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Geographic patterns of genetic diversity of *Pinus mugo* (Pinaceae) in Central European mountains

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Abstract: The genetic diversity within and among twelve populations (379 individuals) of *Pinus mugo* from the Giant Mts., Carpathians and Alps was analyzed using ten chloroplast microsatellite markers. A stepwise mutation model (SMM) for microsatellite loci was used in order to estimate divergence between populations and provenances from three mountain ranges. High levels of genetic diversity and significant differentiation were found among the three population groups. The populations from Giant Mts., Carpathians and Alps were strongly differentiated between each other, while differences among populations within these massifs were much lower. The pattern of genetic structure observed in dwarf mountain pine can be characteristic in conifers with a disjunctive geographic distribution. The significant genetic structuring among isolated parts of the geographic range of the species may be a result of an ancient fragmentation and long lasting geographic isolation between the Giant Mts., Alpine and Tatra populations of *P. mugo*.

Additional key words: Alps, Carpathians, Chloroplast microsatellites, Dwarf mountain pine, Genetic diversity, Giant Mountains, Isolation by distance, Phylogeography

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Introduction

Dwarf mountain pine, *Pinus mugo* Turra (*P. mugo* subsp. *mugo* sensu Christensen 1987), is a prostrate, polycormic shrub occurring in the mountain massifs of central and southern Europe. The species has subalpine character and forms specific plant communities in the subalpine climate-vegetation layer above the upper forest line (Christensen 1987; Ozenda 1988; Poldini et al. 2004; Tsaryk et al. 2006; Sibik et al. 2008). The geographic range of dwarf mountain pine can be easily divided into several centres, namely Alpine, Sudetes, West, East and South Carpathians and others (Jalas and Suominen 1973). Within these main centres *P. mugo* occurs on the massifs, which are sufficiently high that subalpine communities can be formed (Ozenda 1988; Christensen 1987; Tsaryk et al. 2006). Sandoz (1983) hypothesized that the existing fragmented populations of *P. mugo* are small remnants of what had been a very large range in the late Tertiary and during the Quaternary interglacial periods. This biogeographical pattern is thought to be a result of the repeated climatic fluctuations of the Pleistocene and the warming of the Holocene.

The distribution of *P. mugo* expanded during cool periods and regressed during warm periods several times during the Pleistocene (Farcas et al. 1999; Willis et al. 2000; Wolfrath et al. 2001; Ali et al. 2006),

similarly to *P. uncinata* on the Iberian Peninsula (Benito Garzón et al. 2007). As a result, dwarf mountain pine suffered fluctuations in population size and finally became isolated in fragmented populations in subalpine European mountain areas. The expected genetic consequences of these processes are reduced or non-existent gene flow among European moutains populations resulting in vicariant gene pools and random genetic drift (Ellstrand and Elam 1993; Young et al. 1996; Hartl and Clark 2007).

In this paper we investigate the distribution of genetic diversity of dwarf mountain pine. A weak differentiation between populations belonging to different taxa of the *Pinus mugo* complex has recently been described in western Europe using nuclear RAPD markers (Monteleone et al. 2006) and chloroplast microsatellites (Dzialuk et al. 2009; Heuertz et al. 2010; Sannikov et al. 2011). However, little is known about the level and structure of genetic diversity of this species in Central Europe, since previous research in this area was mostly focused on putative hybridization and genetic relationships between *Pinus mugo*, *P. sylvestris*, *P. uliginosa* (Filppula et al. 1992, Neet-Sarqueda 1994, Goncharenko et al. 1995, Lewandowski et al. 2000, Slavov and Zhelev 2004, Wachowiak et al. 2005, Wachowiak and Prus-Głowacki 2008; Jasińska et al. 2010; Wachowiak et al. 2011).

We used chloroplast microsatellites (cpSSRs, chloroplast single sequence repeats) to investigate the haplotypic diversity and differentiation of twelve natural populations of dwarf mountain pine in Central Europe. Our main hypothesis was that *P. mugo*'s disjunct range has led to significant isolation by distance and substantial differentiation within the species. The main aim of the present study was to estimate the diversity within and among 7 populations of *P. mugo* in the Giant Mts. and then to compare them with two populations from the West Carpathians and three from the Alps to test our hypothesis.

