

Evidence of Low Chloroplast Genetic Diversity in Two *Carpinus* Species in the Northern Balkans

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Abstract

Genetic diversity and differentiation in two *Carpinus* species (*C. betulus* and *C. orientalis*) occurring in Romania was investigated by using three chloroplast Simple Sequence Repeat markers (cpSSRs). A total of 96 and 32 individuals were sampled in eighteen *C. betulus* and six *C. orientalis* populations, respectively. A total of four chloroplast haplotypes were observed. Two haplotypes were specific for *C. betulus* and two for *C. orientalis*. Most of *C. betulus* populations were fixed for the predominant haplotype (H1), which was observed in 82% of the individuals. All *C. orientalis* populations were fixed for one haplotype or the other. Populations with haplotype (H3) are spread in southern Romania and the haplotype (H4) was observed at the northern limit of *C. orientalis* natural distribution range. Genetic differentiation among populations was moderate in *C. betulus* ($G_{ST} = 0.422$), compared to the high value observed in *C. orientalis* ($G_{ST} = 1.000$), which can be explained by the occurrence of a distinct haplotype in the peripheral population. R_{ST} values for both species suggest low levels of recurrent gene flow through seeds among populations. Our data on geographic distribution of chloroplast DNA haplotypes may be useful for the identification and conservation of distinct genetic resources of the two *Carpinus* species.

Keywords: *Carpinus betulus*, *C. orientalis*, cpSSR, genetic diversity, phylogeography

Introduction

Genetic diversity is essential for species to evolve and to adapt to changing environmental conditions. Patterns of genetic diversity are influenced by population contractions and expansions during glacial-interglacial fluctuations and by post-glacial migration history (Petit *et al.*, 2005). Phylogeographic studies have provided important evidence on species demographic history and population structure (Petit *et al.*, 2005; White *et al.*, 2007; Xu *et al.*, 2015) revealing that species-specific life history traits and geographic barriers may have a strong influence on the current patterns of population genetic structure (Ortiz-Ramírez *et al.*, 2016).

The genus *Carpinus* (*Betulaceae* family) is represented by two species in Europe (*C. betulus* L. and *C. orientalis* Mill). European hornbeam (*C. betulus* L.) is a temperate hardwood species especially in forests dominated by oak species, where it demonstrates a great ability to compete with oaks at the juvenile stage (Petre *et al.*, 2012). In Romania *C. betulus* occupies around 4.8% of the total forest

cover (IFN, 2012). This species is spread between 100 and 450 (500) m altitude, being rare in mixed beech-fir-spruce mountain forests (Horeanu, 1996). *C. betulus* is considered as a valuable species due to its strong ecological adaptability and increased tolerance to environmental stress, especially in extreme conditions (Șofletea and Curtu, 2007). *C. orientalis* is a major pioneer species in arid regions where it can colonise open and degraded areas (Bergmeier and Dimopoulos, 2008; Čarni *et al.*, 2009). Both species co-occur in southeastern Europe and no natural hybrids between *C. betulus* and *C. orientalis* have been reported; however, controlled crosses indicated that hybrids had inferior survival and growth characteristics, and that adult hybrids were sexually immature (Santamour, 1995; Grivet and Petit, 2003). The northern edge of *C. orientalis* distribution range is the southern part of Romania, but isolated small populations are also present in the northern part of the Moldova region (Șofletea and Curtu, 2007). Most of *C. orientalis* populations in Romania can be considered as marginal populations and their genetic diversity assessment is of great importance, due to ongoing global warming.

Genetic variation of *C. betulus* and *C. orientalis* have been investigated with different DNA markers targeting different polymorphic regions of the chloroplast genome by PCR-RFLP (Restriction Fragment Length Polymorphism) (Postolache *et al.*, 2017) and also by using chloroplast microsatellites (Simple Sequence Repeats; cpSSRs) (Grivet and Petit, 2003; Fărcaș *et al.*, 2006). The chloroplast DNA diversity for *C. betulus* was significantly lower in western Europe compared with southeastern European populations, that harbour nearly all genetic variation and consequently a more detailed analyses in this region is absolutely necessary to quantify the genetic structure and diversity. Beside this, previously published results Petit and Grivet (2003) indicate for very low introgression between these two species. The *C. betulus* populations from Romania have a very distinct Holocene postglacial history with direct consequences on current genetic pattern that needs to be investigated further in order to understand the species diversity and evolutionary history (Grivet and Petit, 2003; Fărcaș *et al.*, 2006). Moreover, the phylogeographic data on *C. orientalis* are very scarce and these data are very important in the conservation and management of this drought-tolerant woody species.

