

## Genetic Analysis of Swiss Stone Pine Populations (*Pinus cembra* L. subsp. *cembra*) from the Carpathians Using Chloroplast Microsatellites

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**Abstract** – The diversity of Swiss stone pine populations (*Pinus cembra* subsp. *cembra*) native to the Carpathians was studied with chloroplast microsatellite markers (cpSSR). Six loci were analyzed in four populations (altogether 148 individuals) and a total number of 22 size variants and 41 combinations referred to as haplotypes were detected. Diversity within populations was found to be high, whereas divergence between the populations was low ( $F_{st} = 0.02$ ). The most variable population with the highest haplotype diversity ( $H = 0.956$ ) originated from the Retezat Mountains (South Carpathians). Multi-stemmed individuals were detected mainly in the Southern part of the Calimani Mountains. They were found to be genetically non-homogenous. It is assumed that these clusters of individuals are the result of plants emerging from seed caches by birds. The great haplotypic variation found in cpSSR loci makes all the populations a useful source for gene conservation purposes. Each population should be considered an important element of the local ecosystem diversity.

**chloroplast DNA microsatellites / size variants / haplotypic diversity**

**Kivonat** – Genetikai diverzitás vizsgálatok a Kárpátok cirbolyafenyő (*Pinus cembra* L. subsp. *cembra*) populációiban kloroplasztisz mikroszatellit markerek felhasználásával. A tanulmányban az európai cirbolya (*Pinus cembra* subsp. *cembra*), Kárpátokban élő, populációinak diverzitását vizsgáltuk kloroplasztisz DNS mikroszatellit markerekkel (cpSSR). 6 SSR lokusz analízisének során négy populációban összesen 21 méret variánst és 41 kombinációt, azaz haplotípust, mutattunk ki (148 egyed). Minden populáció diverzitása magas volt, de a populációk közötti divergencia mértéke alacsonynak bizonyult ( $F_{st} = 0,02$ ). A legnagyobb haplotípus variabilitást a Retyezát havasok populációjában tapasztaltuk, ( $H = 0,956$ ). Iker- és soktörzsű egyedek vizsgálata során kimutattuk, hogy a kéttörzsű egyedek többsége eltérő genotípusú volt. Ezek vélhetően a madarak táplálék raktározó készletéből és együttes csírázásából származtak. A legtöbb ilyen egyedet a Kelemen havasok déli állományában találtuk. A populációk nagy genetikai variabilitása felhívja a figyelmet arra, hogy minden egyes állomány a faj fontos génkészletét képezi, ezért minden kis populáció fokozott védelmét biztosítani kell. A cirbolyafenyő mint jégkorszaki reliktum csakis ily módon maradhat a montán ökoszisztémák diverzitásának meghatározó eleme.

**kloroplasztisz DNS mikroszatellit / méret variáns / haplotípus diverzitás**

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## 1 INTRODUCTION

Swiss stone pine (*Pinus cembra* subsp. *cembra*) is considered to be a glacial relict living in European high mountains, surviving at the timberline and in subalpine forest habitats of the Alps and the Carpathians. The biogeographic distribution of the remaining populations is due to the retreat of individuals in response to the changes of the macroclimate, to the availability of suitable habitats (and some other aspects, in particular to competitive exclusion). Climatic and environmental conditions during the full-glacial facilitated the dispersal of *Pinus* species, including *Pinus cembra*, within Central Europe (Willis et al. 1998). Macroscopic charcoal evidence linked with molluscan paleofauna study supports the presence of *Pinus cembra* even at low elevations of the Carpathian basin (Willis et al. 2000). The wide distribution was interrupted by the global warming of the Holocene when broad scale vegetation changes occurred and this resulted in the withdrawal of pines, including Swiss stone pine, that has retreated to higher elevations. Because of missing macrofossil data, it is hardly possible to resolve the postglacial history of this species. However, some palynological records of the Calimani mountains (Eastern Carpathians) provide evidence for a sporadic occurrence of *Pinus cembra* in the high mountain area since the Boreal-Atlantic age (8000-5000yr. B.P.; Farcas et al. 1999, 2000). It seems that this species suffered a sharp reduction of its range, and population sizes decreased markedly during the postglacial period. Presumably, isolation by distance that largely or completely inhibited gene flow, had some effects on the population structure of the species. However, at the species level, drift in single fragmented populations may even increase among-population variance (Mátyás 2004).

