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# PRELIMINARY RESULTS ON THE GENETIC DIVERSITY OF *CARPINUS BETULUS* IN CARPATHIAN POPULATIONS

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***Abstract:*** *The aim of this study was to obtain data upon the genetic structure of Carpinus betulus in populations from Romania. Four recently characterized nuclear microsatellite markers have been tested in three Carphatian populations. Between 14 and 23 alleles per marker were observed. On average, the number of alleles per locus ($N_a$) and the effective number of alleles ($N_e$) per locus was 1.745 and 1.525, respectively. The expected heterozygosity ($H_e$) for the populations ranged from 0.289 to 0.325. A total of five private alleles were observed in two populations.*

***Key words:*** *Carpinus betulus, hornbeam, genetic diversity, nuclear microsatellites.*

## 1. Introduction

The genus *Carpinus* belongs to the family *Betulaceae* [12] and includes about 35 species distributed throughout large parts of Central and North America, Asia and Europe [10]. There are two European species, *Carpinus betulus* L. (common hornbeam) and *C. orientalis* Mill. (oriental hornbeam), which usually have disjoint distribution areas, due to their different ecological requirements. Thus, the common hornbeam is a mesothermal and mesophilous species, the while oriental hornbeam is thermophilous and xerophylous [11]. These ecological differences between the two species typically result in the separation of their natural distribution. However, at the Southern and/or the lower altitudinal limit of distribution area, the two species coexist. In such situations, hornbeam is a species of interest in forest ecosystems affected by climate change.

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So far, only few molecular studies have been carried out on common hornbeam. Most of them were done to identify chloroplast DNA haplotypes. AFLP (*Amplified Fragment Length Polymorphism*) markers were also applied [2]. Very recently, nuclear Simple Sequence Repeats (SSRs) markers were characterized and developed [8]. This type of genetic markers, also called microsatellites or Short Tandem Repeats (STR) [14], are highly polymorphic and have shown relatively high transferability among related species [13].

*C. betulus* is an octoploid species (2n=8x=64) [4], [5], [7], and consequently the nuclear analyses are more complex than in the diploid species. This is probably one of the reasons for the absence of studies using SSR markers in common hornbeam.

In the Romanian Carpathians, the common hornbeam is only found in natural populations. No plantations with this species were done in the area [11]. This is the first application of the newly developed set of genetic markers for characterizing the genetic structure of *C. betulus* in three natural populations from the Carpathian Mountains.

## 2. Materials and Methods

### 2.1. Plant material

Shoots with buds were collected from: Warthe-Braşov (45°65'N, 25°58'E), Tâmpa-Braşov (45°63'N, 25°59'E) and Colți-Buzău (45°38'N, 26°40'E). The distance between sampled individuals was kept at around 50 m. Fresh shoots with buds were stored in an ultra freezer at temperatures of -60$^0$C, until the time of DNA extraction.

### 2.2. DNA extraction and PCR profiling

Total genomic DNA was extracted from winter buds (100 mg raw material) using the Dneasy 96 Plant DNA isolation Kit [9].

Four primer pairs developed by Prinz and Finkeldey [8] were used to genotype sampled individuals. The amplifications were performed in 14 µl of a reaction mixture consisting of 1,5 µl PCR Puffer, 1,50 µl $MgCl_2$, 1,00 µl dNTPs, 0,20 µl Promega Taq Polymerase, 1,00 µl primer forward, 1,00 µl primer reverse, 6,80 µl $H_2O$ and 1,00 µl DNA. The PCR amplifications were carried out in a Peltier Thermal Cycler (PTC-0200 version 4.0, MJ Research). The PCR *touch down* program was according to Prinz and Finkeldey [8] using the following reaction conditions: initial denaturation for 15 minutes at 95 $^o$C; followed by 10 cycles of denaturation for 1 minute at 94 $^o$C, annealing for 1 minute at 60 $^o$C (-1$^o$), extension for 1 minute at 72 $^o$C; 25 cycles of denaturation for 1 minute at 94 $^o$C, annealing for 1 minute at 50 $^o$C, extension for 1 minute at 72 $^o$C and final extension for 20 minutes at 72 $^o$C. Each reaction was used by two markers (Cb_15 to Cb_27 and Cb_17 to Cb_48a). The PCR products were tested in 1.5 % agarose gel electrophoresis and photographed with UV lights.

