

Bulgarian *Populus tremula*: a genetic evaluation based on chloroplast DNA

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Received: 24 May 2026 / Accepted: 05 June 2026 / Available online: 22 June 2026



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Abstract

The study aimed to assess the genetic diversity and population differentiation of Bulgarian *Populus tremula* L. using chloroplast DNA (cpDNA) markers. Samples were collected from 14 natural populations, and the genetic variation was analysed using PCR–RFLP of six cpDNA regions. A total of 11 cpDNA haplotypes were identified, with the most frequent haplotype occurring in the majority of populations. Haplotypic diversity within populations varied considerably, ranging from monomorphic populations to populations harbouring up to five haplotypes. Overall genetic diversity was moderate to high, while population differentiation was of moderate magnitude, indicating both shared ancestry and local differentiation. Some haplotypes not reported before were identified, indicating distinctive haplotypic compositions and suggesting the presence of regionally important genetic lineages. Comparative analysis with published European data revealed both shared and unique haplotypes. The results are consistent with the hypothesis of Balkans and Bulgaria as part of a complex and important glacial refugium but larger sample set is necessary for more general inferences.

Key words: differentiation, European aspen, genetic diversity, haplotypes, PCR–RFLP, phyllogeography.

Introduction

Poplars (genus *Populus*) are considered ‘model trees’ of the temperate forest tree species. This is due to several advantages possessed by the species of this genus (Ellis et al. 2010). And while the distribution of most European species is restricted to the river banks, there is one notable

exception – European aspen (*Populus tremula* L.) – which is the only European mountain poplar. It possesses greater plasticity and occurs across a wide range of habitats, in the plains at northern latitudes and on the mountain slopes in the southern (Caudullo and de Rigo 2016). The species is considered an important part of the forest in the temperate and bo-

real regions. The wide area of distribution, fast growth and valuable, even though soft timber defines its economic importance. It has been extensively used in the breeding programs of the white poplars section (*Populus*; syn. *Leuce* Duby). An important ecological and evolutionary strategy of *P. tremula* is its clonality, which, combined with the fast growth, allows the species to rapidly colonize appropriate ecological niches.

Almost all poplar species are water-demanding (Slavov and Zhelev 2010) and are expected to face challenges in the light of the climate changes, predicting increasing drought in Central and Southern Europe.

Being an economically important tree species with large distribution, *Populus tremula* has provoked substantial research interest. The studies encompass different subjects like ecology (Kivinen et al. 2020), economic importance and management (Kusbach et al. 2024), disease resistance (Zhang et al. 2014, Verbylaitė et al. 2015), genetic diversity and differentiation (Suvanto and Latva-Krjanmaa 2005, de Carvalho et al. 2010, Politov et al. 2015), phylogeography (Bittkau 2002; Lexer et al. 2005, 2007; Fussi et al. 2010), and genomics (Ingvarsson 2005, Klee-mann et al. 2011, Stölting et al. 2015, Kersten et al. 2016). The hybridization between *P. tremula* and *P. alba* L. has been extensively studied, too (Fussi et al. 2010, Alexandrov and Karlov 2019) both in the mixed stands and during the recolonization process in different parts of its area of distribution, and in relation to breeding activities.

Like in many parts of its natural area of distribution, *P. tremula* in Bulgaria has been neglected species (with some minor exceptions, e.g. Ganchev 1983), in comparison with the other important hard-

woods, like Oaks, Beech and even Black poplars. The information about its genetic structure is practically lacking, which is an important gap because Balkans and Bulgaria, in particular, are considered an important glacial refugium and source for recolonization (Piotti et al. 2017, Gömöry et al. 2020). Therefore, the objective of the present study was to characterize the genetic diversity of *Populus tremula* populations in Bulgaria by mean of chloroplast DNA markers.

Material and Methods

Plant material

Samples were collected from 14 natural populations (Table 1). Both leaves and dormant winter buds were sampled. Samples were taken from either solitary growing individuals situated at least 100 m from other trees of the species, or from trees growing in groups of clonal origin (called hereafter 'clonal groups'). In the case of such sampling, only one tree was sampled in each clonal group. Because the groups were relatively well distinguished on the mountain slopes, the distance among them here was of lesser importance but was, in all cases, not less than 200 m. Twelve of the 14 sampled populations were pure *P. tremula*, while two (Melnik and Krichim), assigned to *P. alba* and/or *P. × canescens*, were used for comparison. Morphological examination classified the samples of Melnik as belonging to *P. alba*, and these of Krichim as a mixture of *P. alba* and *P. × canescens*. Therefore, these two populations were referred to as *P. alba/P. × canescens*. Sample size varied from 6 to 10 trees per population, but the number of successfully genotyped trees was lower – from 2 to 6.