The present genetic structure of the species stands is influenced by the gene-flow patterns (Richardson et al. 2002). In case of whitebark pine (*Pinus albicaulis*) and in stone pine species in general, gene flow is determined by wind-dispersed pollen and by seed dispersed by nutcrackers (*Nucifraga* spp.). The multi-stemmed growth form is attributed to multiple germination of seed caches set up by birds (Linhart – Tomback 1985, Turcek 1961). Thus birds play a crucial role in the natural reproduction and distribution of the stone pine species in Eurasia and North America.

The population genetic structure of Eurasian stone pine species including Swiss stone pine was studied by Goncharenko et al. (1992). Isozyme studies revealed generally high genetic variability in case of all pine populations of the subsection *Cembrae*, but low genetic differentiation between populations. These facts are supported also by the results of Krutovskii et al. (1995). They found that these species have  $F_{st}$  values ranging between 0,02–0,04. While in each study authors included just one population of *P. cembra* – considered peripheral – from the Carpathians (West-Ukraine), they could not characterize the variability level of the Carpathian populations in general. The genetic variability of eleven Swiss stone pine populations was investigated by Szmidt (1982). Based on allozyme frequencies and average heterozygosity, genetic diversity was found to be low within populations, while genetic divergence between populations was considered higher than that calculated for other conifers. He found that the Romanian population from the Retezat Mts., included also in the present study, was the second most outstanding population with regard to its genetic diversity.

To evaluate the present genetic structure of the protected relic populations as well as to characterise diversity patterns of the populations native to different regions of the Carpathians we studied cpDNA microsatellite (cpSSR) haplotype variability of *Pinus cembra* subsp. *cembra*. Paternally inherited cpDNA SSR markers with high levels of variability have proved to be valuable for detecting genetic structure of populations in many conifer species (Echt et al. 1998, Vendramin et al. 1998). Three from the primer pairs designed by Vendramin et al. (1996) for *Pinus thunbergii* have already been applied successfully for species belonging to subsection *Cembrae* of the section *Strobus* (Gugerli et al. 2001). While populations of the

Alps have already been included in molecular studies, evaluation of those occurring in the Carpathians would extend the knowledge about this regionally endangered species.

## 2 MATERIAL AND METHODS

### 2.1 Sampling

148 individuals of four stands belonging to three geographically distinct regions of the Carpathians were analysed (*Table 1*). The distance between the first two populations is less than 20 km, therefore gene flow was between them presumed. Leaf material was collected from individuals that were at least 30 m apart. Exceptions were multistemmed or clusters of young individuals attributed to the germination out of seed caches. These clusters were found mainly in the South Calimani Mts.

*Table 1. Location of sampled populations*

Abbrev.	Population location	Region	Latitude N	Longitude E	No. of samples
Cal-Suc.	Mt. Calimani-North District Suceava	Eastern Carpathians	47° 14'	25° 20'	57
Cal-Mur.	Mt. Calimani-South District Mures	Eastern Carpathians	46° 57'	25° 06'	57
Ret.	Mt. Retezat District Hunedoara	Southern Carpathians	45° 32'	22° 57'	24
Tatra	Mt. Solisko District Prešov	Tatra Mts.	49° 07'	20° 04'	10

### 2.2 DNA extraction and PCR reaction

Genomic DNA was extracted from leaf material dried on silica gel, following the Qiagen Plant Mini kit protocol.