### 2.3. Genotyping of PCR products

The separation was done through capillary electrophoresis in an automated sequencer ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). The mix was composed of 2 µl of the diluted PCR product, 12 µl HiDi Formamide (Applied Biosystems) and 0.01 µl GS 500 Rox (Applied Biosystems) internal size standard. The samples had been denatured for 2 minutes at 90 $^o$C, subsequently stored on ice for about 5 minutes, before capillary electrophoresis. Individual alleles were analyzed using Software-GeneMapper v.

4.0 (the most comprehensive analysis of the available fragments) and Genotyper Version 3.7 NT (Applied Biosystems). GeneMapper Analysis Software v. 4.0 (Applied Biosystems) was used to translate the collected information by the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) into sizing fragment information. The GeneMapper files were further scored with Genotyper v. 4.0 (Perkin Elmer).

### 2.4. Data analysis

The SSR data were transformed into a zero/one matrix and the frequencies of the alleles were estimated using the software GENALEX 6.41 [6]. The genetic variation within populations was rendered in terms of $N_a$ (number of different alleles), $N_e$ (effective number of alleles) and h (expected heterozygosity). Since *C. betulus* is an octoploid species, it is difficult to determine the exact number of alleles present in a particular heterozygote. The analysis of the molecular variance (AMOVA), which is conceptually related to $\Phi_{PT}$, was used to estimate the genetic variation between populations [3], [6].

## 3. Results and Discussions

The number and size of the alleles from the four primers in the three hornbeam populations, are shown in Table 1.

All four markers have a high degree of polymorphism, producing between 14 to 23 alleles. As expected, the number of alleles per individual varied from two to eight, because hornbeam is an octoploid species.

Allele scoring was done visually, to avoid the errors that may occur if this operation had been performed automatically. The peaks were read in descending order with declining putative peak quality (because stutter bands usually occur before main peaks) [7]. The peak patterns are usually coded as dominant data [1] largely comparable to anonymous markers like AFLPs (Amplified Fragment Length Polymorphism) or RAPDs. The presence/absence of alleles per individual was recorded with Genotyper. An example of electropherogram for nuclear markers analyzed for *C. betulus* is shown in Fig. 1.

*Alleles scored in the three hornbeam analyzed populations* Table 1

| Locus | Information peak size range observed (bp) | The number of alleles per individual | | | The number of alleles per population | | | The number of alleles |
|---|---|---|---|---|---|---|---|---|
| | | Warthe | Tâmpa | Colți | Warthe | Tâmpa | Colți | |
| Cb_15b | 75-102 | 2-7 | 4-8 | 3-8 | 14 | 17 | 17 | 17 |
| Cb_17 | 58-98 | 3-6 | 2-6 | 2-7 | 12 | 11 | 11 | 14 |
| Cb_27 | 71-151 | 4-8 | 3-8 | 6-8 | 21 | 18 | 18 | 23 |
| Cb_48a | 136-166 | 2-6 | 4-8 | 3-8 | 12 | 12 | 14 | 14 |

