



Variation in the chloroplast DNA of Swiss stone pine (*Pinus cembra* L.) reflects contrasting post-glacial history of populations from the Carpathians and the Alps

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ABSTRACT

Aim To characterize the genetic structure and diversity of *Pinus cembra* L. populations native to two disjunct geographical areas, the Alps and the Carpathians, and to evaluate the rate of genetic differentiation among populations.

Location The Swiss Alps and the Carpathians.

Methods We screened 28 populations at three paternally inherited chloroplast simple sequence repeats (cpSSRs) for length variation in their mononucleotide repeats. Statistical analysis assessed haplotypic variation and fixation indices. Hierarchical analysis of molecular variance (AMOVA), Mantel test, spatial analysis of molecular variance (SAMOVA) and BARRIER analyses were applied to evaluate the geographical partitioning of genetic diversity across the species' range.

Results Haplotypic diversity was generally high throughout the natural range of *P. cembra*, with the mean value substantially higher in the Carpathians ($H = 0.53$) than in the Alps ($H = 0.35$). The isolated Carpathian populations showed the highest haplotype diversity among the populations originating from the High Tatras (Velka Studena Dolina) and South Carpathians (Retezat Mountains). AMOVA revealed that only 3% of the total genetic variation derived from genetic differentiation between the two mountain ranges. Differentiation among Carpathian populations was higher ($F_{ST} = 0.19$) than among Alpine populations ($F_{ST} = 0.04$). Low, but significant, correlation was found between the geographical and genetic distances among pairs of populations ($r = 0.286$, $P < 0.001$). SAMOVA results revealed no evident geographical structure of populations. BARRIER analysis showed the strongest differentiation in the eastern part of the species' range, i.e. in the Carpathians.

Main conclusions The populations of *P. cembra* within the two parts of the species' range still share many cpDNA haplotypes, suggesting a common gene pool conserved from a previously large, continuous distribution range. Carpathian populations have maintained high haplotypic variation, even higher than Alpine populations, despite their small population sizes and spatial isolation. Based on our results, we emphasize the importance of the Carpathian populations of Swiss stone pine for conservation. These populations comprise private haplotypes and they may represent a particular legacy of the species' evolutionary history.

Keywords

Alps, Carpathians, chloroplast microsatellites, Europe, haplotype diversity, Pinaceae, *Pinus cembra*, post-glacial colonization.

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INTRODUCTION

Among the stone pine species – *Pinus* sect. *Strobus* subsect. *Cembrae* (Critchfield, 1986) – widespread throughout Eurasia, Swiss stone pine (*Pinus cembra* L.) is the only species with its natural range restricted to Europe. It is considered a glacial relict, closely related to *Pinus sibirica* Du Tour, which is widely distributed along the Siberian taiga from the Urals towards northern Mongolia (Goncharenko *et al.*, 1992; Krutovskii *et al.*, 1990, 1995). *Pinus cembra* occurs in two disjunct regions: the continental parts of the Alps (central Alps), which is considered to be its core natural range; and the Carpathian Mountains, where isolated populations exist. This biogeographical pattern is assumed to have resulted from the repeated climatic fluctuations of the Pleistocene and the global warming of the Holocene. During the post-glacial period, the range of *P. cembra* suffered a sharp fragmentation as a consequence of its natural competition with Norway spruce (*Picea abies* (L.) Karst.) which has led to the dominance of spruce in large areas of European mountain forests. As a result, Swiss stone pine populations became isolated and have retreated to marginal habitats of the high mountain area, i.e. at the timber line (Huntley, 1990). Furthermore, Swiss stone pine faced negative anthropogenic impacts (exploitation, hunting of nutcrackers, which are the predominant seed disperser) and became a rare, regionally threatened woody species of Europe during the last few centuries (Marhold & Hindák, 1998).

Isolation and fluctuations in population size increase the population divergence because of reduced gene flow and random genetic drift (Hartl & Clark, 1997). These processes presumably influence the spatial genetic structure by decreasing within-population variation and by increasing between-population differentiation (Ellstrand & Elam, 1993; Eckert *et al.*, 2008). However, the impact of these processes depends strongly on the time elapsed since fragmentation (in terms of generations), and respective effects might be slowed down in long-lived tree species with long generation turnover (Hamrick *et al.*, 1992). Thus, even small sized, isolated relict populations which faced recent fragmentation may still hold important reserves of genetic variability. The present-day genetic diversity of these populations is likely to reflect the accumulated history of population fluctuations rather than the present population size (Landergrott *et al.*, 2001; Eckert *et al.*, 2008).

Swiss stone pine occupies the high mountain ecotones of the Alps and Carpathians and tends to represent the climax forest type, forming mixed stands with Norway spruce (*Picea abies*) or European larch (*Larix decidua* Mill.). However, clusters of individuals frequently grow as pioneers and inhabit steep rocky outcrops close to the tree line or even at higher elevations. Among the five-needle (Haploxylon) pine species, *P. cembra* belongs to the closed-cone pines with wingless, zoochorous seeds. Like all other pines it is monoecious, wind-pollinated and considered to be predominantly outcrossing. The species' characteristic population structure, frequently showing multi-stemmed clusters of individuals, is the consequence of a bird–

tree mutualistic relationship. While stone pine seeds are the main food source for European nutcrackers (*Nucifraga caryocatactes* L.), the wingless seeds often germinate simultaneously from seed caches set up by the birds. Thus, bird-mediated seed dispersal strongly influences the distribution and niche properties of stone pine (Tomback & Linhart, 1990).