Six mononucleotide microsatellite loci (SSR) from the chloroplast DNA (Pt36480, Pt26081, Pt63718, Pt 30204, Pt87268, Pt15169) were screened for length variation. PCR amplifications were performed according to Vendramin et al. (1996) using a Perkin Elmer 9600 thermal cycler with the following profile: 5 min. at 95 °C, 5 min at 80 °C, 25 cycles of 1 min. at 94 °C, 1 min. at 55 °C, 1 min. at 72 °C, with a final extension step at 72 °C for 8 min. The success of the amplification was tested on a 1.4% agarose gel.

### 2.3 Fragment analysis

Amplified fragments were multiplexed by size. Two or three fragments of different size ranges were pooled and loaded along with internal size standards (50, 100, 150, 200 bp), and external standards were used. Size detection was performed on an Alf Express Fragment Analyser (Amersham) using Reprogel Long Read acrylamide gel. Each run contained a control individual. Fragments that differed in size by 1 bp could be reliably distinguished and assigned to different categories for the same samples run on different gels. Results were analysed with Fragment Manager 1.2 (Amersham). Several samples were run twice to increase the accuracy.

## 2.4 Statistical analysis

Haplotypic diversity, estimated by  $H = (n/n-1)(1-\sum p_i^2)$ , where  $p$  refers to the haplotype frequencies, and  $n$  to the number of individuals per population (Nei 1987), effective number of haplotypes was estimated by  $n_e = 1/\sum p_i^2$ , frequency of the most common haplotype and number of different size variants were calculated (Table 3). Partitioning of molecular variance and also fixation indices ( $F_{st}$ ) were estimated by an analysis of molecular variance (AMOVA). Comparison of pairs of population samples were conducted using the genetic distance approach based on haplotype frequency under the infinite allele model (IAM), as well as using the stepwise mutation model (SMM) (Slatkin 1995) in a second approach. These procedures were performed using Arlequin software (v. 2.0; Schneider et al. 2000).

## 3 RESULTS

Five out of six cpSSR loci analysed were polymorphic, giving a total of 22 different size variants. The distribution of the size variants in the studied populations is presented in Figure 1. Rare variants were present mainly in the Retezat Mts. as well as in the Calimani-Suceava population.

