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GENETIC DIVERSITY OF PEDUNCULATE OAK (QUERCUS ROBUR L.) IN PREJMER NATURAL RESERVE

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Abstract: *Genetic diversity is very important for species survival and ensures its continuous evolution under changing environments. Here, we analyse the genetic structure of Quercus robur population from Prejmer Natural Reserve. Two categories of genetic markers were used: isozymes and microsatellites. High levels of genetic diversity have been revealed (He = 0.284 for isozymes and He = 0.769 for microsatellites, respectively). These values are comparable with those reported for other populations distributed throughout European distribution of the pedunculate oak. No indications of inbreeding have been found for this relatively isolated oak forest.*

Key words: *genetic diversity, Quercus robur, isozymes, microsatellites.*

1. Introduction

Genetic diversity is essential for species long-term survival and adaptation under changing environments. As species encounter new stresses from habitat loss, pollution and climate change, management actions are needed to conserve their gene pool [4].

Prejmer natural reserve was founded approximately 50 years ago in order to protect the natural forest located in the floodplain of the Olt River, in the vicinity of Brasov city. This site was included recently in the Natura 2000 network. The keystone species of this unique forest ecosystem is the pedunculate oak (*Quercus robur* L.). Although in the past, the pedunculate oak was the dominant tree species on the actual territory of Romania, nowadays this species occupies only 2% of national wooded area. Its present natural range is very fragmented, consisting of many isolated stands [14]. The genetic diversity of several Romanian populations of *Q. robur* was estimated with codominant markers [15] or in a mixed oak forest in west-central Romania [1]. Pedunculate oak diversity was also investigated at chloroplast DNA level [7], [10], showing that the present oak stands have their phylogenetic origin in Balkan and Italian peninsula.

The aim of this study is to evaluate genetic diversity of the main tree species of Prejmer natural ecosystems by using two types of genetic markers: isozymes and microsatellites.

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2. Materials and Methods

2.1. Sample collection

A total of 32 adult individuals of *Quercus robur* L. were sampled in 2010 at Prejmer (45°44'N, 25°44'E, altitude 510 m). The most widespread species is *Fraxinus excelsior* L., in expansion in this forest. The pedunculate oak population are at higher altitude in this part of Romania. The specific local climatic condition marked by low winter temperature, late frosts and hydromorphic soils determines classifications of this population as ecotypic [11].

Tree identification was done using leaf morphological characters [14]. Geographical position of each tree was recorded using a GPS. The distance between individuals was at least 50 m in order to prevent the sampling of related individuals. Winter buds were collected from each tree. The material was frozen at –60 °C until analysis.

2.2. Isozymes analysis

The enzymes were extracted from buds and separated from crude homogenate by horizontal starch gel electrophoresis [19] using two different buffer systems: Ashton and Tris-Citro.

Twelve enzyme systems were analyzed (Enzyme Commission number, the acronym and controlling locus are given in parenthesis): aconitase (4.2.1.3; *Aco-A*), formate dehydrogenase (1.2.21; *Fdh-A*), glutamate-dehydrogenase (1.4.1.2; *Gdh-A*), glutamate oxaloacetate transaminase (2.6.1.1; *Got-B*), isocitrate dehydrogenase (1.1.1.42; *Idh-B*), leucine aminopeptidase (3.4.11.1; *Lap-B*), malate dehydrogenase (1.1.1.37; *Mdh-B*), menadione reductase (1.6.99.2;*Mnr-A*), phosphoglucoisomerase (2.7.5.1; *Pgm-B*), phosphoglucose isomerase (3.5.1.9; *Pgi-B*), 6-phosphogluconate dehydrogenase (1.1.1.44; *6-Pgdh-B*), *shikimate dehydrogenase* (1.1.1.25; *Skdh-A*). The alleles were labeled with numbers from the cathode to the anode. The zymograms interpretation was carried out following Konnert et al. [6] and an example is shown in Figure 1.

2.3. DNA isolation and microsatellite analysis

DNA was extracted using the QiagenDNeasy96 Plant Mini Kit following the manufacturer protocol, but without liquid nitrogen for material disruption, as described by Toader et al. [16].

All trees were genotyped using five genomic microsatellite loci (gSSRs): ssrQrZAG 11, ssrQrZAG36, ssrQrZAG96, ssrQrZAG112 [12], ssrQpZAG110 [5] and two expressed sequence tag microsatellites (EST-SSRs): PIE040 and GOT004 [3].