The aim of this study was to analyse the chloroplast DNA variation in two sister species of hornbeam (*C. betulus* and *C. orientalis*) in Romania (Northern Balkan Peninsula) using cpSSRs markers, that can bring detailed information on species genetic structure, which could be applied in developing site-oriented conservation strategy and sustainable management of *Carpinus* spp. genetic resources. The major objective of this work is to characterize the genetic structure of *Carpinus* spp. in Romania and to test whether Carpathian Mountains acted as a major geographic barrier during the Holocene postglacial recolonization history. More specifically we aimed: i) to assess the chloroplast DNA genetic variation within and among *Carpinus* spp. populations using cpSSRs; ii) to analyse the geographic distribution of chloroplast haplotypes and infer direct consequences on the management and conservation of *Carpinus* spp. genetic resources.

Materials and Methods

Sampling and DNA isolation

Shoots and leaves were collected from 18 populations of *C. betulus* and six populations of *C. orientalis* covering the species distribution range in Romania (Table 1, Fig. 1). The material was stored in an ultrafreezer at temperatures of -60°C until used for DNA extraction.

Genomic DNA was extracted from biological specimens using the CTAB method described by Doyle and Doyle (1987) with minor modifications. The DNA was quantified using NanoDrop 8000 (Thermo Scientific, Wilmington, USA, 2008) and extracted DNA was stored at -20°C .

Chloroplast microsatellites analysis

Due to the lack of intraspecific variation observed in *Carpinus* spp. we initially tested ten universal chloroplast microsatellites (*ccmp1*, *ccmp2*, *ccmp3*, *ccmp4*, *ccmp5*, *ccmp6*, *ccmp7*, *ccmp8*, *ccmp9* and *ccmp10*) developed by Weising and Gardener (1999). Nine out of ten primers showed the amplification products, but only three primers (*ccmp4*, *ccmp7* and *ccmp10*) showed fragment length polymorphism. DNA was diluted (1:30) prior to PCR amplification. PCR reactions were performed in a total volume of 15 μl of a reaction mixture consisting of 1 \times PCR Buffer (Promega), 2.5 mM MgCl_2 , 200 μM of each of the four dNTPs, 1 unit of *Taq* DNA Polymerase (Promega), 0.2 μM of each primer added to 2 μl of genomic DNA. PCR amplifications were performed in a *Corbett Palm-Cycler CG1-96* with the following conditions: an initial denaturation for 15 minutes at 95°C followed by 30/35 cycles of denaturation for 1 minute at 94°C , annealing for 1 minute at 50°C , extension for 1 minute at 72°C and a final extension for 20 minutes at 72°C (30 cycles for *ccmp4* and 35 cycles for *ccmp7* and *ccmp10*). The amplification of chloroplast SSRs was performed using fluorescence dyed forward primers (Metabion) for genotyping purpose, namely 6-FAM/Blue (*ccmp7* and *ccmp10*) and HEX/Green (*ccmp4*).