An isozyme study by Krutovskii *et al.* (1995) on stone pine species of the subsection *Cembrae* reported a generally high degree of within-population genetic variation and low divergence among populations. Since their study included only one population of *P. cembra* from the Ukrainian Carpathians, they could not provide information about the population genetic structure of *P. cembra*. Further studies using isozymes were performed on five populations of *P. cembra*: three from the eastern Carpathians and two from the Alps (Belokon *et al.*, 2005). These authors confirmed the generally high level of intrapopulation genetic variability in *P. cembra*, which is higher in Carpathian than in Alpine populations. *F*-statistics showed again a low degree of population divergence ($F_{ST} = 0.04$). A molecular study based on six chloroplast microsatellites, or simple sequence repeats (cpSSRs), supported the high level of intrapopulation variability of the Carpathian populations (mean haplotypic diversity $H = 0.917$; Höhn *et al.*, 2005). However, until now there has been no other molecular study that has evaluated the genetic diversity of *P. cembra* using a reasonably large sample size and including populations from both mountain regions of the species' natural range.

Palaeoecological data suggest that the Carpathian region can be considered one possible refugial area for *P. cembra* during the last glaciation, from where it may have recolonized the eastern Alps (Lang, 1994). A similar recolonization pathway has also been proposed for *P. abies* by Gugerli *et al.* (2001b), and was recently confirmed by Tollefsrud *et al.* (2008). Additionally, south-eastern Alpine refugia are also likely to have existed for *P. cembra* (Lang, 1994; Burga & Perret, 1998) and might have contributed to the haplotypic diversity in the eastern Alps (Gugerli *et al.*, 2001a). According to such a post-glacial premise and the results of previous studies on *P. cembra*, we hypothesized that: (1) the Alps and the Carpathians are likely to comprise distinct (i.e. genetically differentiated) compositions of cpDNA haplotypes, as a consequence of their different histories; (2) both the Alps and the Carpathians have served to preserve high levels of haplotypic diversity; and (3) the genetic differentiation should be higher among the isolated populations (Carpathians) than among the central ones (Alps). To test these hypotheses, highly variable cpSSRs were used on population samples from both main areas within the species' natural range.

MATERIALS AND METHODS

Population sampling

We sampled 28 populations, 19 of which were from the Swiss Alps ($n = 593$) and nine from the Carpathians ($n = 238$;

Table 1 Locations and sample sizes (*n*) of the study populations of *Pinus cembra* from the Alps and the Carpathians. Geographical coordinates are given in decimal degrees.

Locality	Abbreviation	Region	Country	Co-ordinates (latitude N; longitude E)	<i>n</i>
<i>Swiss Alps</i>					
Alp Sadra	Sad	Val Müstair	Switzerland	46.36; 10.20	34
Arvengarten	Arv	Berner Oberland	Switzerland	46.36; 7.58	34
Col du Pillon	Pil	Les Diablerets	Switzerland	46.20; 7.12	33
Flumserberg	Flu	St Galler Oberland	Switzerland	47.04; 9.14	30
Fôret de Derbellec	Der	Val d'Anniviers	Switzerland	46.15; 7.36	34
Fôret du Lapé	Lap	Alpes Fribourgeoises	Switzerland	46.32; 7.13	34
God Baselgia	Bas	Lower Engadin	Switzerland	46.42; 10.07	34
God Tamangur	Tam	Lower Engadin	Switzerland	46.40; 10.21	32
Kreuzboden	Kre	Saastal	Switzerland	46.07; 7.57	34
Letziwald	Let	Avers	Switzerland	46.28; 9.30	34
Mürtschenalp	Mue	Glarus	Switzerland	47.02; 9.09	32
Neuenalp	Neu	Churfirsten	Switzerland	47.10; 9.21	18
Rautialp	Rau	Glärnisch	Switzerland	47.04; 9.00	33
Saflischtal	Saf	Binntal	Switzerland	46.19; 8.08	18
Sagiwald	Sag	Berner Oberland	Switzerland	46.26; 7.38	34
Sardonaalp	Sar	St Galler Oberland	Switzerland	46.55; 9.17	34
Seebergalp	See	Simmental	Switzerland	46.34; 7.26	34
Siviez	Siv	Val de Nendaz	Switzerland	46.07; 7.19	34
Stazerwald	Sta	Upper Engadin	Switzerland	46.30; 9.53	31
<i>Carpathians</i>					
Morskie Oko	Mor	High Tatras	Poland	49.20; 20.08	18
Velka Studena Dolina	Vel	High Tatras	Slovakia	49.17; 20.20	27
Kedryn, Forest Reserve	Ked	Ukrainian Carpathians	Ukraine	48.42; 24.00	21
Borsa	Bor	Rodnei Mts, Eastern Carp.	Romania	47.58; 24.63	14
Neagra Sarului	Nea	Calimani Mts, Eastern Carp.	Romania	47.17; 25.28	56
Negoiu	Neg	Calimani Mts, Eastern Carp.	Romania	47.10; 25.20	57
Cindrel	Cin	Cindrel Mts, South Carp.	Romania	45.58; 23.80	15
Gentiana	Gen	Retezat Mts, South Carp.	Romania	45.38; 22.87	16
Gemele	Gem	Retezat Mts, South Carp.	Romania	45.37; 22.83	20

Table 1). Needles were collected from individuals that were at least 30 m apart, except in small populations where distances were shorter in some cases. Fresh, frozen or silica-dried needles were used to extract genomic DNA.

DNA extraction and microsatellite analysis

The Qiagen Plant Mini kit protocol (Qiagen, Hilden, Germany) was used for the Carpathian samples. Samples from the Alps were extracted with the Qiagen 96-well blood kit following the extraction protocol described in Sperisen *et al.* (2000).