Materials and methods

Plant material, DNA extraction and scoring of PCR products

Needle samples were collected from 12 populations of *Pinus mugo* distributed throughout three mountain ranges in Central Europe: The Giant Mts. (Sudety mountains), Tatra Mts. (W Carpathians) and the Alps. About 30 shrubs were sampled in each population, resulting in a total sample size of 379 individuals (Table 1). As *P. mugo* rootbounds easily by layering (Tsaryk et al. 2006), individuals were sampled along transects through populations, at distances of at least 30–40 m one from another, to avoid duplicate sampling the same genet (Boratyńska et al. 2005). After collection, fresh needles were preserved in 70% ethanol, then stored at –20°C until total genomic DNA was extracted following the protocol by Doyle and Doyle (1990) using 50 mg of needle tissue after grinding with Mixer Mill (MM301, Retsch). The DNA concentrations were estimated using a DNA calculator (BioPhotometer, Eppendorf). All samples were amplified using ten primer pairs, corresponding to ten cpSSR loci: Pt26081, Pt36480, Pt45002, Pt71936, Pt15169, Pt30204 (Vendramin et al. 1996), PCP1289, PCP41131, PCP87314, PCP102652 (Provan et al. 1998) using multiplex PCR amplification conditions described elsewhere (Dzialuk et al. 2009). The fluorescence labelled PCR products were run on an automated sequencer (ABI 310, Applied Biosystems) and the raw data were scored using GENESCAN software ver. 3.7 (Applied Biosystems). The allele binning was carried out using the least square method described by Idury and Cardon (1997), implemented in a Pascal/Delphi computer program (Chybicki unpubl.).

Genetic structure and population parameters estimates

Genetic diversity

For each individual sample, the haplotype was defined as the unique combination of size variants across the microsatellite regions. We calculated chloroplast haplotype variation within populations by estimating the number of haplotypes (N_h), number of private haplotypes (N_p), the effective number of haplotypes (N_e), the unbiased haplotype diversity (H_e), the proportion distinguishable (*PD*), which is the ratio of haplotypes relative to the total number of individuals analyzed in the population (Ellstrand and Roose 1987). In addition, we computed the average distance $\overline{D}_{sh}^2$, as defined by Vendramin et al. (1998), which assumes a stepwise mutation model (SMM) for microsatellite loci, to estimate divergence between haplotypes within populations, and haplotype richness after rarefaction to a uniform sample size of 30, in order to compare the genetic variability across populations taking into account differences in sample size using the software Contrib 1.02 (Petit et al. 1998).

Genetic differentiation

Population differentiation was calculated by F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995) using the analysis of molecular variance (AMOVA) implemented in Arlequin ver. 3.11 software (Excoffier et al. 2005). Analyses were conducted based on all populations pooled as well as assuming population substructuring based on isolation into Giant Mts., Tatra Mts. and Alps. To obtain significance levels for variance components, 1000 permutations

Table 1. Geographic location and genetic diversity estimates for the *Pinus mugo* populations

Code	Location	Latitude E/ Longitude N	Altitude (m)	N	N_h	N_p	A_R (30)	N_e	H_e	$\overline{D}_{sh}^2$	PD(%)
GM 1	Równia below Śnieżka	50°44'44"/15°47'41"	1400–1420	32	22	4	20.05	17.66	0.97	6.47	68.8
GM 2	between Łabski Szczyt and Szrenica	50°47'40"/15°33'15"	1350–1450	31	21	3	19.58	17.47	0.97	4.70	67.7
GM 3	slopes of Śnieżka above Kocioł Łomniczki	50°44'40"/15°47'50"	1300–1500	32	19	5	17.42	16.00	0.97	8.03	59.4
GM 4	Kocioł Małego Stawu near Samotnia	50°44'41"/15°47'34"	1350–1400	31	21	7	19.52	14.79	0.96	6.14	67.7
GM 5	Czarny Kocioł Jagniątkowski	50°47'05"/15°35'30"	1300–1400	33	20	1	17.80	13.79	0.96	9.28	60.6
GM 6	Wielki Kocioł Snieżny	50°46'55"/15°34'00"	1400–1450	32	21	7	19.12	15.06	0.96	6.58	65.6
GM 7	Śląskie Kamienie	50°46'40"/15°36'10"	1410–1420	32	23	5	20.87	16.52	0.97	5.94	71.9
TM 1	Dolina Pięciu Stawów Polskich	49°13'09"/20°03'05"	1680–1710	33	23	11	20.51	16.75	0.97	7.05	69.7
TM 2	N slopes of Grześ–Wołowiec ridge	49°13'07"/19°45'50"	1600–1650	33	25	15	22.08	17.29	0.97	6.56	75.8
A 1	NW slopes of Kreuzspitze Mt	47°31'30"/10°55'12"	1859–1900	30	28	26	27.00	26.47	0.99	7.66	93.3
A 2	SW slopes of Hochkonig Mt	47°26'00"/13°05'00"	1500	30	26	21	25.00	23.68	0.99	5.38	86.7
A 3	Passo di Pramollo	46°32'45"/13°15'35"	1530	30	23	16	22.00	19.57	0.98	6.56	76.7
Mean				31.6	22.7			17.92	0.97	6.70	72.0
Total				379	168				0.99		