Table 1. Studied populations of *P. tremula*.

Location (population abbreviation)	Geographic coordinates	Sample size ¹⁾
<i>Populus tremula</i> L.		
Konyavska Mts (KON)	42°23' N; 22°48' E	10 (6)
Smolyan (SMO)	41°36' N; 24°40' E	7 (2)
Teshel (TES)	41°40' N; 24°20' E	10 (3)
Vitosha – Chuypetlovo (VT2)	42°32' N; 23°15' E	8 (3)
Vitosha – Dragalevtzi (VT1)	42°36' N; 23°15' E	10 (3)
Chepelare (CHE)	41°46' N; 24°42' E	6 (3)
Ihtiman (IHT)	42°26' N; 23°55' E	6 (3)
Devin (DEV)	41° 45' N; 24°21' E	8 (3)
Strandzha-east – Marina ryaka (MAR)	42°07' N; 27°46' E	9 (3)
Strandzha-west – Brashlyan (BRA)	42°03' N; 27°25' E	8 (3)
Yundola (YUN)	42°07' N; 23°55' E	7 (3)
Rudozem – Ardino (RDA)	41°32' N; 24°56' E	6 (3)
<i>Populus alba</i> L./<i>P. × canescens</i> (Aiton) Sm.		
Krichim	41°58' N; 24°27' E	8 (3)
Melnik	41°29' N; 23°25' E	9 (3)

Note: ¹⁾ Numbers outside parentheses indicate the total number of sampled trees, whereas numbers in parentheses indicate the number of successfully genotyped individuals used in subsequent analyses. Population abbreviations given in parentheses are used in Table 6.

Laboratory analyses

DNA extraction was done with Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany) from approximately 30 mg of dry leaves, following the instructions of the producer. Polymerase chain reactions (PCR) and restriction procedures (PCR–RFLP) followed these described by Lexer et al. (2005) and Fussi

et al. (2010). Six primer pairs and five enzymes or combinations of enzymes were used for the PCR–RFLP procedures (Table 2). Amplification and restriction products were visualized in 2.5 % agarose gel stained with ethidium bromide. Fragments were scored visually, with the exception for the fragment II (rpl16R1516+rpl16F71R). One primer of this fragment was marked with fluorescent dye (D2,

Table 2. Primers, restriction enzymes and variation observed.

Fragment number	Forward primer	Reverse primer	Observed length (bp)	Restriction enzymes	Variable fragments	Characters
I	trnDP	trnTP	1066	HinfI	2	a/a
II	rpl16R1516	rpl16F71R	1180	EcoRI+HhaI	1	a
III	rpl16ex1f	rps3r2	800	EcoRI+HhaI	1	a,b
IV	rps3f2	ccmp10R	1020	HhaI+SspI	1	a,b,c,d
V	atpBSAM	rbclSAM	850	EcoRI	1	a,b
VI	ccmp10R	trnHM	600	MspI	2	a,b,c,d,e/b,c

Note: Letters (a–e) denote individual polymorphic characters (restriction-site polymorphisms) used to define chloroplast DNA haplotypes.

D3 or D4, Sigma-Proligo, USA), and the restriction products were analysed on a Beckman CEQ 8000 automatic sequencer for exact length determination (Fussi et al. 2010). For the other fragments, variants were labelled in alphabetical order according to their size, starting from the largest one. Haplotypes were identified as unique combinations of the variants of different fragments.

Data analysis

Diversity values (gene diversity and haplotypic richness) and distance measures were calculated. Gene diversity (diversity of unordered alleles, h_T) is frequency dependent and accounts for relative diversity in a population. Haplotypic richness is equal to allelic richness in the case of chloroplasts. Haplotypic richness (diversity of ordered alleles, v_T) is dependent on the sample size. Both measures, h_T and v_T , were calculated using the softwares HAPLODIV (Pons and Petit 1996) and CONTRIB (Petit et al. 1998). Testing the

existence of phylogeographic structure was done by the software PERMUT (Pons and Petit 1996).

Results and Discussion

The six possible combinations of PCR primers and restriction enzymes resulted in 18 variants identified for all 8 variable fragments. There was only one variant in each of fragments I-1, I-2 and II, two – in fragment III, four – in fragment IV, two – in fragment V, five – in fragment VI-1, and two – in fragment VI-2. These variants were combined into 11 different cpDNA haplotypes (Table 3).