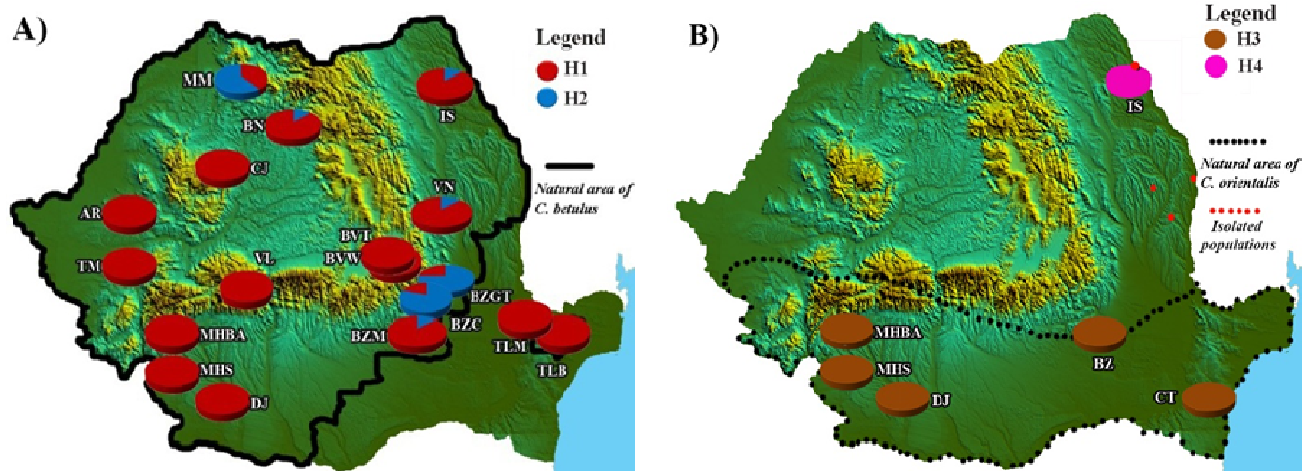


Fig. 1. Distribution of *C. betulus* (A) and *C. orientalis* (B) chloroplast DNA haplotypes in Romania

Table 1. Geographic location of populations

No	Population	Abbreviation	Latitude	Longitude	Sample size	Species
1	Apa Sărată-Maramureș	MM-CB	47°40'	23°29'	5	<i>C. betulus</i>
2	Teaca-Bistrița-Năsăud	BN-CB	46°52'	24°32'	5	
3	Roșcani-Iași	IS-CB	47°26'	27°24'	5	
4	Panciu-Vrancea	VN-CB	46°04'	27°01'	5	
5	Babadag-Tulcea	TLB-CB	44°51'	28°41'	5	
6	Măcin-Tulcea	TLM-CB	45°14'	28°11'	5	
7	Warthe-Brașov	BVW-CB	45°39'	25°34'	5	
8	Tâmpa-Brașov	BVT-CB	45°38'	25°35'	5	
9	Gura Teghii-Buzău	BZGT-CB	45°32'	26°28'	8	
10	Măgura-Buzău	BZM-CB	45°16'	26°33'	8	
11	Colți-Buzău	BZC-CB	45°22'	26°23'	5	
12	Lotrisor-Vâlcea	VL-CB	45°17'	24°12'	7	
13	Leamna-Dolj	DJ-CB	44°18'	23°42'	5	
14	Baia de Aramă-Mehedinți	MHBA-CB	44°57'	22°45'	5	
15	Stârmina-Mehedinți	MHS-CB	44°30'	22°46'	3	
16	Lugoj-Timișoara	TM-CB	45°42'	21°58'	5	
17	Săvârșin-Arad	AR-CB	46°00'	22°13'	5	
18	Huedin-Cluj	CJ-CB	46°56'	22°49'	5	
Total					96	
1	Roșcani-Iași	IS-CO	47°26'	27°24'	6	<i>C. orientalis</i>
2	Murfatlar-Constanța	CT-CO	44°09'	28°23'	6	
3	Cândești-Buzău	BZ-CO	45°16'	26°40'	5	
4	Leamna-Dolj	DJ-CO	44°17'	23°43'	5	
5	Baia de Aramă-Mehedinți	MHBA-CO	44°57'	22°45'	5	
6	Stârmina-Mehedinți	MHS-CO	44°29'	22°45'	5	
Total					32	

Amplified fragments were analysed on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter) using an internal size standard (CEQ 400, Beckman Coulter). Polymorphisms were defined by different length variants of detected microsatellite fragments.

Data analysis

The programme PERMUT cpSSR (Pons and Petit, 1996) was used to calculate the parameters of chloroplast DNA diversity (h_s = haplotype diversity within populations based on unordered haplotypes, h_T = total haplotypic diversity statistics based on unordered alleles, G_{ST} = differentiation among populations based on unordered alleles and R_{ST} = differentiation among populations based on ordered alleles).