Abbreviations: (GM) Giant Mts., (TM) Tatra Mts., (A) Alps, (N) sample size, (N_h) number of haplotypes, (N_p) number of private haplotypes, (A_R (30)) haplotypic richness (after rarefaction to a uniform sample size of 30), (N_e) effective number of haplotypes, (H_e) unbiased haplotype diversity, ($\overline{D}_{sh}^2$) within population genetic distance between tree haplotypes, *PD* proportion distinguishable.

were carried out by resampling individuals among populations. SPADE software (Chao and Shen 2010) was used to measure the actual differentiation among populations (*D*) according to Jost (2008).

Grouping of populations

Using geographical information, a spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002) was performed to identify most differentiated groups of populations that are as spatially clustered as possible. SAMOVA was performed with the aid of Samova 1.0 software (Dupanloup et al. 2002) to define groups of populations that are geographically homogeneous and maximally differentiated from each other. The method is based on a simulated annealing procedure that aims at maximizing the proportion of total genetic variance due to differences between K groups of populations (largest F_{CT} value). We examined the results for between two and eleven groups of populations based on F_{ST} and R_{ST} performing 100 independent simulated annealing processes, to check the degree of relatedness between populations through the consistency of groupings in each level and to compare relatedness with the geographic distribution of those populations.

Several different kinds of analyses were employed to further explore the possible structure in the *P. mugo* populations. First, a cluster grouping without consideration of the geographical location of populations, was computed based on a genetic distance matrix analysis. For the unweighted pair-group method analysis (UPGMA), Nei's genetic distance (1972) was calculated for each pair of populations using PowerMarker (Liu and Muse 2005). The dendrogram was constructed with the aid of programs CONSENSE and DRAWTREE of the package PHYLIP (Felsenstein 2003) and R^2 value was calculated (Kalinowski 2009), as the proportion of variation in the genetic distance matrix that is explained by the tree. Statistical support for the clusters was assessed by means of 1,000 bootstrap replicates over loci. Grouping of the populations was also carried out by a principal coordinates analysis (PCA) using the program GenAlEx (Peakall and Smouse 2005). The pairwise genetic differentiation among the populations was estimated with *D* (Jost 2008). The Monmonier's algorithm applied on a Delanaunay triangulation was used to define zones of maximum genetic change and genetic barriers within the network of *P. mugo* populations. This analysis was performed with the software BARRIER 2.2 (Manni et al. 2004) based on 100 bootstrap matrices of Goldstein's pairwise genetic distances $(\delta\mu)^2$ to obtain statistical confidence for the predicted barriers. The possible presence of phylogeographic structure, i.e. whether alleles within populations were more related than alleles in the overall sample, was evaluated by comparing R_{ST} to pR_{ST} (permuted) after 10,000 random permutations using the SPAGeDi program. If R_{ST} was significantly higher than pR_{ST}, then allele size mutations contributed to population differentiation and can be interpreted as phylogeographical structure (Hardy and Vakemans 2002). Finally, spatial genetic structure was assessed by testing the significance of isolation by distance (IBD) by using a Mantel test with 9,999 random permutations of the relationship between the matrix of geographic and genetic distances using the procedure of Smouse et al. (1986) implemented within the program GenAlEx 6 (Peakall and Smouse 2005).