The highest number of haplotypes – five – was detected in the population from the Konyavska Mountain (Table 4). Three haplotypes were identified in two populations (Teshel and Chepelare), two haplotypes were found in six populations, and five populations were monomorphic, each containing only a single haplotype. The most frequent haplotype is haplotype

Table 3. Defining of the 11 chloroplast haplotypes identified in *Populus tremula* using PCR-RFLP.

Haplotype No	Variable fragments							
	I-1	I-2	II	III	IV	V	VI-1	VI-2
H1	a	a	a	a	a	a	a	b
H3	a	a	a	a	b	a	b	b
H4	a	a	a	a	b	a	c	b
H5	a	a	a	a	b	b	b	b
H6	a	a	a	a	b	b	d	b
H9	a	a	a	a	c	b	b	b
H10	a	a	a	a	c	b	c	b
H12	a	a	a	a	c	b	e	b
H14	a	a	a	a	d	b	d	b
H16	a	a	a	b	a	a	a	c
H17	a	a	a	b	a	a	b	b

Note: Letters (a–e) denote different restriction fragment variants observed for each polymorphic cpDNA marker. Chloroplast haplotypes were defined by the combination of variant states across all variable fragments.

Table 4. Distribution of haplotypes by populations.

Population	Haplotype No											No of analysed individuals
	1	3	4	5	6	9	10	12	14	16	17	
Konyavska Mts			2				1	1	1	1		6
Krichim ¹⁾	1	2										3
Smolyan			1				1					2
Teshel			1		1	1						3
Vitosha			2									2
Dragalevtzi			3									3
Chepelare			1			1			1			3
Melnik ¹⁾		3										3
Ihtiman				1							1	2
Devin			2					1				3
Strandzha–Marina reka			1			2						3
Strandzha–Brash-lyan			3									3
Yundola			1			2						3
Rudozem–Ardino			3									3

Note: ¹⁾ *P. alba*/*P. × canescens*.

no. 4, which was detected in 11 out of the 14 populations. Haplotype 9 was found in four populations. Haplotypes 3, 10, 12, and 14 were detected in two populations each, while haplotypes 1, 5, 6, 16, and 17 were found in only one population each.

More haplotypes were found by Fussi et al. (2010) in the populations from Central Europe (3 to 10), and only 2 in a Swedish population. However, it should be outlined considerably larger sample sizes in their study. Our results are comparable with those of Salvini et al. (2001), who reported between two and four haplotypes per population (six in total) in their study on *P. tremula* in Italy.

The total haplotypic diversity (h_T and v_T) was higher as compared to within-population diversity. It was higher when all 14 populations were included in the statistical analysis (0.736 and 0.743) and slightly lower when analysis was performed only on pure *P. tremula* (0.636 and 0.638).

Within-population diversity ranged from 0.359 to 0.496. These values are significantly lower than those reported by Fussi et al. (2010) – 0.772 for within-population diversity and 0.898 for total genetic diversity. The values obtained in the present study are comparable to those reported by Salvini et al. (2001) – 0.55 for h_S and 0.70 for h_T .

Genetic differentiation coefficient based on ordered alleles (N_{ST}) had larger value than the differentiation coefficient based on unordered alleles (G_{ST}) only when all populations were included in the analysis, while N_{ST} was lower when the analysis was run only on *P. tremula* populations only (Table 5).

Contributions of the different populations to genetic diversity within populations (hk) ranged from 0 to 1, with a mean value of 0.495, indicating relatively high within-population diversity. Total genetic diversity (h_T) ranged from 0.538 to 1.00,

Table 5. Level of diversity and differentiation of ordered and unordered alleles calculated by the software PERMUT (Pons and Petit 1996).

All 14 populations				Only <i>Populus tremula</i>			
Unordered alleles		Ordered alleles		Unordered alleles		Ordered alleles	
h_s	0.495 (0.107)	v_s	0.359 (0.092)	h_s	0.464 (0.123)	v_s	0.496 (0.132)
h_T	0.736 (0.086)	v_T	0.743 (0.086)	h_T	0.638 (0.098)	v_T	0.636 (0.095)
G_{ST}	0.327 (0.121)	N_{ST}	0.517 (0.151)	G_{ST}	0.273 (0.168)	N_{ST}	0.221 (0.164)

Note: h_s and v_s – average haplotypic diversity within populations; h_T and v_T – total haplotypic diversity; G_{ST} – nucleotide differentiation, N_{ST} – coefficient of genetic differentiation based on ordered (phylogenetically related) haplotypes, considering both haplotype frequencies and genetic distances among haplotypes.

with a mean value of 0.735 (Table 6).