Three mononucleotide cpSSRs (Pt26081, Pt36480, Pt63718; Vendramin *et al.*, 1996), shown to be variable in previous studies, (Gugerli *et al.*, 2001a; Höhn *et al.*, 2005) were screened for length variation. Chloroplast DNA is paternally inherited in all pine species for which segregation analysis has been performed (Neale & Sederoff, 1989; Petit & Vendramin, 2007) and this has been substantiated for *P. cembra* by the haplotypic diversity in half-sibling seed lots (M. Rüegg & F. Gugerli, WSL, Birmensdorf, Switzerland, unpublished data). Polymerase

chain reaction (PCR) amplifications were performed according to Vendramin *et al.* (1996), with the following thermal 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 fragments were sized on a 96-capillary Megabace 1000 automatic sequencer (GE Healthcare, Chalfont St Giles, UK). The fluorescently labelled PCR products were separated by capillary electrophoresis, with a 400-bp size standard (GE Healthcare). Alleles were sized using FRAGMENT PROFILER 1.2 (GE Healthcare).

Data analysis

Based on combinations of the individuals' cpSSR size variants, considered as haplotypes, the number of haplotypes per population was determined. The frequency of the most common haplotype, the effective number of haplotypes and haplotypic variability (unbiased haplotypic diversity) were calculated, the latter according to $H = (n/n - 1)(1 - \sum p_i^2)$, where p refers to the haplotype frequencies and n to the number of sampled individuals per population (Nei, 1987).

We used analysis of molecular variance (AMOVA) to estimate the hierarchical partitioning of molecular variation and to calculate fixation indices (F -statistics; Wright, 1951). Mantel matrix correlations based on Slatkin's linearized F_{ST} and Euclidean distance between pairs of populations (in km) were applied to all populations and within each of the two mountain ranges. Analyses were performed using ARLEQUIN 3.0 (Excoffier *et al.*, 2005). We tested for a difference between mean haplotypic diversity in the Alps and the Carpathians using a t -test, after confirming that data were normally distributed and showed even variances. The test was performed with PAST (Hammer *et al.*, 2001).

The possible presence of geographical structure was evaluated by performing two tests. First, we used spatial analysis of molecular variance (SAMOVA) which, based on a simulated annealing procedure, defines groups of populations that are geographically homogeneous and maximally differentiated from each other (Dupanloup *et al.*, 2002). The program iteratively seeks the composition of a user-defined number K of groups of geographically adjacent populations that maximizes F_{CT} , i.e. the proportion of total genetic variance due to differences among groups of populations. The program was run for 10,000 iterations for $K = \{2 \dots 15\}$ groups from each of 100 random initial conditions. Second, the presence of genetic barriers among populations was tested with the Monmonier algorithm implemented in the BARRIER 2.2 software (Manni *et al.*, 2004). The program identifies geographical boundaries of abrupt change in genetic differences between pairs of populations based on a network obtained by Delaunay triangulation (for technical details see the software manual). Virtual points were added to the original triangulation in order to allow the connection between the populations of Negoiu and Cindrel from the Carpathians.

RESULTS

The three polymorphic cpSSR loci studied displayed four (Pt2626081, Pt36480) and five (Pt63718) size variants, combining into 20 haplotypes. All populations were dominated by a single haplotype that reached the highest frequency in each population (value range 0.44–0.97; Table 2). In all populations except for Borsa (East Carpathians), the same haplotype was most frequent (Fig. 1). For all genetic parameters considered, populations of the High Tatras and the South Carpathians – Retezat Mountains – displayed the highest diversity values (Table 2).

The number of haplotypes per population and haplotypic diversity varied substantially among populations, with haplotypic diversity being generally high within both mountain ranges. However, mean haplotypic variation was significantly higher in the Carpathian populations ($H = 0.53$) than in the Alpine populations ($H = 0.35$; $t = 2.6488$; $P(\text{same mean}) = 0.0355$; Fig. 1). Among the 16 Carpathian haplotypes detected, seven were specific for the area, while among the 13 haplotypes of the Alps, only four were not found in the Carpathians. Two Alpine populations (Seeberg, Sagiwald), two

Table 2 Measures of genetic variation within 19 Alpine and nine Carpathian populations of *Pinus cembra* ($n = 14\text{--}57$ per population; see Table 1), based on three chloroplast microsatellite loci.

Locality	No. of haplotypes	Frequency of most common haplotype	Effective number of haplotypes*	Haplotypic diversity
<i>Swiss Alps</i>				
Alp Sadra	3	0.824	1.43	0.313
Arvengarten	6	0.738	1.79	0.456
Col du Pillon	3	0.939	1.13	0.119
Flumserberge	3	0.833	1.40	0.296
Fôret de Derbellec	5	0.824	1.45	0.320
Fôret du Lapé	2	0.971	1.03	0.058
God Baselgia	4	0.706	1.87	0.475
God Tamangur	7	0.548	2.89	0.675
Kreuzboden	6	0.794	1.54	0.370
Letziwald	7	0.647	2.90	0.559
Mürtschenalp	2	0.875	1.28	0.225
Neuenalp	4	0.556	2.49	0.634
Rautialp	5	0.758	1.68	0.418
Saflischtal	2	0.944	1.11	0.111
Sagiwald	4	0.824	1.44	0.319
Sardonaalp	3	0.912	1.19	0.169
Seeberg	4	0.882	1.27	0.222
Siviez	2	0.971	1.06	0.058
Stazerwald	4	0.806	1.49	0.341
Mean	–	–	1.610	0.350
SE	–	–	0.140	0.040
<i>Carpathians</i>				
Morskie Oko	6	0.444	3.59	0.764
Velka Studena Dolina	8	0.444	3.90	0.772
Borsa	4	0.786	1.58	0.395
Neagra Sarului	3	0.803	1.48	0.333
Negoiu	3	0.859	1.33	0.255
Kedryn	3	0.524	2.46	0.623
Cindrel	2	0.867	1.30	0.247
Gentiana	7	0.500	3.28	0.711
Gemelele	8	0.550	2.94	0.694
Mean	–	–	–	0.532
SE	–	–	–	0.080

*The effective number of haplotypes accounts for the uneven sample size per population.

populations from the High Tatras (Morskie Oko, Velka Studena Dolina) and one population from the South Carpathians (Gentiana) harboured private haplotypes.

