at ZAG112 and ZAG96, respectivelly) mainly because of different pattern in allele frequencies (Figure 1). In addition, these loci discriminated also very well between the same species in other European studies [30], [41]. On the other hand, low differentiation (F_{ST}=0.028) was reported at ZAG96 between these species in Turkey [48], near a putative glacial refugia. Interestingly, ZAG112 locus had the highest interspecific F_{ST} value between *Q. pubescens* and *Q. frainetto* [8].

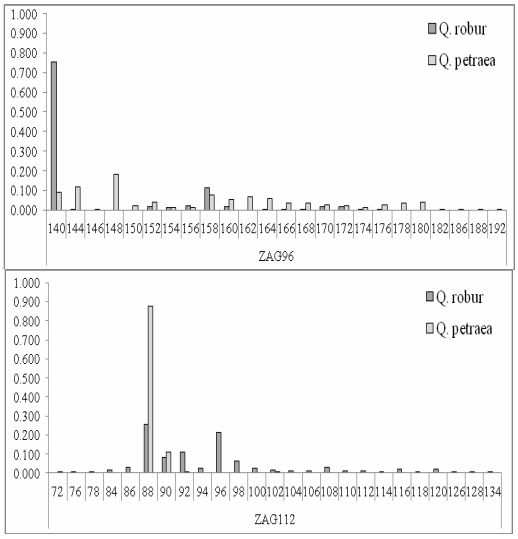


Fig. 1. *Distribution of allele frequencies at ZAG96 and ZAG110 loci*

The four populations cluster according to species rather than geographic origin (Figure 2). The genetic distance between *Q. petraea* populations is slightly smaller than that between *Q. robur* populations, which is consistent with the geographic distances among populations. Moreover, in accordance with AMOVA, the genetic variation between *Q. petraea* and *Q. robur* is nearly 21 fold higher than the variation among populations within species (6.2% vs. 0.3%). However, the majority of genetic variation is explained within populations (93.5%).

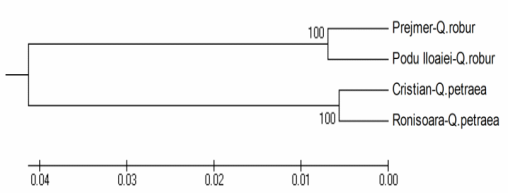
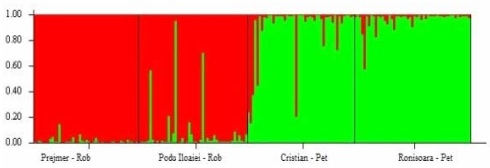


Fig. 2. *UPGMA dendrogram based on Nei's minimum genetic distances between Q. robur and Q. petraea populations at ten microsatellite loci*

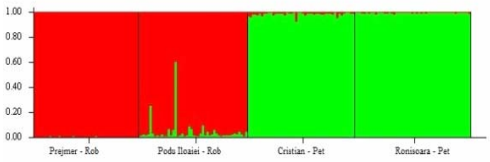
In the Bayesian analysis, the uppermost level of structure corresponds to two clusters, one for each species. The admixture coefficient (Q), corresponding to the assignment probability of each individual to a cluster, was used to infer the species status. There was a clear correspondence between the genetic cluster and the species designation. When no prior information was used in the assignment procedure, 95.8% of the individuals had the highest admixture coefficient (Q > 0.50) for the genetic group corresponding to their phenotype (Figure 3a). The percentage reaches 99.5% if information about the sampling localities is considered in the admixture model (Figure 3b).

A tree from Podu Iloaiei population (*Q. robur*) was the only individual that showed a different genetic assignment compared to morphological traits. This situation is due

to a high number of alleles (44%) from the multilocus genotype that are more frequent in *Q. petraea*, compared to few alleles (11%) that are found more in *Q. robur*. As an example, the allele 253 bp at ZAG11 locus has a relative frequencies of 0.011 and 0.199 in *Q. robur* and *Q. petraea*, respectivelly.



a.



b.

Fig. 3. *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. robur (Rob) and Q. petraea (Pet) are separated by black lines and identified at the bottom*

Among the individuals that were not classified as pure species (i.e. 8.9%) using the blind procedure, only three individuals (one *Q. robur* and two *Q. petraea*) could be the result of natural hybridization between purebreeds. On the other hand, most of admixed trees (4 and 10 trees from *Q. robur* and *Q. petraea* populations, respectivelly) had admixture coefficients that could indicate an introgressive hybridization. However, only a part of them (64%, 3 and 6 trees from *Q. robur* and *Q. petraea* populations, respectivelly) showed the direction of introgression towards the species they most resemble. The rest of admixed trees indicated the direction of introgression to the opposite species. The latter situation seems to be not supported due to the sampling in pure stands. If these stands were composed by only purebreeds individuals, than the formation of hybrids and introgressive individuals could not be possible. However, both species are mentioned to occur in the proximity of all four populations sampled [43] and hybridization events are possible to occur.

Quite frequent natural hybridization combined with low differentiation in most nuclear loci requires special measures in order to increase the performance of genetic assignment in white oaks stands. First, genetic markers have to be evaluated and sorted according to their interspecific discrimination, in order to accurately infer species in a cost-effective manner. However, in case of limited interspecific differentiation a high number of loci may be required for reliable assignment of hybrids and especially backcrosses [46]. Second, sampling schemes must be balanced, because otherwise problems can occur to genetic structure identification [31]. Third, a benchmark with pure trees (at least 100 reference individuals) belonging to all taxa with regional or national distribution must be constituted. Such an approach would have a better resolution in species delineation based on genetic criteria and a more accurate identification of the admixed trees, even in stands with one parental species missing.

3. Conclusions

In spite of the fact that *Q. robur* has a more fragmented natural habitat than *Q. petraea* in Romania, similar values of genetic diversity measures were observed in both species. Two out of ten

microsatellite loci discriminated very well between the two species. The molecular analysis demonstrates that *Q. robur* and *Q. petraea* can be unambiguously designated at population level. Using ten 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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