A hierarchical analysis of molecular variance (AMOVA) was conducted to estimate the distribution of genetic variation among and within populations using GenAlEx version 6.5 (Peakall and Smouse, 2012). The variance components were tested statistically by non-parametric randomization tests using 999 permutations. Genetic distances (Nei, 1987) between pairs of populations were calculated with HAPLOTYPE ANALYSIS© version 1.05 (Eliades and Eliades, 2009).

The software SPAGEDI version 1.5 (Hardy and Vekemans, 2002) was used to test for the presence of a phylogeographic pattern by permuting allele sizes among alleles. If the observed R_{ST} is higher than the R_{ST} after permutations, we may infer that there is a phylogeographic pattern.

The relationships among found cpSSR haplotypes were inferred based on the median-joining network algorithm (Bandelt et al., 1999). Maximum-parsimony analysis was conducted using the software NETWORK version 5.0 (available at <http://www.fluxus-engineering.com/sharenet.htm>). NETWORK identifies the number of mutational

steps that separate a given set of haplotypes and accounts for haplotype frequency at a given node.

To evaluate the population phylogenetic relationships among populations an unweighted pair group average network (UPGMA) was constructed using Euclidean paired group algorithm of PAST software (Hammer et al., 2001). One thousand bootstrapped replicated matrices of pairwise Nei's genetic distances (1987) were calculated using the same program.

The association between the pairwise genetic distance (Nei, 1987) and geographic distances (GGD) was analyzed with Mantel test (Mantel, 1967) using GenAlEx version 6.5 software (Peakall and Smouse, 2012).

Results

Chloroplast DNA haplotypes

Following the genotyping of the 128 individuals with three cpSSRs, 8 variants were identified. Thus, only two variants (and thus haplotypes) were found in the two species, which are completely different in this analysis.

One of the three chloroplast microsatellite regions showed polymorphism in *C. betulus*, that displayed two size variants at *ccmp7* (Table 2). The size variants from all three analyzed chloroplast microsatellites were combined into two haplotypes for *C. betulus*, with H1 being found in all eighteen populations, while H2 was found in seven populations (Fig.1.A).

In *C. orientalis* two size variants were observed at *ccmp10* among three analyzed microsatellite regions (Table 2). By combining size variants of chloroplast SSRs, two haplotypes were identified in six populations. Five populations of *C. orientalis* from southern Romania are monomorphic (haplotype H3, while haplotype H4 was detected only in an isolated population located in north-eastern part of Romania (Fig.1.B).

Table 2. Observed chloroplast DNA haplotypes in *C. betulus* and *C. orientalis*

SSR marker	<i>C. betulus</i>		<i>C. orientalis</i>		No. of variants
	H1	H2	H3	H4	
<i>ccmp4</i>	118 bp	118 bp	129 bp	129 bp	2
<i>ccmp7</i>	158 bp	157 bp	159 bp	159 bp	3
<i>ccmp10</i>	110 bp	110 bp	117 bp	115 bp	3
No. of individuals	79	17	26	6	

Genetic diversity

Genetic differentiation among *C. betulus* populations was moderate ($G_{ST} = 0.422$ and $R_{ST} = 0.422$) compared with *C. orientalis* ($G_{ST} = 1$ and $R_{ST} = 1$) (Table 3).

The analysis of molecular variance (AMOVA) for *C. betulus* showed that 42% of the haplotypic variation was due to differences among populations, whereas 58% to differences within populations. In *C. betulus* only 11 out of 18 populations are represented by one haplotype and 7 populations are mixed by two haplotypes with three populations significantly dominated by H2 (MM, BZC and BZGT) (Fig. 1).

The permutation test performed in SPAGEDI showed that the observed R_{ST} was equal to the R_{ST} after permutations, indicating an absence of a phylogeographic pattern for both species. Indeed, the dominance of two haplotypes in the Carpathians was also suggested by Postolache et al. (2017), based on the polymorphic bands observed in two combinations (atpH-atpI/Hinfl and trnC-trnD/TaqI). However, a geographic pattern of genetic variation may be observed from the haplotype frequency map in *C. betulus* and *C. orientalis* (Fig. 1). Because our study found only two variants in each of the two species, the results should be interpreted with caution.