F -statistics, using AMOVA based on the number of different alleles (infinite allele model, IAM), showed that almost 13% of the total genetic variance was due to differences among populations ($F_{ST} = 0.127$). However, the respective value for the genetic differentiation between the two mountain ranges was low but significant ($F_{CT} = 0.03$, $P < 0.01$). We found significantly higher genetic divergence among populations

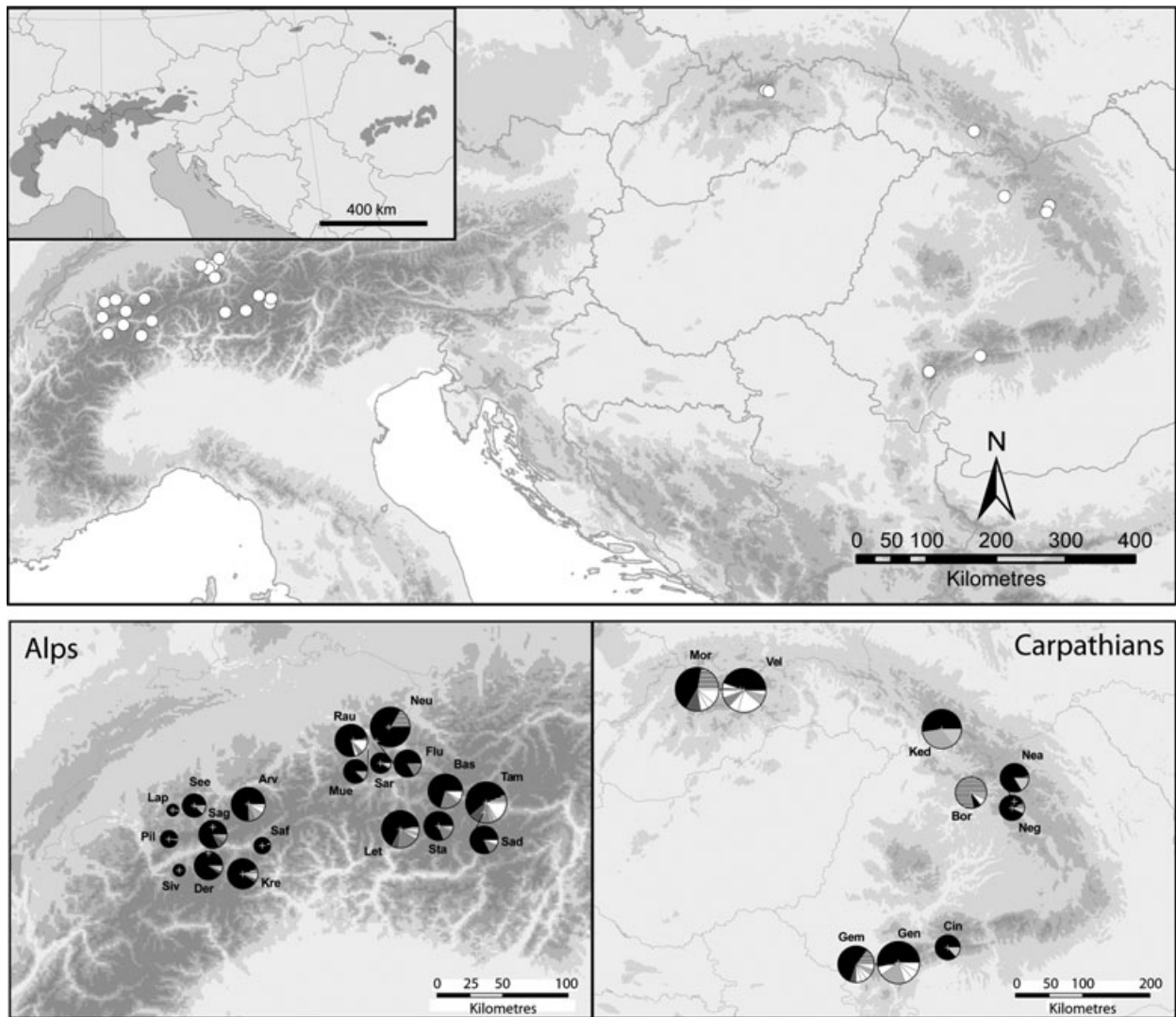


Figure 1 Geographical distribution (top), composition and frequency of chloroplast microsatellite haplotypes (bottom) in *Pinus cembra* in the Alps and the Carpathians. The species' natural range is indicated by the grey shaded areas in the inset of the top panel (distribution map courtesy to Euforgen, http://www2.bioversityinternational.org/Networks/Euforgen/Euf_Distribution_Maps.asp). Pie charts represent haplotype frequencies. Rare haplotypes (white) are not differentiated by shading in the pie charts. For abbreviations see Table 1.

within the Carpathians ($F_{ST} = 0.19$) than among populations within the Alps ($F_{ST} = 0.04$; Table 3).

The overall Mantel test showed a relatively low but significant correlation between genetic and geographical distances ($r = 0.286$, $P < 0.001$). A higher positive correlation was found within the Alps ($r = 0.328$, $P < 0.001$). Although genetic differentiation was higher among the Carpathian populations, it was not positively correlated with the geographical distance among populations ($r = -0.289$, $P = 0.982$).