Differentiation (G_{ST}) is of moderate magnitude for the system of populations. For each population, a surrogate of this measure was calculated following the method of Petit et al. (1998). The values indicating the contribution of each population to the total diversity is used to identify the most genetically distinct populations for conservation purposes. The

mean differentiation index ($DGst$) indicated a relatively high level of differentiation – 0.33. Differentiation among individual populations ranged from 0.074 to 0.719. The lowest values were observed for the Teshel population, while the highest were observed for the Melnik population. Fussi et al. (2010) reported a significantly lower degree of differentiation (0.147); however, in our case both populations of *Pop-*

Table 6. Contribution of the different populations to the total genetic diversity and differentiation calculated by using the software CONTRIB (Petit et al. 1998).

Pop.	Nh	Nps	hk (s.e.)	DHs	DHt	DGst	Ct(k)
KON	5	4	0.933 (0.122)	0.697	0.816	0.146	0.018
KRI	2	1	0.667 (0.314)	0.574	0.949	0.395	0.048
SMO	2	1	0.667 (0.314)	0.574	0.671	0.144	-0.015
TES	3	1	1.000 (0.272)	0.728	0.786	0.074	0.011
VT2	1	1	0.000 (0.000)	0.267	0.538	0.505	-0.045
VT1	1	0	0.000 (0.000)	0.267	0.538	0.505	-0.045
CHE	3	4	1.000 (0.272)	0.728	0.782	0.069	0.011
MEL	1	0	0.000 (0.000)	0.267	0.949	0.719	0.048
IHT	2	2	0.667 (0.314)	0.574	1.000	0.426	0.060
DEV	2	3	0.667 (0.314)	0.574	0.671	0.144	-0.015
MAR	2	0	0.667 (0.314)	0.574	0.761	0.245	0.006
BRA	1	0	0.000 (0.000)	0.267	0.538	0.505	-0.045
YUN	2	2	0.667 (0.314)	0.574	0.761	0.245	0.006
RDA	1	1	0.000 (0.000)	0.267	0.538	0.505	-0.045

Note: Nh – number of haplotypes; Nps – number of polymorphic sites; hk – gene diversity within population k ; s.e. – standard error; DHs – divergence of the respective population from the remaining ones based on intrapopulation diversity; DHt – divergence based on among-population diversity; $DGst$ – divergence based on total genetic differentiation; $Ct(k)$ – contribution of the respective population to total genetic diversity (see Petit et al. 1998 for details).

ulus alba are included, whereas in the cited study the differentiation for *P. alba* showed similar values – 0.303. Low differentiation values were also reported by Salvini et al. (2001) – 0.22. The index describing the contribution of each individual population to the others, based on allelic richness ($Ct(k)$), presented in the last column (Table 6), is of lesser informational value when chloroplast markers are used, because genetic variation in chloroplast markers is generally lower than that of nuclear markers (Yang et al. 2017).

Chloroplast RFLP markers could be of much greater importance for phylogeographic studies and for identifying migration routes and glacial refugia in Europe (Birks and Willis 2008) and also on Balkans (see Postolache et al. 2017).

The geographic distribution of haplotypes does not reveal any strict patterns (Fig. 1). The lowest diversity is ob-

served on Vitosha, where only a single identical haplotype was detected in both populations. Somewhat surprisingly, the Strandzha populations also show a low level of diversity – one is monomorphic, and only one additional haplotype was detected in the other. Strandzha is a region that remained unaffected by the last glaciations, and therefore higher genetic, including haplotypic, diversity would be expected there. For example, for *Carpinus betulus* L. it was considered an important microrefugium within the larger Balkan refugium (Postolache et al. 2017). The populations from Krichim and Melnik, which belong to the *Populus alba*/*P. × canescens* group, are specific in this respect. In these populations, two haplotypes were detected that are absent in *P. tremula* populations (haplotypes no. 1 and 3) and could potentially be used for species identification. The proper status