Results

Size variants and haplotypes

Among ten cpSSR loci analyzed, only PCP 102652 was monomorphic (allele 112 bp) and thus was excluded from further analyses. From 3 to 8 size variants were identified at each locus, yielding a mean of 5.7 and the effective number of alleles (Ne) ranged from 1.95 to 4.11, with an average of 2.74. Of the 51 alleles detected, 8 were unique to particular populations: three private alleles in population A 2, and one in each of populations A 1, A 3, GM 1, GM 6, TM 1. The 51 size variants at the nine polymorphic cpSSRs combined into 168 different haplotypes out of 4,233,600 mathematically possible combinations. Only the most frequent haplotype (H1 on Fig. 1) had a frequency over 5%. The majority of haplotypes (72.6%) were private, 13.10% were detected twice. Nineteen most abundant haplotypes were common to only 164 (43.27%) individuals (Table 2). The number of private haplotypes was high in Alps (26–16) and in Tatra Mts. (11–15). The lowest N_p was obserwed in GM 5 (1). Similar, the highest rarefried allelic richness was observed in Alps (22–27) but the lowest in Giant Mts. (17.42–20.87). Haplotype diversity was very high for all populations, with a mean H_e of 0.97 (Table 1). The estimates of the effective number of haplotypes (N_e) varied greatly among populations, ranging from a minimum of 13.79 in GM 5 to 26.47 in A 1, with a mean of 17.92. The "proportion distinguishable" was high, ranging from 59.4% in population GM 3 to 93.3% in population A 1. Values of mean genetic distances between haplotypes within populations $\overline{D}^2_{sh}$ varied from a minimum of 4.70 in GM 2 to 9.28 in GM 5, with a mean of 6.70.

Phylogeographic structure

The permutation procedure did not reveal the existence of a phylogeographic structure in the total sample (R_{ST} of 0.137 > pR_{ST} of 0.083, P = 0.263). However, the overall Mantel test showed significant positive correlation between genetic and geographic dis-