SAMOVA results revealed progressively decreasing F_{CT} values (Table 4) with increasing numbers of groups and did not show a clear geographical grouping of the populations. In fact, each new group delimited was represented by only one population, except at $K = 8$ where two populations formed a

new group. According to the first seven delimited groups, divergent populations were identified only in the Carpathian part of the natural range. The population separations of the BARRIER analysis were largely congruent with the SAMOVA results and showed the strongest genetic separation in the eastern part of the species' range (Fig. 2). The first genetic discontinuity was detected in the eastern Carpathians, with F_{ST} c. 0.6, and encircling the Borsa population of the Rodnei Mountains. The second separation had an F_{ST} value only slightly higher than 0.2. All the remaining discontinuities were supported by lower F_{ST} values (< 0.2). The separation between the central (Alps) and eastern populations (Carpathians), i.e. between the easternmost Alpine population God Tamangur and the western High Tatra population Morskie Oko, appeared only as the eighth 'barrier' ($F_{ST} = 0.048$).

Table 3 Results of the analyses of molecular variance (AMOVA) for *Pinus cembra* in the Alps (19 populations; $n = 18\text{--}34$) and the Carpathians (9 populations; $n = 14\text{--}57$), based on chloroplast haplotype variation assessed at three microsatellite loci.

Source of variation	d.f.	SS	% variation	
<i>Alps and Carpathians</i>				
Among mountain ranges	1	4.250	3.05**	F_{CT} 0.030
Among populations within ranges	26	28.003	9.73***	
Within populations	816	203.212	87.23***	
Total	843	235.464	0.285	F_{ST} 0.127
<i>Alps</i>				
Among populations	18	9.227	4.10***	F_{ST} 0.041
Within populations	581	126.828	95.90***	
<i>Carpathians</i>				
Among populations	8	18.775	19.36***	F_{ST} 0.193
Within populations	235	76.385	80.64***	

Significance tests based on 1023 permutations.

d.f., degrees of freedom; SS, sums of squares.

** $P < 0.01$; *** $P < 0.001$.

DISCUSSION

Our study on the genetic variation of *P. cembra*, based on a large population sample covering the two disjunct parts of the species' range, showed a generally high level of haplotypic variation of cpDNA in most of the populations. These results confirm our expectations of the preserved cpDNA diversity in *P. cembra* on the large scale. Apparently, Carpathian populations have maintained high haplotypic diversity, even higher than Alpine populations, despite their small population sizes and a high degree of spatial isolation. However, our results do not necessarily contradict expectations, as the frequency of support reported for reduced diversity at the range margins for pine species was only 54% among 24 studies reviewed by Eckert *et al.* (2008). Moreover, the diversity pattern of

populations suggests that the abundant centre model does indicate a decrease in variation at the margins of the species' geographical range, but highlights the need to distinguish between the effects of historical vs. current variation in population size and isolation. According to the fixation index (F_{ST}), almost 13% of the total genetic variation was due to differences among populations. This value is in line with those estimated for other pine species with disjunct distribution areas (Powell *et al.*, 1995; Bucci *et al.*, 1998; Petit *et al.*, 2005). Other factors to be taken into account when considering the genetic structure of the paternally inherited cpDNA are the high mobility of wind-borne pollen, the subsequent bird-mediated seed dispersal (embryo with paternal haplotype) and the long generation turnover of the species, all of which may contribute to diminish the effects of spatial isolation.

As expected, owing to the greater degree of isolation, we found higher genetic differentiation among Carpathian populations as compared to the central populations from the Alps. However, this low level of differentiation among populations from the Alps might be increased if the entire Alpine range of the species was considered. Yet we also observed lower genetic diversity within the Swiss Alpine peripheral populations compared with the central populations (data not shown), which is confirmed by a more detailed study (Gugerli *et al.*, in press). These results are in agreement with and support the earlier isozyme study of Belokon *et al.* (2005). Although significant genetic differentiation between the two distinct parts of the natural range of *P. cembra* in Europe was estimated, its degree was lower than we found among populations within the two mountain systems. The low differentiation between the Alpine and Carpathian populations ($F_{CT} = 0.03$) indicates that, despite the distinct spatial separation, the two subpopulations of *P. cembra* within the natural range still share many cpDNA haplotypes, particularly one dominant and widespread haplotype (black in Fig. 1). A similar coincidence of joint cpSSR haplotypes has already been found between *P. cembra* and the closely related *P. sibirica* (Gugerli *et al.*, 2001a). The latter also shows great morphological proximity to *P. cembra* (Belokon *et al.*, 2005) or is occasionally considered as a subspecies of *P. cembra* (Meusel *et al.*, 1965).

Table 4 Fixation indices (F_{CT}) of *Pinus cembra* population groupings obtained with a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.*, 2002) as a function of the user-defined number K of groups of populations. Bold populations in the grouping indicate the newly separated populations at a given level of K .

K	F_{CT} ($P < 0.05$)	Grouping
2	0.519	{Borsa} {rest}
3	0.392	{Borsa/ Gentiana } {rest}
4	0.334	{Borsa/Gentiana/ Morskie Oko } {rest}
5	0.292	{Borsa/Gentiana/Morskie Oko/ Velka Studena Dolina } {rest}
6	0.264	{Borsa/Gentiana/Morskie Oko/Velka Studena Dolina/ Kedryn } {rest}
7	0.241	{Borsa/Gentiana/Morskie Oko/Velka Studena Dolina/Kedryn/ Gemenele } {rest}
8	0.220	{Borsa/Gentiana/Morskie Oko/Velka Studena Dolina/Kedryn/ Gemenele/Cindrel/Neuenalp } {rest/ Gemenele }
9	0.207*	{Borsa/Gentiana/Morskie Oko/Velka Studena Dolina/Kedryn/ Gemenele/Cindrel/Neuenalp } {rest}
10	0.188	{Borsa/Gentiana/Morskie Oko/Velka Studena Dolina/Kedryn/Gemenele/Cindrel/Neuenalp/ Saffischtal } {rest}

*Gemenele had been re-included into the main large group in the configuration of $K = 8$.