More information about the primer pairs, repeat motif and allele length is given in Table 1. Two multiplex PCRs (five gSSRs and two EST-SSRs) were run independently in a *Corbett* Thermal Cycler. The cycling profile consisted of an initial denaturation

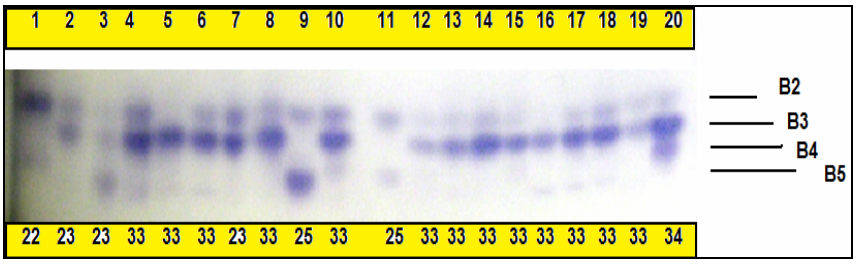

Fig. 1. *Electrophoretic zymograms for locus Pgm-B for twenty trees*

Characteristics of the seven microsatellite investigated loci Table 1

Microsatellite marker	Locus	Nucleotide motif	Linkage group	Primer concentration [µM]	Allele size [bp]
Genomic SSRs (gSSRs)	ssrQrZAG11	di	10	0.34	242-289
	ssrQrZAG39	di	5	0.28	105-169
	ssrQrZAG96	di	10	0.25	140-180
	ssrQpZAG110	di	8	0.18	205-243
	ssrQrZAG112	di	12	0.09	82-112
EST-SSRs	GOT004	di	2	0.20	266-306
	PIE040	tri	-	0.33	171-190

step of 3 minutes followed by 31 cycles of 50 s denaturation at 94 ºC, a 40 s annealing step at 52 ºC for gSSRs (57 ºC for EST-SSRs), a 1 min 20 s elongation step at 70 ºC and a final extension step at 70 ºC for 12 min. Forward primers were labeled with fluorescent dye at the 5'end. Fragment size polymorphisms were detected and scored using a Beckman CQ 8800 capillary electrophoresis system with Size Standard 400. The products were then analyzed using Beckman Analysis Software using default parameters and PAver.1 dye correction. An example for electropherogram interpretation is shown in Figure 2.

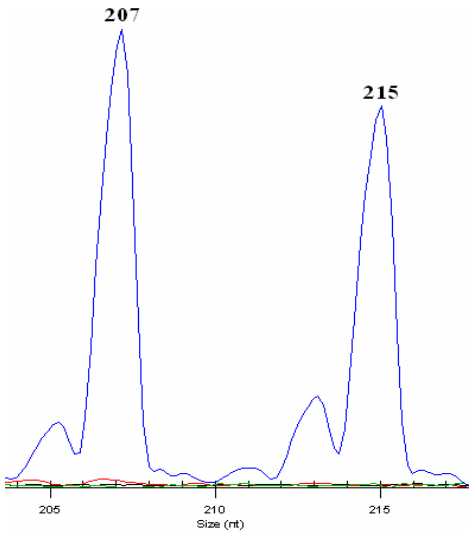

Fig. 2. *Electropherogram for a heterozygote tree. The genotype is 207/215*

2.4. Statistical data analysis

The following standard parameters of population genetics were calculated using the computer software GenAlEx version 6.4 [9]: number of alleles (*Na*), effective number of alleles ($Ne = 1/[1-He]$), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index ($F = 1-Ho/He$) [18]. Using the same software, deviations from Hardy-Weinberg equilibrium were checked by χ^2 test, pooling genotypes whenever expected frequencies were less than 0.05. Microsatellite loci were tested for genotyping errors due to non-amplified alleles, short allele dominance and the scoring of stutter peaks using MICRO-CHECKER 2.2.0.3 [17].

3. Results and Discussion

Eleven out of the isozyme loci and all microsatellite markers were polymorphic in our sample (Table 2). As expected [2], the microsatellite regions were much more variable than the enzyme coding loci. This situation may be explained by the fact that the majority of the microsatellite (genomic SSRs) are not parts of functional genes, and the mutations occurred are not relevant for adaptation.