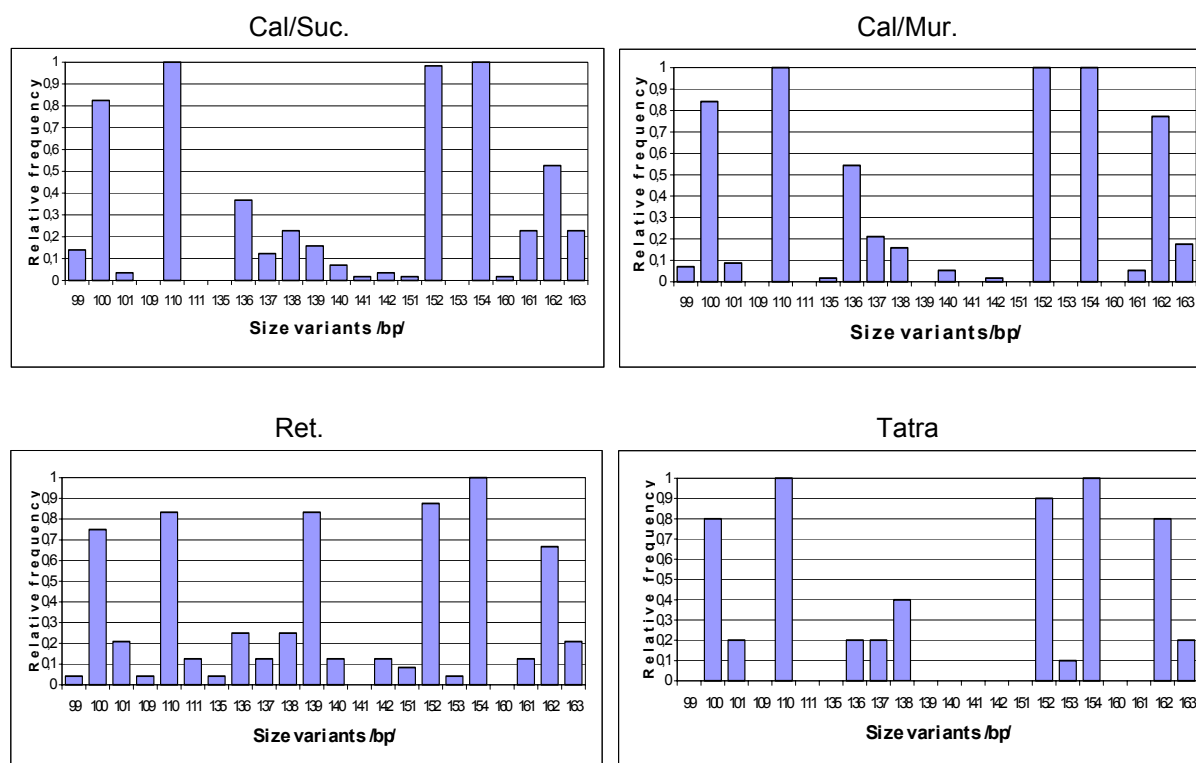


Figure 1. Distribution of size variants in the studied populations: locus 1 (3 size variants), locus 2 (3 size variants), locus 3 (8 size variants), locus 4 (3 size variants), locus 5 (1 size variant), locus 6 (4 variants)

The most variable locus was Pt15169, having altogether eight variants, followed by Pt30204 with four size variants. We detected 41 different combinations of size variants i.e. haplotypes. No significant correlation was found between the number of size variants and population size. There was also no significant correlation between the number of haplotypes and the population sample size. Frequency and distribution of haplotypes are reported in Table 2.

Table 2. Frequency and distribution of haplotypes in the four studied populations

Haplotype label	Cal-Suc.	Cal-Mur.	Ret.	Tatra	Average	Private haplotype for populations
1	0.018	0.018	0.042	0	0.020	
2	0.035	0	0	0	0.014	Cal-Suc.
3	0.053	0	0.042	0	0.027	
4	0.018	0	0	0	0.007	Cal-Suc.
5	0.053	0	0	0	0.020	Cal-Suc.
6	0.053	0	0.058	0	0.034	
7	0.105	0.123	0	0.200	0.101	
8	0.018	0	0	0	0.007	Cal-Suc.
9	0.018	0	0	0	0.007	Cal-Suc.
10	0.193	0.351	0.167	0.100	0.243	
11	0.018	0	0	0	0.007	Cal-Suc.
12	0.070	0.018	0	0	0.034	
13	0.035	0.018	0	0	0.020	
14	0.035	0.175	0.083	0.200	0.108	
15	0.018	0.105	0.042	0	0.014	
16	0.035	0.018	0	0	0.020	
17	0.018	0	0	0	0.007	Cal-Suc.
18	0.035	0	0.042	0	0.020	
19	0.035	0.053	0.042	0	0.041	
20	0.018	0	0	0	0.007	Cal-Suc.
21	0.070	0	0	0	0.068	Cal-Suc.
22	0.018	0	0	0	0.007	Cal-Suc.
23	0.018	0.018	0	0	0.014	
24	0.018	0	0.125	0	0.027	
25	0	0.018	0	0.100	0.014	
26	0	0.018	0	0	0.007	Cal-Mur.
27	0	0.018	0	0	0.007	Cal-Mur.
28	0	0.018	0	0	0.007	Cal-Mur.
29	0	0.018	0	0	0.007	Cal-Mur.
30	0	0.018	0	0	0.007	Cal-Mur.
31	0	0	0.042	0	0.007	Ret.
32	0	0	0.042	0	0.007	Ret.
33	0	0	0.042	0	0.007	Ret.
34	0	0	0.042	0	0.007	Ret.
35	0	0	0.042	0	0.007	Ret.
36	0	0	0.083	0	0.014	Ret.
37	0	0	0.042	0	0.007	Ret.
38	0	0	0	0.100	0.007	Tatra
39	0	0	0	0.100	0.007	Tatra
40	0	0	0	0.100	0.007	Tatra
41	0	0	0	0.100	0.007	Tatra
Count	24	16	16	8	41	