Phylogenetic relationships between haplotypes

The genetic relationships between all haplotypes show a clear (Fig. 2) separation of haplotypes specific for *C. betulus* and those identified in *C. orientalis*. Within the network, H1 is the ancestral one and H2 appears to be derived from H1. On the other hand, H3 detected in *C. orientalis* most probably evolved from H4, however these results need to be interpreted with caution due to limited variants found.

The UPGMA dendrogram based on Nei's distances (1987) supports geographic grouping of haplotypes by species (Fig. 3). All *C. betulus* populations were clustered together with subsequent subclustering of populations in two groups, each group being specific for one of the two haplotypes. On the other hand, *C. orientalis* populations are clustered into two distinct subclusters, with one of subcluster represented by population Roșcani, in which a unique haplotype was observed for *C. orientalis*. It is also important to note that branches of the major clusters correspond to distinct geographic regions.

Table 3. Levels of genetic diversity and differentiation in *Carpinus* spp.

Species	Sample size	Number of haplotypes	h _s (SE)	h _T (SE)	G _{ST} (SE)	R _{ST} (SE)
<i>C. betulus</i>	96	2	0.160 (0.051)	0.277 (0.087)	0.422 (0.094)	0.422 (0.093)
<i>C. orientalis</i>	32	2	0.000 (0.000)	0.333 (0.222)	1.000 (NC)	1.000 (NC)

h_T = total genetic diversity, h_s = genetic diversity within populations, G_{ST} and R_{ST} = genetic differentiation, SE – standard errors, NC = non-significant

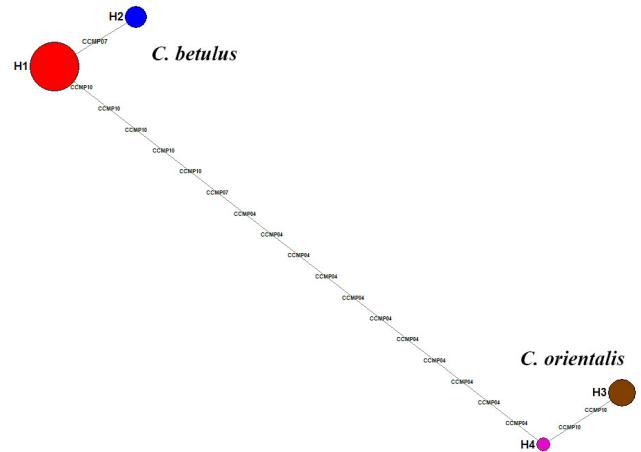


Fig. 2. Maximum parsimony phylogenetic network among four chloroplast haplotypes observed in three chloroplast SSRs loci. Size of circles corresponds to number of individuals of a given haplotype

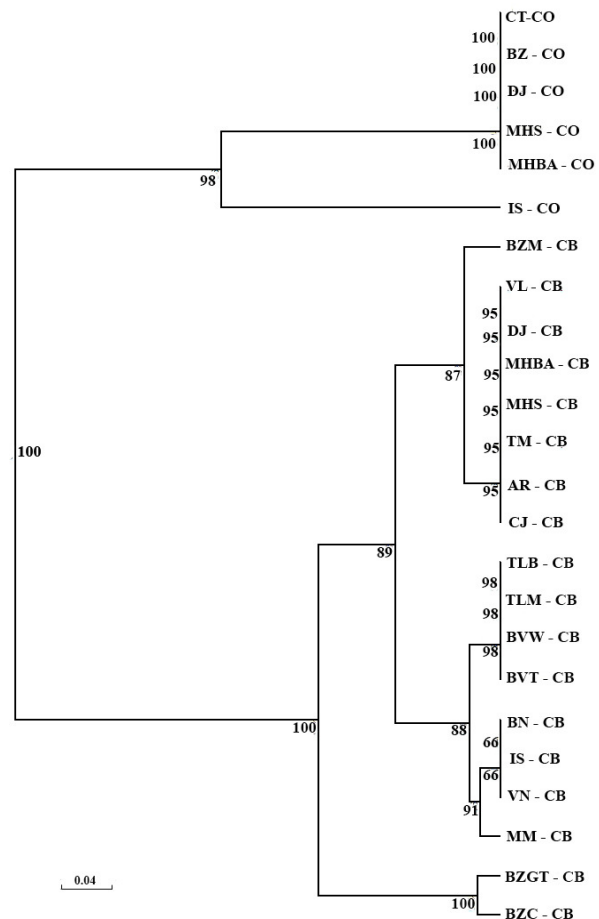


Fig. 3. UPGMA dendrogram based on genetic distances (Nei, 1983) among populations (*C. betulus* - CB; *C. orientalis* - CO)