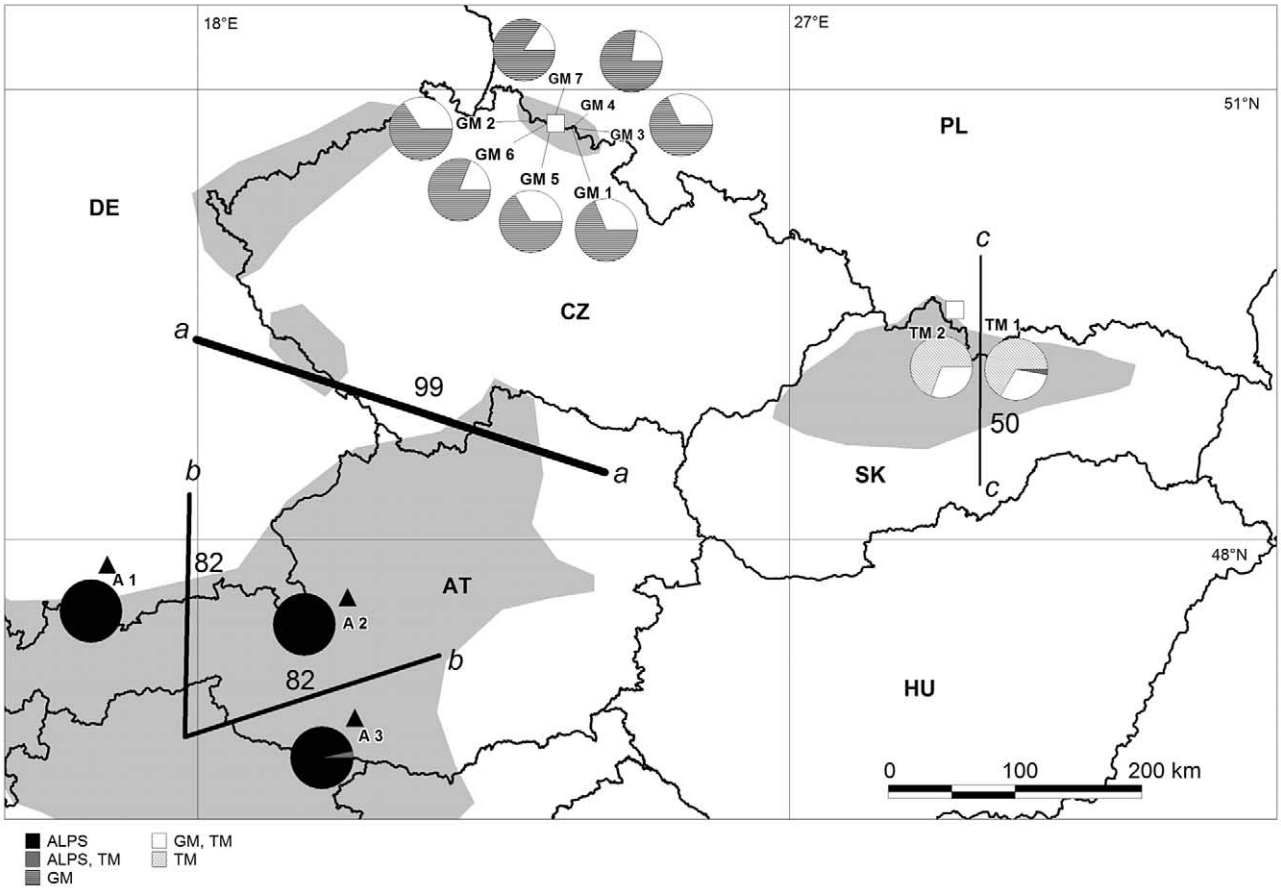

Fig. 1. Map of haplotypic distribution and genetic boundaries computed on 100 bootstrap $(\delta\mu)^2$ genetic distance matrices of the sampled populations of *Pinus mugo* (codes as in Table 1). Symbols (squares and triangles) show genetically different groups according to spatial analysis of molecular variance (SAMOVA) based on R_{ST} index and a $K = 2$. The robustness of computed barriers is shown as a percentage of supporting resampled bootstrap matrices and the thickness of each edge. The shaded area represents the native range of the *P. mugo*

tances among the *Pinus mugo* populations ($r^2 = 0.630$; $P = 0.002$). This isolation by distance structure was not present when tested within Giant Mts. separately (data not shown).

The actual differentiation among populations (*D*) was high (0.60). However, the analysis of molecular variance (AMOVA) based both on F_{ST} and R_{ST}, showed that the proportion of genetic variation attributable to differences among populations was fairly low (8.26 and 13.74%, respectively), but significant. Most of the total genetic variation (91.74 and 86.26%, respectively) was distributed within populations (Table 3). The hierarchical AMOVA showed that a significant amount of genetic variation (11.86 and 19.64%, respectively) was due to differences among the three mountain ranges and that a very small amount (0.55 and 0.56% of the total, respectively) was due to differences among populations within ranges.

The results of a Spatial Analysis of Molecular Variance (SAMOVA) revealed a maximum F_{CT} value of 0.27 with a partition into two geographic groups corresponding to the Giant-Tatra Mts. and Alps (Fig. 1, Table 5). A more detailed SAMOVA analysis in the Giant-Tatra Mts. group showed that F_{CT} began to plateau when K=2, identified TM 1 population as a separate cluster (F_{CT}=0.037, $P < 0.001$). A clear geographic structure was also obtained when simply grouping all haplotypes into one of the five groups: Alps, Alps-Tatra Mts., Giant Mts., Giant Mts.-Tatra Mts. and Tatra Mts. (Fig.1). The distinction was evident between the Giant Mts. and the Tatra Mts. populations, as well as a remarkable divergence of the Alpine populations. This result was corroborated by pairwise *D* analyses (Table 4) and the principal coordinate analysis (PCA), where the two first factors explain 84.32% of the total variation (Fig. 2). Similarly, in a UPGMA dendrogram based on Nei's (1972) genetic distances, three clades can be observed, consisting of populations from Giant, Tatra Mts. and Alps (Fig. 3), with high bootstrap support and R^2=0.923. A remarkable difference among populations, as shown by branch lengths, rivals the differences among clades and presumably derives from the high proportion of private haplotypes identified. The Monmonier's algorithm identified three genetic boundaries. By definition, barriers correspond to

zones of most abrupt genetic change in space. The first barrier (a in Fig. 1), separated the Alpine from all other populations. The second barrier (b in Fig. 1) separated population A2 from other alpine populations. The third barrier (c in Fig. 1) separated populations from Tatra Mts. The presence of these genetic

Table 2. Frequencies of the nineteen most common haplotypes (H1-H19) for twelve *P. mugo* populations (acronyms as in Table 1)

