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Geographic distribution of quantitative traits variation and genetic variability in natural populations of *Pinus mugo* in Central Europe

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Abstract: Divergence in genetic as well as phenotypic structures can be expected in species with disjunctive geographic ranges and restricted gene flow among isolated populations. Dwarf mountain pine has such a disjunctive geographic range in the mountains of Central Europe. We hypothesised that populations of *Pinus mugo* from the Giant Mts. differ from Alpine and Carpathian populations to a greater extent than differentiation within these regions; furthermore, these differences would be detectable at both the genetic and phenotypic levels. To verify this hypothesis, the diversity and differentiation within and among eleven populations from the Giant Mts., Carpathians and Alps were analysed using 19 isozyme isozyme loci, 17 needle and 15 cone morphological characters. Moreover, the data on 10 chloroplast microsatellites used in the previous study, were reanalysed. The differences between the three regions were greater than among populations within them. The microsatellites and isozymes clearly differentiated between regions, while in the multivariate analyses of cone and needle characters the Alpine and Carpathian populations were intermingled but distinct from those sampled in the Giant Mts. The significant genetic structuring among regions may result from an ancient fragmentation and long lasting geographic isolation between the Giant Mts., Alps and Tatras. The populations from the Giant Mts., the northernmost within the geographic range of *P. mugo*, presented lower level of genetic variation then those from the Alps and Carpathians. The pattern of genetic structure observed in dwarf mountain pine may be characteristic of wind-pollinated trees with a disjunctive geographic distribution

Additional key words: genetic diversity, isozymes, isolation by distance (IBD), phenotypic diversity, chloroplast microsatellites

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Introduction

The major topics in evolutionary biology and conservation genetics is determining the level of genetic diversity within population and the differentiation between populations. Factors such as natural selection, genetic drift and mutations promote evolution by increasing differentiation among populations, while gene flow is an obstacle to such differentiation. It is generally accepted that genetic and morphological divergence of taxa starts with differentiation of their populations resulting from spatial isolation, which prevents, or at least strongly reduces, gene flow (Abbott et al. 2008; Comes et al. 2008). For this reason it can operate much more easily among populations within particular regions but not among regions. The level of differences between regions could additionally be intensified due to genetic drift and bottlenecks effects (Hampe and Petit 2005).

Pines are wind pollinated and produce large amounts of pollen (Koski 1970; Sugita et al. 1999; Sjögren et al. 2008). In spite of this and very effective pollen dispersal (Johansen 1991; Sjögren et al. 2008), the division of geographic range should hinder the exchange of genetic material between populations, because the effective pollen transport distance is much shorter than the potential one. Even within the same stand of *P. sylvestris*, transport of pollen is limited (Burczyk and Chalupka 1997; Smouse et al. 2001).

The dwarf mountain pine *P. mugo* Turra (= *P. mugo* subsp. *P. mugo* sensu Christensen 1987) is a prostrate, polycormic shrub which occurs in the mountain massifs of Central and Southern Europe and forms specific plant communities in the subalpine climate-vegetation layer above the upper forest line (Ozenda 1988; Jirásek 1996; Poldini et al. 2004; Tsaryk et al. 2006). Inside its major occurrence centres, *P. mugo* can be found on the massifs, which are sufficiently high that subalpine communities of the species can be developed (Ozenda 1988; Christensen 1987; Tsaryk et al. 2006). Its lowest localities, however, can be found much below the tree line, but only under special site conditions (Gostyńska-Jakuszewska 1976; Christensen 1987). The geographic range of *P. mugo* is disjunctive, divided into several dozen parts (Jalas and Suominen 1973; Tsaryk et al. 2006) that have been isolated from each other from the moment the Holocene climate started to warm (Obidowicz 1996; Willis et al. 2000; Wolfrath et al. 2001; Rybníček and Rybníčková 2002; Latałowa et al. 2004).

The area of distribution of *P. mugo* repeatedly spread during periods with cool temperatures and regressed during warm periods of the Pleistocene (Willis et al. 2000; Wolfrath et al. 2001; Latałowa et al. 2004); this is similar to the case of *P. uncinata* on the Iberian Peninsula (Ramil-Rego et al. 1998; Benito Garzón et al. 2007) and follows the general role proposed by Hewitt (1996). The reduced gene flow between regions during warm periods, random genetic drift and/or possible founder effects influenced the spatial genetic and morphological structure of the populations (Young et al. 1996; Hampe and Petit 2005). The strength of particular genetic and demographic processes was probably modified by cold versus warm periods of Pleistocene (Hewitt 2000). These hypotheses seem to find confirmation in the variation of *P. mugo*. The isoenzymatic differentiation of *P. mugo* complex between the East Carpathians and Swiss Alps was found to be high (Sannikov et al. 2011), but it could also result from taxonomic differences between Carpathian (*P. mugo* s. str.) and alpine (possibly *P. uncinata* influenced) populations. A high level of differences in chloroplast microsatellite loci between the Carpathians, Sudetes and Alps was recently described (Dzialuk et al. 2012), but rather low at selected nucleotide loci (Wachowiak et al. 2013). The significant morphological differences between *P. mugo* from the Carpathians, Sudetes and Abruzzi Mts. were also found (Staszkiewicz and Tyszkiewicz 1976; Boratyńska et al. 2005; Boratyńska and Boratyński 2007).

The populations of *P. mugo* in the Giant Mts. could also be younger from the Alpine and Carpathian ones, established as result of the founder events, at the end of last glacial period and forming the "leading edge" of the species geographic range (Hampe and Petit 2005). In this case they should present the low level of within-population diversity and high level of differentiation.