Discussion

Our data confirmed previous results that the two *Carpinus* species do not share common chloroplast haplotypes (Grivet and Petit, 2003). The non-overlapping and non-sharing of haplotypes between *C. betulus* and *C. orientalis* suggest that a much more ancient separation of these two taxa occurred perhaps even before quaternary glaciations, as discussed for other related species (Hewitt, 1999). Indeed, sequences of the nuclear ribosomal internal transcribed spacer (ITS) suggest an extensive biogeographic track between southeastern Europe and eastern Asia and most importantly indicate for two distinct clades of *Carpinus* where *C. betulus* is grouped in a different clade than *C. orientalis* (Yoo and Wen, 2002). This result suggests that these two species have their origin in eastern Asia considered a refugium for the section *Carpinus*. Paleobotanic fossils of the section *Carpinus* have been reported for Europe from the Middle Eocene Eckfeld flora (Wilde and Frankenhäuser, 1998). Additionally, each species has a different ploidy level with $2n = 8x = 64$ for *C. betulus* (Petrova, 2006) and $2n = 2x = 16$ in *C. orientalis* (Santamour, 1995). The differentiation among *C. orientalis* populations was very high and reached a value of 1, which is due to the fact that populations are fixed for one haplotype or another. Our data indicates fixation of haplotype H3, which was found in five out of the six populations. The high differentiation detected in *C. orientalis* populations can be explained by strong fragmentation of populations that are isolated over long distances. High values of genetic differentiation was also observed in *Corylus avellana* ($G_{ST} = 0.89$) with low dispersal abilities and which is member of the same family *Betulaceae* (Petit et al., 2003; Leinemann et al., 2013). The low values of differentiation similar to those observed in our study for *C. betulus* populations were reported for *Castanea sativa* ($G_{ST} = 0.43$ - Fineschi et al., 2000) and *Betula pendula* ($G_{ST} = 0.424$ - Palmé, 2003; Leinemann et al., 2013). It was estimated that, on average, the woody angiosperms present a differentiation index between populations of 0.73 (Petit, 1999). The relatively low genetic differentiation among *C. betulus* populations may be explained by historical effective seed dispersal over short but also over long distances during postglacial recolonisation (Heuertz et al., 2004).

The geographic distribution of cpDNA haplotypes in *C. betulus* reveals a phylogeographic pattern, which is comparable with a recent study on hornbeam by Postolache et al. (2017) based on PCR-RFLP analysis. Haplotype H2 is preponderant in populations located on the external side of the southeastern Carpathians arch (Gura Teghii - 75% and Colți - 80%). In this area, palynological analysis revealed early records of *C. betulus* at lower altitudes in the Subcarpathian region (Magyari, 2002; Willner et al., 2009). The same haplotype H2 was also observed in a quite distant *C. betulus* population located in the north-west part of Romania (Apa Sărată), the Maramureș region, where it has a relative frequency of 60%. It is worth to mention the high frequency of H2 in the population (MM) from northern part of Romania and in two populations from the Curvature Carpathians (BZC and BZGT). These

populations are very close to the 'suture zones' identified in the Ukrainian and in the Curvature Carpathians, which resulted as a consequence of contact between different recolonization routes (Postolache et al., 2017). Delineated postglacial recolonisation routes by Postolache et al. (2017), confirm our phylogeographic pattern and explain the observed geographic distribution of haplotypes (H1 and H2).

The existence of a northern latitude refugium for *C. betulus* was previously mentioned in the seminal work of Willis (1996). Indeed, this was also suggested by Magyari (2002) who mentioned the existence of a glacial refugium for *C. betulus* in the North Hungarian Middle Mountains, based on palynological data. More recently, Mitka et al. (2014) also indicated a possible refugium in the Northern Carpathians, and raised the question of whether post-glacial recolonisation would have been plausible from this northern refugium. However, based on the recent findings (Postolache et al., 2017), we can suggest that haplotype H2 most probably originated in the south-eastern part of Bulgaria. The glacial refugium for haplotype H1 was most probably located in the mountains of Rhodope and Pirin, while the glacial refugium for H2 was located separately in the Strandzha Mountains, very close to the Black Sea coast. The post-glacial re-colonization routes B and C that correspond to haplotypes H1 and H2, respectively, were described in detail by Postolache et al. (2017).