Haplotype	Population											
	GM 1	GM 2	GM 3	GM 4	GM 5	GM 6	GM 7	TM 1	TM 2	A 1	A 2	A 3
H1	2	2	2	5	1	5	2	–	–	–	–	–
H2	3	2	2	2	5	2	1	1	–	–	–	–
H3	–	1	3	2	4	2	4	–	–	–	–	–
H4	1	1	3	1	1	3	4	–	–	–	–	–
H5	2	2	3	1	–	–	–	–	1	–	–	–
H6	3	2	2	1	1	–	–	–	–	–	–	–
H7	3	–	2	–	2	1	–	–	–	–	–	–
H8	1	3	2	1	–	–	–	1	–	–	–	–
H9	2	–	–	–	1	2	1	–	2	–	–	–
H10	2	1	–	–	2	1	1	–	–	–	–	–
H11	–	–	2	–	3	–	–	1	–	–	–	–
H12	–	–	1	3	1	–	1	–	–	–	–	–
H13	–	–	–	–	1	–	2	2	1	–	–	–
H14	–	–	–	–	–	–	–	–	–	–	2	3
H15	–	–	–	–	–	–	–	–	5	–	–	–
H16	1	2	–	–	–	–	1	1	–	–	–	–
H17	1	1	1	–	1	–	1	–	–	–	–	–
H18	–	–	–	–	–	–	–	5	–	–	–	–
H19	1	3	–	–	–	1	–	–	–	–	–	–

Table 3. Analysis of molecular variance (AMOVA) based on F_{ST} and R_{ST} among *P. mugo* populations: (a) assuming no population structuring, (b) assuming population structuring based on isolation in 3 groups: Giant Mts., Tatra Mts. and Alps

	Source of variance	df	Variance component	Variation (%)	*P*
F_{ST}					
a)	Among populations	11	0.2285	8.26	P<0.001
	Within populations	367	2.5375	91.74	P<0.001
b)	Among groups	2	0.3436	11.86	P<0.001
	Among populations within groups	9	0.0159	0.55	P<0.001
	Within populations	367	2.5375	87.59	P<0.001
R_{ST}					
a)	Among populations	11	1.0726	13.74	P<0.001
	Within populations	367	6.7341	86.26	P<0.001
b)	Among groups	2	1.6574	19.64	P<0.001
	Among populations within groups	9	0.0471	0.56	P<0.001
	Within populations	367	6.7341	79.80	P<0.001

Table 4. Geographic distance (km, lower diagonal) and genetic differentiation (*D*, upper diagonal) between *P. mugo* populations (acronyms as in Table 1)

	GM 1	GM 2	GM 3	GM 4	GM 5	GM 6	GM 7	TM 1	TM 2	A 1	A 2	A 3
GM 1	0	0.000	0.000	0.244	0.072	0.087	f0.384	0.843	0.740	1.000	1.000	1.000
GM 2	17.8	0	0.000	0.216	0.332	0.145	0.327	0.739	0.899	1.000	1.000	1.000
GM 3	0.2	18.0	0	0.085	0.000	0.163	0.028	0.828	0.882	1.000	1.000	1.000
GM 4	0.2	17.7	0.3	0	0.324	0.054	0.145	0.862	0.943	1.000	1.000	1.000
GM 5	14.9	2.8	15.1	14.8	0	0.252	0.022	0.763	0.903	1.000	1.000	1.000
GM 6	16.5	1.6	16.7	16.4	1.8	0	0.000	0.892	0.834	1.000	1.000	1.000
GM 7	14.0	3.9	14.2	13.9	1.1	2.6	0	0.823	0.878	1.000	1.000	1.000
TM 1	348.4	365.9	348.2	348.5	363.1	364.5	362.0	0	0.794	1.000	1.000	0.962
TM 2	330.7	348.1	330.4	330.7	345.2	346.6	344.2	20.9	0	1.000	1.000	1.000
A 1	503.8	495.5	503.8	503.6	496.6	495.1	496.6	699.8	679.4	0	0.877	0.837
A 2	417.8	414.7	417.8	417.7	414.9	413.9	414.6	551.9	532.1	162.9	0	0.024
A 3	502.7	501.5	502.6	502.5	501.4	500.5	501.0	586.9	568.6	208.1	99.6	0

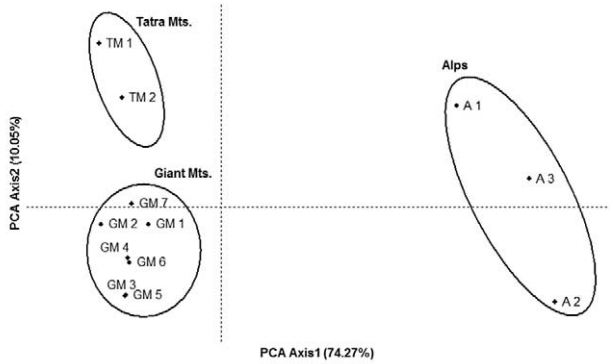

Fig. 2. Principal coordinates analysis of 12 *Pinus mugo* populations in Central Europe based on pairwise Nei's (1972) genetic distances (*Ds*). Population abbreviations are the same as in Table 1