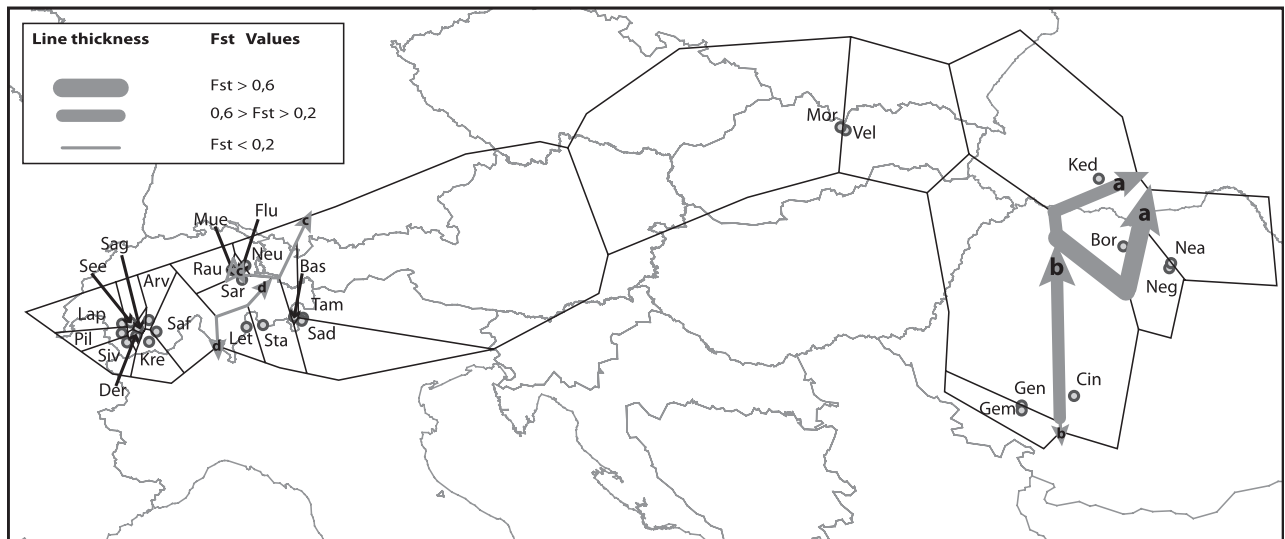


Figure 2 Map of genetic discontinuities (barriers) computed using F_{ST} population pairwise matrices. Dots represent the populations analysed. Black lines indicate the spatial triangulation performed by BARRIER 2.2 (Manni *et al.*, 2004). Grey lines represent the first four barriers generated. The line thickness is proportional to the F_{ST} values that support them. The ends of the barriers are indicated by arrowheads. Barriers are ordered from 'a' (first barrier) to 'd' (fourth barrier). For abbreviations see Table 1.

The low degree of substructuring was also confirmed by the BARRIER analysis. We interpret this pattern as the result of a formerly connected distribution that was gradually separated in the course of climate warming and the resulting range contraction during the late Holocene. Moreover, historical events linked to large fluctuations of the species' distribution range along the Carpathians since the last full glacial might have led to a more pronounced genetic structure among Carpathian populations, as inferred from palynological records. Macroscopic charcoal evidence linked with a molluscan palaeofauna study also suggests that *P. cembra* was widely distributed during the Last Glacial Maximum even in the lowlands of the Carpathian Basin (Willis *et al.*, 2000) and suffered a subsequent fragmentation of its range as a consequence of competitive exclusion or a loss of suitable habitats around 5000 yr BP (Farcas & Tantau, 1999). Presumably, the size of populations colonizing the higher elevations decreased markedly and prevented extensive gene flow between the stands. If so, different population genetic patterns could have been preserved from the formerly large and continuous population and this is now reflected in the persistence of a large number of region-specific haplotypes. The specific structuring of genetic diversity in subalpine/alpine species such as *P. cembra* may be further supported by the topographical constitution of the Carpathians. While timberline ecotones are scattered across this mountain range, like islands in a sea of low-elevation habitats (Gugerli *et al.*, 2008), similar habitat types occur rather contiguously in the Alps. Mraz *et al.* (2007) found a genetic structure in *Hypochoeris uniflora*, a herbaceous perennial of subalpine/alpine grasslands, which was similar to that observed in *P. cembra*. Likewise, the genetic structure found in the extremely long-lived *Carex curvula*, a dominant species of acidic alpine grasslands in the Central European mountain system, led Puşcaş *et al.* (2008) to the same

conclusion on post-glacial migration processes as ours for *P. cembra*. This coincidence may reflect the particular habitat configuration available to these taxa, while other Alpine/Carpathian plant species from higher elevations show rather different genetic structures (e.g. Ronikier *et al.*, 2008).

The lack of correlation between geographical and genetic distance within the Carpathians could have several explanations, for example multiple independent colonization events within a meta-population framework (Hilfiker *et al.*, 2004). Again, the differences in topography between the Carpathians and the Alps may in part explain the lack of isolation by distance in the former range. The Alps have a rather linear arrangement and significant isolation by distance is expected under this configuration, whereas the Carpathian mountains form an arc (Fig. 1). Accordingly, Euclidean distances between Carpathian populations do not well represent presumed dispersal routes, which necessarily follow the distribution of available habitat. However, such an alternative explanation requires further investigation.