The effective number of alleles also reflects the difference in the level of polymorphism between the two types of marker, 6.50 for microsatellite loci and only 1.64 for isozyme loci on average.

The genetic diversity, measured as the expected heterozygosity (*He*), is three times higher for microsatellites than for isozymes. However, four isozyme loci, Lap-B, Gdh-A, Aco-A and Pgm-B, showed higher values of expected heterozygosity than one microsatellite locus, ssrQrZAG96. The reduction in variability at this microsatellite is very likely caused by selection effects [8].

Both types of markers indicate on average a slight excess of homozygotes (positive values for *F*). At least for isozyme this deficit of heterozygote is mainly due to three enzyme coding loci: Idh-B, Pgm-B and Skdh-A. Significant deviations ($p < 0.05$) from Hardy-Weinberg proportions were also observed at two of these loci: Idh-B and Skdh-A. Higher values of fixation index were also observed at two microsatellite markers, ssrQrZAG11 and GOT004. Since the majority of the investigated loci show negative or close to zero values for *F*-index, there is no sign for inbreeding in this apparently isolated population of pedunculate oak. The positive values of *F*-index may be caused by null alleles (even at isozyme loci), possible selection effects in favor of homozygote genotypes or scoring errors (mainly at microsatellites).

Summary of genetic variation Table 2

Locus	***N***	***Na***	***Ne***	***Ho***	***He***	***F***
Got-B	32	4	1.17	0.156	0.148	–0.056
Idh-B	32	2	1.95	0.281	0.488	0.423*
Pgm-B	32	4	2.04	0.344	0.509	0.325
Pgi-B	32	3	1.13	0.125	0.119	–0.049
6-Pgdh-B	32	2	1.03	0.031	0.031	–0.016
Mnr-A	32	4	1.10	0.094	0.091	–0.032
Gdh-A	32	4	2.72	0.656	0.632	–0.038
Lap-B	32	4	3.14	0.469	0.682	0.312
Fdh-A	32	2	1.03	0.031	0.031	–0.016
Skdh-A	32	4	1.17	0.094	0.148	0.366*
Aco-A	32	3	2.14	0.469	0.534	0.122
Mdh-B	32	1	1.00	0.000	0.000	–
Mean	**32**	**3.08**	**1.64**	**0.229**	**0.284**	**0.122**
ZAG112	32	14	5.77	0.813	0.827	0.017
ZAG39	31	18	8.54	0.839	0.883	0.050
ZAG96	32	6	1.64	0.375	0.392	0.042
ZAG110	28	13	4.87	0.750	0.795	0.056
ZAG11	28	18	11.97	0.786	0.916	0.143
PIE040	30	7	3.07	0.733	0.674	–0.088
GOT004	30	18	9.63	0.767	0.896	0.144
Mean	**30.143**	**13.43**	**6.50**	**0.723**	**0.769**	**0.052**

Sample size (*N*), number of alleles (*Na*), effective number of alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index (*F*); * $p < 0.05$

At all seven microsatellites loci MICRO-CHECKER found no evidence for scoring error due to stuttering and no evidence for large allele dropout. Evidence for null alleles was indicated two (ssrQrZAG11 and GOT004) out the seven loci, because of general excess of homozygotes for most allele size classes. This result explains the highest *F* values among microsatellites at ssrQrZAG11 and GOT004 loci.

The estimated values of genetic diversity of *Quercus robur* from Prejmer Forest are consistent with those reported in other studies carried out with the same category of markers. Thus, in Romania at Bejan Forest, Curtu et al. [1] calculated a value of 0.243 for isozyme gene diversity and 0.750 for 6 microsatellite loci.

In a study by Streiff *et al.* [13] a *Quercus robur* population located in the north-west of France showed higher genetic diversity values than at Prejmer Forest: 0.34 for isozymes and 0.87 for microsatellites, respectively. Muir and Schlötterer [8] analysed six *Quercus robur* populations, covering the species distribution in Europe, with 20 microsatellite loci and found only a difference for genetic diversity as compared with Prejmer Forest: 0.79.

However, caution is required when comparing data provided by different sets of markers and sample sizes.

4. Conclusions

This is the first study aiming at estimating the level of genetic diversity for the keystone species of the forest ecosystem located at Prejmer. The calculated values indicate that this pedunculate oak population still possess high levels of genetic diversity, which are comparable with those reported for other populations distributed throughout its European distribution.

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