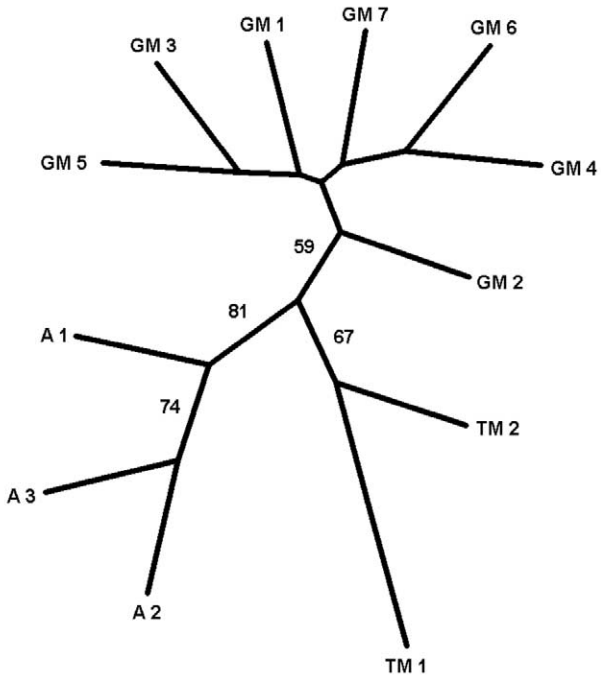

Fig. 3. UPGMA tree for *Pinus mugo,* based on Nei (1972) genetic distances computed from cpSSR haplotype frequencies. Bootstrap support based on 1000 permutations (only values above 50) are indicated in each node

Table 5. Fixation indices (F_{CT}) corresponding to groups of populations (in curly brackets) inferred by SAMOVA algorithms in 12 populations of *Pinus mugo* in Central Europe. Bold populations in the grouping indicate the newly separated populations at given number of groups (*K*)

K	Population groupings	F_{CT}	*P*
2	{A1 / A2 / A3} {rest}	0.270	0.008
3	{A1 / A3} {**A2**} {rest}	0.260	0.003
4	{A1} {A2} {**A3**} {rest}	0.253	0.004
5	{A1} {A2} {A3} {**TM1**} {rest}	0.218	0.002
6	{A1} {A2} {A3} {TM1} {**GM7**} {rest}	0.195	<0.001
7	{A1} {A2} {A3} {TM1} {GM7} {**GM4**} {rest}	0.178	0.002
8	{A1} {A2} {A3} {TM1} {GM7} {GM4} {**GM6**} {rest}	0.167	0.002
9	{A1} {A2} {A3} {TM1} {GM7} {GM4} {GM6} {**GM2**} {rest}	0.162	0.002
10	{A1} {A2} {A3} {TM1} {GM7} {GM4} {GM6} {GM2} {**TM2**} {rest}	0.159	0.001
11	{A1} {A2} {A3} {TM1} {GM7} {GM4} {GM6} {GM2} {TM2} {**GM3**} {rest}	0.158	0.020

barriers was confirmed by analysis with single overall matrix (data not shown).

Discussion

Genetic diversity

The main objective of this study was to assess the level and patterns of genetic variation among *Pinus mugo* populations originating from Central Europe. Our study showed a generally high level of haplotypic variation of cpDNA (He = 0.97), similar to or higher than in other conifers in Central Europe or the Mediterranean Basin (Gómez et al. 2005; Robledo-Arnuncio et al. 2005; Terrab et al. 2006, 2007) and other mountain pine species (Dzialuk et al. 2009; Heuertz et al. 2010; Sannikov et al. 2011). Our results indicate groupings between populations according to geographic distances, which means that geography determines not only genetic relationships between taxa of the *P. mugo* complex in the western Europe (Heuertz et al. 2010), but is also a strong determinant of genetic structure within dwarf mountain pine in central Europe.