The two haplotypes detected in *C. orientalis* are located in two distinct geographic areas: haplotype 3 being specific for southern part of Romania, while haplotype 4 was observed in an isolated population located at the northern limit of species range. Haplotype H3 is more present in the area of forested steppe lands, located in the southern part of the Carpathians and may suggest that those populations might have a common origin most probably as a result of recolonisation from southern Balkan refugia (Grivet and Petit, 2003). The presence of a distinct haplotype (H4) in the Roșcani population may be explained by a different glacial refugium origin. Based on this finding we may suggest that Roșcani population deserve high priority for conservation and can be included in the genetic resources of this species. Interestingly, haplotype H4 that was identified 200 kilometres farther north than the nearest southern *C. orientalis* populations (Jideni, Ramnicu Sărat). Isolated *C. orientalis* populations are also found in Vaslui (Bogdana, Lupesti, Stănileşti) (Chifu et al., 2006), at a distance of 100-110 km from Roșcani population, therefore this population would deserve more attention in future studies with both chloroplast and nuclear SSRs. Roșcani population that harbour haplotype H4 consists of a small number of trees, that was possibly larger in the past although there is no evidence of recent bottleneck (based on nSSRs analysis (data not shown)). The origin of this unique haplotype is difficult to be explained, but it is worth to mention that this *C. orientalis* population is located at the limit of species natural distribution range in highly fragmented habitats.

Previous studies have also shown that *C. betulus* populations from Romania harbour outstanding genetic polymorphism compared with western European populations (Grivet and Petit, 2003). Indeed, we observed seven polymorphic *C. betulus* populations, out of 18, using

only three cpSSR markers. All six populations of *C. orientalis* are fixed for one of the two detected haplotype. To our knowledge, this is the first study on *C. orientalis* genetic structure of populations from Romania. The small number of observed haplotypes in both species may suggest that only few glacial refugia of *Carpinus* species have existed in southern Balkans during the Late Glacial Maximum (LGM). We may thus expect that populations of *Carpinus* species from southern Balkans may harbour unique haplotypes, which was confirmed in a recent study (Postolache et al., 2017), but those populations did not succeed to expand and to migrate because of tough competition with early successional trees species (Grivet and Petit, 2003). Additionally, the high heterogeneity of mountain landscape across Balkan Peninsula may hinder expansion northwards of *Carpinus* species. Reduced genetic chloroplast DNA variation was also observed in beech (Demesure et al., 1996), which is also considered a late-successional tree species and also in hazel (Palmé, 2002; Leinemann et al., 2013). Most of these studies suggest that long glacial episodes have a profound effect on geographic organization of chloroplast DNA variation. The analysis of chloroplast genome in two *Carpinus* species (*C. betulus* and *C. orientalis*) revealed a distinct geographic distribution of cpDNA haplotypes, which emphasize that hornbeam forests have undergone idiosyncratic postglacial migration. The interspecific phylogeography of *Carpinus* spp. succeeded to reveal hornbeam populations with peculiar genetic structure (e.g. Roșcani), which may have important consequences on the management and conservation of *Carpinus* spp. genetic resources in Romania.

Conclusions

Only a limited number of chloroplast haplotypes was observed in both *Carpinus* species across Romania. The geographic distribution of chloroplast haplotypes suggests that Carpathian Mountains may have played an important role during the Holocene postglacial recolonization history of *Carpinus* species. Our study confirms that both *Carpinus* species do not share common haplotypes, which may suggest a much more ancient separation of these two taxa. A very rare chloroplast DNA haplotype was identified in an isolated peripheral population of *C. orientalis*, which deserve a high priority for conservation. The geographic structure of chloroplast genetic variation in *Carpinus* species appears to be primarily a reflection of past (postglacial) migration history, whereas more recent adaptive and stochastic processes have occurred at more local scales.

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