To summarize, haplotype no.10 was the most frequent (0.243) with a high dominance in each population. 36.6% (15) of the haplotypes were common in the populations, while the remaining 63.4 % (26) – considered as private haplotypes – only occurred in one population.

The private haplotypes were represented by low frequency values, ranging between 0.018–0.1. No pairs of populations were composed of the same haplotypes. The number of private haplotypes were highest in Calimani-Suceava and Retezat, 10 (41.66%), and 7 (43.79%), respectively.

Gene diversity (unbiased haplotypic diversity), was high in all populations, with a mean value of 0.917. Most of the H values ranged above 0.90 except for Calimani-Mures (*Table 3*).

*Table 3. Measures of cpSSR variation within populations*

Population label	No of samples	No of size variants	Effective no. of haplotypes ( $n_e$ )	Frequency of the most common haplotype ( $f_a$ )	Haplotypic diversity (H)
Cal-Suc.	57	18	12.94	0.192	0.939
Cal-Mur.	57	15	5.14	0.350	0.819
Ret.	24	20	12.02	0.166	0.956
Tatra	10	13	7.14	0.100	0.955
Mean		16.5	9.31	0.20	0.917

Indicated by all genetic parameters, this population had the lowest within-population diversity. Taking all parameters into account, the most variable and divergent population was that of the Retezat Mts. The extremely high within-population variability of Retezat Mts. may be one of the reasons for the genetic differentiation of this population based on isozyme, analysis reported by Szmidt (1982). The second most variable population was the Tatra population, followed by that from the Calimani-Suceava, genetic parameters being close to those of the Retezat Mts.

AMOVA confirmed low genetic differentiation between populations (*Table 4*). Genetic divergence based on  $F_{st}$  as well as  $R_{st}$  (not shown) estimates shows low values, ( $F_{st} = 0.02$ ).

*Table 4. AMOVA results according to genetic distance,  $F_{st}$*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	3	4.854.8	0.02367	2.76
Within populations	144	119.923	0.83280	97.24
Total	147	124.777	0.85647	
Fixation index	$F_{st} = 0.0276$			

Pairwise comparison of populations according to genetic distances are presented in *Table 5*.

*Table 5. Pairwise  $F_{st}$  (below the diagonal) and  $R_{st}$  (above the diagonal) values*

Population label	Cal-Suc	Cal-Mur	Ret.	Tatra
Cal-Suc		0.098 +	0.008	0
Cal-Mur	0.037 +		0.196 +	0.123
Ret.	0.015	0.043 +		
Tatra	0.016	0.026	0	

Significance shown: “+” (significance level: 0,05)

Based on pairwise  $F_{st}$  (IAM) as well as on pairwise  $R_{st}$  (SMM), significant differences were detected between the populations of the Calimani-Mures and Retezat, as well as between the two sites of the Calimani Mountains. Although genetic differentiation resulted in higher values between the populations of Calimani-Mures and Retezat, the non-significant differentiation between Calimani-Suceava and Retezat suggests that no discernible correlation

exists between genetic and geographical distance. Pairwise estimation produced negative values for the Tatra. These values are not interpretable, are considered to be zero.

### 3.1 Multi-stemmed individuals

During material collection multi-stemmed individuals were detected in populations of the Calimani Mts., three in the south (Calimani-Mures), and one in the north (Calimani-Suceava). A group of cluster-forming individuals probably of same age was also studied (Table 6). All of them were growing on rocky surface. Three twin-stemmed out of four multi-stemmed growing individuals were genetically different. Among cluster forming individuals, one was genetically different.