Phylogeographic implications

The great genetic differences among populations of *P. mugo* from the three distant centres of the species distribution confirm the long period of their spatial isolation. The present day geographic range of mountain dwarf pine is discontinuous and divided onto several isolated populations (Jalas and Suominen 1973, map 169; Tsaryk et al. 2006). High genetic distances suggest the lack of gene exchange among populations from the Sudetes, Alps and Tatras. The species probably survived the Last Glacial Maximum (LGM) in different refugia, without contact. The refugial areas for mountain plants of sub-alpine vegetation layer, in which *P. mugo* is included (Ozenda 1988, Boratyński 1994), have been poorly recognized, when compared to the central-European tree species of lowland-montane (e.g. Magri et al. 2006; Bhagwat and Willis 2008) and/or high-montane, alpine vegetation layers (e.g. Schönswetter et al. 2005; Ronikier et al. 2008). By interpolating between these two groups of plants, we can suggest that *P. mugo* survived LGM in different, spatially isolated refugia in the Sudetes, Carpathians and Alps (e.g. Obidowicz 1996; Farcas et al. 1999; Jankovská 2001; Wolfrath et al. 2001; Latałowa et al. 2004; Birks and Willis 2008), without or with very strongly restricted exchange of genetic material, in spite of pollination by wind. In Central Europe, the production and dispersal of pollen has been studied for *P. sylvestris,* which is closely related to *P. mugo* (Lewandowski et al. 2000). Scots pine produces a great amount of pollen (Sarvas 1962, Chałupka and Fober 1977; Koski 1987), which is dispersed over long distances (Johansen 1991). The production and transport of *P. mugo* pollen are comparable (Sjögren et al. 2008) and suggest that gene flow by pollen transport among populations of *P. mugo* in the Alps, Sudetes and Tatras is possible, but with a very low probability of effective pollination due to geographic distance (even lower, when taking into account the common rule in conifer pollination "first come – first served", Sarvas 1972). Also, seed transport by birds is rather unlikely.

Differences between Alpine, Sudetan and Carpathian populations of *P. mugo* also suggest different migration patterns during Pleistocene climate oscillations. Species macrofossils have been reported from the forest of the end of LGM and early Holocene from the low parts of the Carpathians, from altitudes of about 600m (Obidowicz 1996; Rybníček and Rybníčková 2002). Pollen attributed to *P. mugo* has also been reported from other parts of the Carpathians (e.g. Farcas et al. 1999), but generally, has not been distinguished from that of *P. sylvestris*, making direct interpretation of several palynological reports impossible (Latałowa et al. 2004). However, the very high percentages of *Pinus* pollen, determined as "*sylvestris*" or "*diploxylon*" type were frequently interpreted as presence of *P. mugo*, especially in records from the LGM and early Holocene in mountainous regions outside the *P. sylvestris* geographical range (Farcas et al. 1999; Latałowa et al 2004; Ali et al. 2006). This suggests a broader area of distribution of *P. mugo* during cold periods of Pleistocene, as was proposed for closely related *P. uncinata* in the Iberian Peninsula (Ramil-Rego et al. 1998; Robledo-Arnuncio et al. 2005; Benito Garzón et al. 2007). The differences found among populations of *P. mugo* from the three centres compared in this study suggest, however, longer period of isolation, than during Holocene. Comparing these differences (Fig. 1, 2 and 3), the more possible seems the connection of populations from the Giant Mts. and the Tatras during one (or more) Pleistocene glacial periods, but probably earlier than Last Glacial. However, this needs to be verified, as macro-fossil remnants are unknown to date.

The genetic differences among populations of *P. mugo* from the Giant Mts. appeared smallest, when compared to those from the Tatras and Alps. Alpine populations had the largest among-population genetic distances, which is, of course, related to longer geographic distances between them (Fig. 1), but may also result from other refugial areas of alpine-subalpine plants, recognized in the Alps itself (Schönswetter et al. 2005; Ronikier et al. 2008; Heuertz et al. 2010). The morphological and anatomical differences between populations of *P. mugo* in the Giant Mts. were also small (Boratyńska et al.

2005; Sobierajska and Boratyńska 2008; Sobierajska et al. 2010).

Conclusions

The populations of *P. mugo* from Giant Mts., Carpathians and Alps were strongly differentiated between each other, while differences among populations within these massifs were much lower. The genetic structuring among isolated parts of the geographic range of the species may be a result of an ancient fragmentation and long lasting geographic isolation between the Giant Mts., Alpine and Tatra populations of *P. mugo*.

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