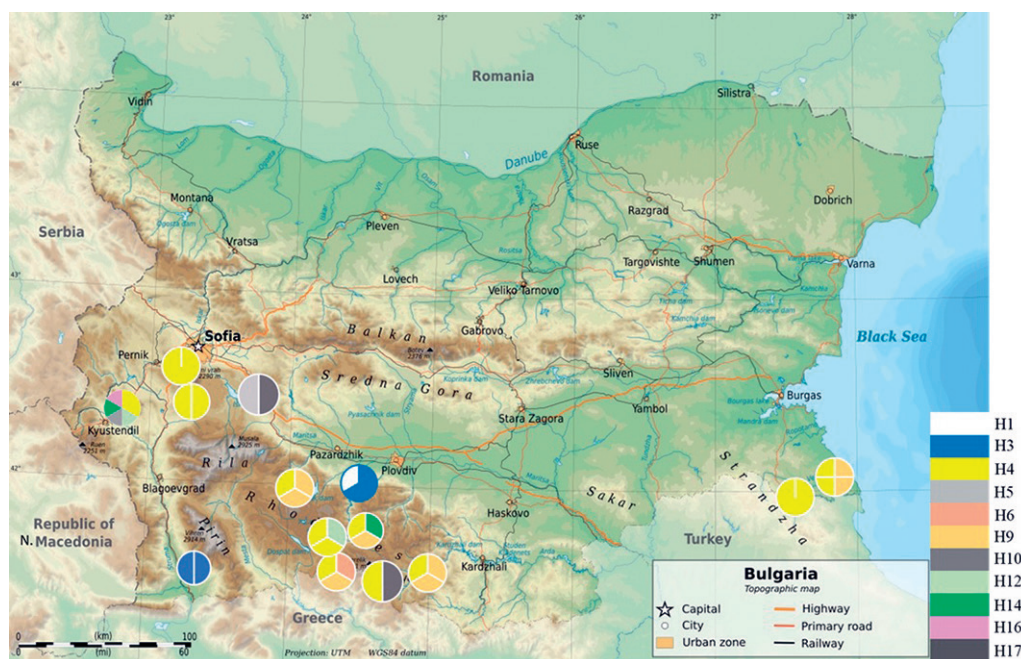


Fig. 1. Geographic distribution of chloroplast PCR-RFLP haplotypes in Bulgaria.

of populations of *P. alba* and *P. canescens* could be clarified by applying broader set of markers, as recommended by Fossati et al. (2004). However, we must state that the small sample size used in the present study does not allow general inferences.

When the results of the present study are placed in an international context (see Fig. 3 in Fussi et al. 2010), it becomes evident that five of the haplotypes detected in Bulgaria have also been found in other parts of Europe. Haplotype no. 4, which is the most frequent under our conditions (present in 11 populations), has been reported from Croatia; haplotype no. 5 (found only in Ihtiman in our study) has been reported from the Czech Republic, Slovakia, Hungary, and Italy; haplotypes nos 10 and 12 have been reported from Italy; and haplotype no. 17 (found only in Ihtiman) has been reported from the Czech Republic (Fussi et al. 2010). The most frequent haplotype in the study by Fussi et al. (2010), designated as no. 21, was not detected in our populations. This preliminary overview indicates that differences exist between populations from Bulgaria and those from other parts of Europe, the causes of which require further clarification. If the 'refugium within a refugium' hypothesis is adopted (Abellán and Svenning 2014), the most likely candidates among the studied populations are those from Konyavska Mountain and the region of the Central Rhodopes. The Ihtiman population also shows unique haplotypes and is probably influenced by Sredna Gora and the Balkan Range, but this issue requires further investigation.

Conclusions

The results of the present study, although based on a relatively small sample size,

allow the following conclusions:

This is the first study of Bulgarian *Populus tremula* using PCR-RFLP chloroplast markers. The number of haplotypes identified was comparable to that reported in regional European studies. Approximately half of the haplotypes previously recorded elsewhere in Europe were detected in Bulgaria, whereas the remaining haplotypes appear to be restricted to the studied material. They represent different genetic lineages, whose origin whose origin still remains to be studied.

Haplotype diversity within populations was higher than that among populations, which is consistent with patterns commonly reported for forest tree species, including poplars.

The observed haplotype diversity is consistent with the hypothesis that the Balkan Peninsula, including present-day Bulgaria, served as a glacial refugium for forest tree species.

Populus tremula and *P. alba*/*P. × canescens* exhibited clearly different patterns of haplotype variation, demonstrating the discriminatory power and potential usefulness of the chloroplast markers employed in this study.

Acknowledgements

This work was supported by projects KP-06-Austria/9 and BG 06/2023 under the Bulgaria–Austria Bilateral Programme for Scientific Cooperation. We thank two anonymous reviewers for their constructive comments and suggestions.

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