We also registered very low values of genetic diversity in some populations in both geographical regions. Even some large and well-connected populations from the Alps (for example, Siviez and Alp Sadra) and some of the Carpathian populations (Cindrel, Negoiu and Borsa) displayed low variation. This could be attributed to the generally low seed production within some populations, or could be because the seedlings germinated from seeds that have been sired by paternal trees of the same cpDNA haplotype (correlated paternity). The latter may even represent bi-parental inbreeding, thus also affecting the nuclear genome. On the other hand, the low haplotypic diversity detected in the Carpathians might be attributed to anthropogenic factors, for example intensive timber exploitation. Alternatively, the specific haplotype composition of the Borsa population, with one

haplotype frequency strongly exceeding that of the other populations (mean frequency value for the Carpathians 0.14 and for Borsa 0.78), may indicate a potential refugial area, with little expansion after the glacial retreat. According to the BARRIER analysis, Borsa showed the strongest differentiation even from the geographically proximate Carpathian populations (Fig. 2). As expected, the small population from the Cindrel Mountains, represented by a very low census size, also exhibited extremely low cpDNA variation, a probable sign of a random sampling effect (bottleneck, founder event) that is probably linked to strong inbreeding in the area. Progeny analyses, for which recently developed polymorphic nuclear markers are required (Salzer *et al.*, 2009), could establish whether strong inbreeding has affected the genetic diversity of the population in this particular location.

In conclusion, our study demonstrates the importance of considering the Carpathian populations of *P. cembra* for conservation. They represent a particular legacy of the species' evolutionary history. All these populations should be given high conservation priority because they contribute to the species' total genetic diversity. If these populations should disappear, their respective history would be lost irrevocably.

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REFERENCES

- Belokon, M.M., Belokon, Yu.S., Politov, D.V. & Altukhov, Yu. P. (2005) Allozyme polymorphism of Swiss stone pine *Pinus cembra* L. in mountain populations of the Alps and the Eastern Carpathians. *Russian Journal of Genetics*, **41**, 1268–1280.
- Bucci, G., Anzidei, A., Madaghiale, A. & Vendramin, G.G. (1998) Detection of haplotypic variation and natural hybridization in *halepensis*-complex pine species using chloroplast simple sequence repeat (SSR) markers. *Molecular Ecology*, **7**, 1633–1643.
- Burga, C.A. & Perret, R. (1998) *Vegetation und Klima der Schweiz seit dem jüngeren Eiszeitalter*. Ott, Thun, Switzerland.
- Critchfield, W.B. (1986) Hybridization and classification of the white pines (*Pinus* section *Strobus*). *Taxon*, **35**, 647–656.
- Dupanloup, I., Schneider, S. & Excoffier, L. (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571–2581.
- Eckert, C.G., Samis, K.E. & Loughheed, S.C. (2008) Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. *Molecular Ecology*, **17**, 1170–1188.
- Ellstrand, N.C. & Elam, D.R. (1993) Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics*, **24**, 217–242.
- Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.
- Farcas, S. & Tantau, I. (1999) Analyse palynologique du profil tourbeux 'Intre Afini' (Monts Calimani). *Acta Palaeontologica Romaniaica*, **2**, 157–162.
- Goncharenko, G.G., Padutov, V.E. & Silin, A. (1992) Population structure, gene diversity, and population differentiation in natural populations of Cedar pines (*Pinus* subsect *Cembrae*, Pinaceae) in the USSR. *Plant Systematics and Evolution*, **182**, 121–134.
- Gugerli, F., Senn, J., Anzidei, M., Madaghiale, A., Büchler, U., Sperisen, C. & Vendramin, G.G. (2001a) Chloroplast microsatellites and mitochondrial *nad1* intron 2 sequences indicate congruent phylogenetic relationships of Swiss stone pine (*Pinus cembra*), Siberian stone pine (*P. sibirica*) and Siberian dwarf pine (*P. pumila*). *Molecular Ecology*, **10**, 1489–1497.
- Gugerli, F., Sperisen, C., Büchler, U., Magni, F., Geburek, T., Jeandroz, S. & Senn, J. (2001b) Haplotype variation in a mitochondrial tandem repeat of Norway spruce (*Picea abies*) populations suggests a serious founder effect during postglacial re-colonization of the western Alps. *Molecular Ecology*, **10**, 1255–1263.
- Gugerli, F., Tribsch, A., Niklfeld, H., Mirek, Z., Ronikier, M., Englisch, T., Zimmermann, N., Taberlet, P. & IntraBioDiv Consortium (2008) Relationships among levels of biodiversity and the relevance of intraspecific diversity in conservation – a project synopsis. *Perspectives in Plant Ecology, Evolution and Systematics*, **10**, 259–281.
- Gugerli, F., Rüegg, M. & Vendramin, G.G. (in press) Gradual decline in genetic diversity in Swiss stone pine populations (*Pinus cembra*) across Switzerland suggests postglacial re-colonization into the Alps from a common eastern glacial refugium. *Botanica Helvetica*.
- Hammer, Ř., Harper, D.A.T. & Ryan, P.D. (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, http://palaeo-electronica.org/2001_1/past/past.pdf.
- Hamrick, J.L., Godt, J.W. & Sherman-Broyles, S.L. (1992) Factors influencing levels of genetic diversity in woody plants. *New Forests*, **6**, 95–124.
- Hartl, D.L. & Clark, A.G. (1997) *Principles of population genetics*, 3rd edn. Sinauer Associates, Sunderland, MA.