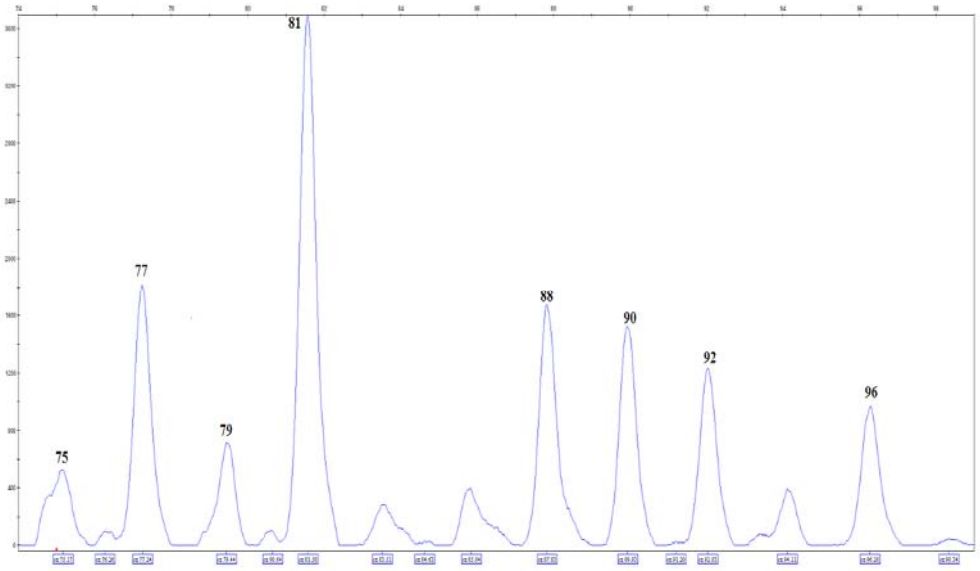


Fig. 1. *Allele scoring at marker Cb_ 15a for individual 15 from Colți-Buzău population*

The number of alleles per population ranged from 11 for the marker Cb_17 in Tâmpa-Braşov population to 21 for the marker Cb_27 in Warthe-Braşov population. Averaged across loci, a total of 17 alleles per marker were identified. The highest number of alleles was observed at the marker 27_Cb (23 alleles). The number of observed alleles per marker was lower than the one reported by Prinz and Finkeldey [9]. Private alleles were detected only in Warthe-Braşov population (four alleles) and Tâmpa-Braşov (one allele). In Colți-Buzău population, no private alleles were found. In the two populations from Brasov area, with a distance of 3-4 km from one another, a different number of alleles for the markers Cb_15b and Cb_27 were observed. These differences between the two closest populations might be due to the low sample size, especially in Warthe-Braşov population, in which, indeed, the lowest gene diversity was found ($H_e$ = 0.289).

Similar values of genetic diversity within populations were estimated (Tab. 2). The analyzed nuclear markers revealed a low genetic diversity between populations, with a $\Phi_{PT}$ value of 0.065 (P = 0.001). In the two populations from Braşov area, the values were identical for $N_a$ and slightly different for $N_e$. For both parameters, the population Colți-Buzău showed slightly higher values. The level of gene diversity in the three populations of our study ($H_e$ = 0.309) was similar to the analysis performed by AFLP markers in Săvârşin populations, situated in South-West Romania ($H_e$ = 0.3197) [2].

Table 2

Values of genetic parameters in *C. betulus* populations

| Location | Population | N | | $N_a$ | $N_e$ | $H_e$ |
|---|---|---|---|---|---|---|
| Braşov | Warthe | 32 | Mean | 1.735 | 1.475 | 0.289 |
| | | | SE | 0.083 | 0.039 | 0.019 |
| Braşov | Târnpa | 36 | Mean | 1.735 | 1.538 | 0.313 |
| | | | SE | 0.083 | 0.041 | 0.021 |
| Buzău | Colți | 30 | Mean | 1.765 | 1.564 | 0.325 |
| | | | SE | 0.079 | 0.042 | 0.020 |
| Total | | 98 | Mean | 1.745 | 1.525 | 0.309 |
| | | | SE | 0.047 | 0.024 | 0.012 |

Note: N - sample size; $N_a$ - observed number of alleles per locus; $N_e$ - effective number of alleles per locus; $H_e$ - gene diversity; SE – standard error.

## 4. Conclusion

A relatively large number of alleles per marker were identified in the three *C. betulus* populations. Similar values of genetic parameter were estimated. Our preliminary results suggest the utility of these markers for characterizing the genetic structure of this octoploid tree species.

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