Table 6. Haplotypes of twin-stemmed growing and cluster forming individuals

Label of pairs of individuals	Haplotype	Population
51/51a	152/110/100/161/154/139	Cal-Suc.
	152/110/100/161/154/140	
17/18	152/110/100/162/154/136	Cal-Mur.
	152/110/100/162/154/136	
2/3	152/110/100/162/154/136	Cal-Mur
	152/110/100/162/154/138	
12/13	152/110/100/162/154/137	Cal-Mur.
	152/110/100/162/154/139	
Cluster of 7/8/9/10	152/110/100/162/154/136	Cal-Mur.
	152/110/100/162/154/138	
	152/110/100/162/154/136	
	152/110/100/162/154/136	

## 4 DISCUSSION

In this investigation haplotypic diversity of populations of the Carpathians has been studied. CpSSR markers revealed large polymorphism within populations. The five polymorphic loci analyzed in this study indicated a high variability level, resulting in a great number of haplotypes within each population. Private haplotypes of the populations were also detected. The low frequency of private haplotypes and the higher frequency values of the common haplotypes resulted in low differentiation between populations ( $F_{st} = 0.02$ ). These values are similar to the results obtained based on isozyme studies carried out by Krutovskii et al. (1995) in case of other stone pine species. However, these authors analyzed just one Carpathian population: our results seem to confirm their supposition that populations along the range of *Pinus cembra* in the Carpathians have generally high variability. The comparatively low genetic variability of the population in the Southern Calimani (Calimani-Mures) may be explained by the low number and scattered occurrence of individuals clustering predominantly on rocky surfaces. Private haplotypes which were detected in old individuals of more than 200 years of age, growing in narrow deep valleys, provide evidence for a once existing large population in this area forming for a wide, continuous distribution of stone pine in the Calimani Mts. This was supported also by palynological records that ascertain the species' presence in increasing quantity from 'Picea-Quercus mixture pollen horizon' of the Atlantic age, up to the surface (Farcas 1999). However, nutcrackers counterbalance the drastic decrease in the population size along the southern part, providing permanent seed

supply even from the north. Multi-stemmed clusters of individuals found genetically not identical represent an important aspect of the population diversity.

Pairwise genetic differentiation of the populations resulted in low values even between geographically distant populations. Species with a scattered range, such as *Pinus cembra*, are expected to show low within-population genetic diversity due to genetic drift and limited gene flow. In these populations isolation following the withdrawal of species range in the Holocene did not produce inbreeding that would appear in genetic structure. Possible reasons can be attributed to the longevity of stone pine, to the effective selection mechanism eliminating inbred embryos and individuals, and/or the possibility of gene flow between populations (wind dispersed pollen and animal mediated seed transfer). Even if gene flow does not occur in present, the genetic pattern reflects processes of the recent past up to few thousand years ago when stone pine was probably more widely distributed in the upper mountain zone. Due to postglacial climate conditions, natural competition between spruce and stone pine was decisive in favour of spruce dominance, and since then stone pine has retreated to marginal habitats. Presumably, natural processes were accelerated also by human activities in the last centuries. Clear-cut or burned areas in the mountain forest regions were naturally regenerated by spruce and not colonized by stone pine being already at the edge of its ecological tolerance. Such conditions seem to occur in the southern part of the Calimani Mts.

Because of the limited number of populations available in this study, results and conclusions have to be taken with caution. More extensive investigations covering additional populations should be made in the future to prove whether the genetic structure described above is proper for the whole distribution range of *Pinus cembra* in the Carpathians.

The large haplotypic variation found in cpSSR loci renders all populations a useful source for gene conservation purposes. Each scattered small population should be considered a proper genetic resource of the species and an important element of the local ecosystem diversity, which may justify the species' protection. There is a compelling claim for protection of all those habitats that are strongly influenced by human activities, resulting in extensive fragmentation of the distribution which has been already profoundly shaped previously by biogeographical events.

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