- Hilfiker, K., Holderegger, R., Rotach, P. & Gugerli, F. (2004) Dynamics of genetic variation in *Taxus baccata*: local versus regional perspectives. *Canadian Journal of Botany*, **82**, 219–227.
- Höhn, M., Ábrán, P. & Vendramin, G.G. (2005) Genetic analysis of Swiss stone pine populations (*Pinus cembra* L. subsp. *cembra*) from the Carpathians using chloroplast microsatellites. *Acta Silvatica et Ligniensi Hungarica*, **1**, 39–47.
- Huntley, B. (1990) European post-glacial forests: compositional changes in response to climatic change. *Journal of Vegetation Science*, **1**, 507–518.
- Krutovskii, K.V., Politov, D.V., Altukhov, Y.P. & Yu, P. (1990) Interspecific genetic differentiation of Eurasian pines for isoenzyme loci. *Genetika*, **26**, 694–707.
- Krutovskii, K.V., Politov, D.V. & Altukhov, Y.P. (1995) Isozyme study of population genetic structure, mating system and phylogenetic relationships of the five stone-pine species (subsection *Cembrae*, section *Strobus*). *Population genetics and genetic conservation of forest trees* (ed. by P. Baradat, W.T. Adams and G. Müller-Starck), pp. 279–304. SPB Academic Publishing, Amsterdam.
- Landergott, U., Holderegger, R., Kozłowski, G. & Schneller, J.J. (2001) Historical bottlenecks decrease genetic diversity in natural populations of *Dryopteris cristata*. *Heredity*, **87**, 344–355.
- Lang, G. (1994) *Quartäre Vegetationsgeschichte Europas*. Gustav Fischer, Jena.
- Manni, F., Guérard, E. & Heyer, E. (2004) Geographic patterns of (genetic, morphologic, linguistic) variation: how barriers can be detected by 'Monmonier's algorithm'. *Human Biology*, **76**, 173–190.
- Marhold, K. & Hindák, F. (1998) *Zoznam nižších a vyšších rastlín Slovenska. [Checklist of non-vascular and vascular plants of Slovakia]*. Veda, Bratislava.
- Meusel, H., Jäger, E. & Weinert, E. (1965) *Vergleichende Chorologie der Zentraleuropäischen Flora*. Gustav Fischer, Jena.
- Mraz, P., Gaudeul, M., Gielly, L., Choler, P., Taberlet, P. & IntraBioDiv consortium (2007) Genetic structure of *Hypochaeris uniflora* (Asteraceae) suggests vicariance in the Carpathians and rapid post-glacial colonization of the Alps from an eastern Alpine refugium. *Journal of Biogeography*, **34**, 2100–2114.
- Neale, D.B. & Sederoff, R.R. (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theoretical and Applied Genetics*, **77**, 212–216.
- Nei, M. (1987) *Molecular evolutionary genetics*. Columbia University Press, New York.
- Petit, R.J. & Vendramin, G.G. (2007) Plant phylogeography based on organelle genes: an introduction. *Phylogeography of southern European refugia – evolutionary perspectives on the origins and conservation of European biodiversity* (ed. by S. Weiss and N. Ferrand), pp. 23–97. Springer, Dordrecht.
- Petit, J.R., Duminil, J., Fineschi, S., Hampe, A., Salvini, D. & Vendramin, G.G. (2005) Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molecular Ecology*, **14**, 689–701.
- Powell, W., Morgante, M., McDevitt, R., Vendramin, G.G. & Rafalski, J.A. (1995) Polymorphic simple sequence repeat regions in chloroplast genomes: application to the population genetics of pines. *Proceedings of the National Academy of Sciences USA*, **92**, 7759–7763.
- Puşças, M., Choler, P., Tribsch, A., Gielly, L., Rioux, D., Gaudeul, M. & Taberlet, P. (2008) Post-glacial history of the dominant alpine sedge *Carex curvula* in the European Alpine System inferred from nuclear and chloroplast markers. *Molecular Ecology*, **17**, 2417–2429.
- Ronikier, M., Cieślak, E. & Korbecka, G. (2008) High genetic differentiation in the alpine plant *Campanula alpina* Jacq. (Campanulaceae): evidence for glacial survival in several Carpathian regions and long isolation between the Carpathians and the Eastern Alps. *Molecular Ecology*, **17**, 1763–1775.
- Salzer, K., Sebastiani, F., Gugerli, F., Buonamici, A. & Vendramin, G.G. (2009) Isolation and characterization of polymorphic nuclear microsatellite loci in *Pinus cembra* L. *Molecular Ecology Resources*, **9**, 858–861.
- Sperisen, C., Gugerli, F., Büchler, U. & Mátyás, G. (2000) Comparison of two rapid DNA extraction protocols for gymnosperms for application in population genetic and phylogenetic studies. *Forest Genetics*, **7**, 133–136.
- Tollefsrud, M.M., Kissling, R., Gugerli, F., Johnsen, Ø., Skroppa, T., Cheddadi, R., van der Knaap, P., Latalowa, M., Terhürne-Berson, R., Litt, T., Geburek, T., Brochmann, C. & Sperisen, C. (2008) Genetic consequences of glacial survival and postglacial colonization in Norway spruce: combined analysis of mitochondrial DNA and fossil pollen. *Molecular Ecology*, **17**, 3134–3150.
- Tombäck, D.F. & Linhart, Y.B. (1990) The evolution of bird-dispersed pines. *Evolutionary Ecology*, **4**, 185–219.
- Vendramin, G.G., Lelli, L., Rossi, P. & Morgante, M. (1996) A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. *Molecular Ecology*, **5**, 595–598.
- Willis, K.J., Rudner, E. & Sümegi, P. (2000) The full-glacial forests of central and south-eastern Europe. *Quaternary Research*, **53**, 203–213.
- Wright, S. (1951) The genetical structure of populations. *Annals of Eugenetics*, **15**, 323–354.

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