The aim of the study was verification of both hypotheses using isoenzyme markers as well as morphological traits of cones and needles. The cpDNA markers were reanalysed and results from all four data sets were compared using similar statistics.

Material and methods

Plant material

Samples were collected in three geographic regions: the Giant Mts. (Sudetes), the Alps and the Tatra Mts. (W Carpathians) from six, two and three populations, respectively (Table 1). Material for cpDNA, isoenzymatic and morphological examinations was collected from the same individuals, 30 or more, in each population, at distances of about 30–40 m from each other to avoid possible duplicate sampling from the same genet, as *P. mugo* frequently spreads vegetatively (Prus-Głowacki et al. 2005; Tsaryk et al. 2006). Ten two-year-old dwarf shoots with needles, ten one-year-old needles and ten cones with seeds were sampled separately from each individual. The young needles for DNA analyses were dried and stored at −20°C (for details see Dzialuk et

Table 1. Geographic location of the tested *Pinus mugo* populations and basic climatic data retrieved from DIVA GIS: AMT – Annual Mean Temperature; AP – Annual Precipitation; TMin – average minimal temperature of the coldest month; TMax – average maximal temperature of the warmest month; MTCQU – mean temperature of the coldest quarter (December, January, February); MTW – mean temperature of the warmest quarter (June, July, August); PWM – precipitation in the wettest month; PWQU – precipitation in the wettest quarter (June, July, August)

Code	Location	Voucher specimens	Latit. (N)/ Longit. (E)	Altitude [m]	AMT	TMin	TMax	MTCQU	MTWQU	AP	PWM	PWQU
GM 1	Sudetes, Giant Mts. Równia below Śnieżka	KOR 48739	50°44'44"/ 15°47'41"	1400–1420	3.64	−8.6	16.3	−5.0	11.8	882	108	320
GM 2	Sudetes, Giant Mts. between Łabski Szczyt and Szrenica	KOR 01465 KOR 41988	50°47'40"/ 15°33'15"	1350–1450	2.43	−8.8	14.1	−5.5	10.2	984	115	338
GM 3	Sudetes, Giant Mts. slopes of Śnieżka above Kocioł Łomniczki	KOR	50°44'40"/ 15°47'50"	1300–1500	3.64	−8.6	16.3	−5.0	11.8	882	108	320
GM 4	Sudetes, Giant Mts. Kocioł Małego Stawu near Samotnia	KOR	50°44'41"/ 15°47'34"	1200	3.64	−8.6	16.3	−5.0	11.8	882	108	320
GM 5	Sudetes, Giant Mts. Czarny Kocioł Jagniątkowski	KOR	50°47'05"/ 15°35'30"	1180	3.08	−8.7	15.2	−5.2	11.1	928	111	326
GM 6	Sudetes, Giant Mts. Wielki Kocioł Snieżny	KOR	50°46'55"/ 15°34'00"	1250	2.63	−8.8	14.5	−5.4	10.5	971	114	336
TM 1	Carpathians, Tatra Mts. Dolina Pięciu Stawów Polskich	KOR 41987	49°13'09"/ 20°03'05"	1680–1710	1.04	−10.9	13.2	−7.0	8.9	1440	195	526
TM 2	Carpathians, Tatra Mts. N slopes of Grześ–Wołowiec ridge	KOR	49°13'07"/ 19°45'50"	1600–1650	1.12	−11.0	13.5	−7.0	9.1	1433	195	523
A 1	Alps, NW slopes of Kreuzspitze Mt		47°31'30"/ 10°55'12"	1850–1900	3.15	−8.4	15.8	−4.5	10.7	1134	149	417
A 2	Alps, SW slopes of Hochkonig Mt		47°26'00"/ 13°05'00"	1500	2.51	−10.5	16.6	−5.8	10.6	1467	175	515
A 3	Passo di Pramollo	KOR	46°32'45"/ 13°15'35"	1530	3.47	−9.4	17.6	−4.7	11.5	1208	138	407

al. 2012). The seeds were extracted from the cones and stored at −20°C until further isoenzymatic analyses. The empty cones were used for biometrical analyses. The length of ten two-year-old needles, each from a different dwarf shoot, were measured immediately after collection and then put into 70% alcohol to preserve them until anatomical analyses.

Laboratory treatment

Isozymes

Due to particular feature of the conifer seeds, each mother tree was analysed using no less than 10 macrogametophytes, to reconstruct it diploid genotype (Pastorino and Gregorius 2002). The tissue was homogenised using Tris/HCl, (pH 7.5) homogenising buffer containing 4% PVP (K-15), 0.07% Na-EDTA, 0.2% DTT and 0.13% albumin. The separation of isozymes on starch gels was conducted using two buffer systems. The first was in electrode buffer of pH 8.2 containing 0.3 M boron acid, 0.06 M lithium hydroxide and in gel buffer balanced with citric acid to pH 8.2, containing 0.03 M Tris and 10% electrode buffer (Ridgeway et al. 1970). The second system was in electrode buffer of pH 7.5 containing 0.013 M Tris and 0.043 M citrate acid, and the gel buffer was prepared by a 1:10 dilution of electrode buffer in distilled water (Siciliano and Shaw 1976). The electrophoresis has been conducted using currents with an amperage of 60 mA and voltage of 250 V for the first and 120 V for the second buffer system.

Thirteen enzyme systems were studied (Enzyme Commission number and locus abbreviations are put in parentheses): alcohol dehydrogenase (EC 1.1.1.1, *Adh*), fluorescent esterase (EC 3.1.1.2, *Fle*), glutamate dehydrogenase (EC 1.4.1.2, *Gdh*), glutamate oxalo-acetate transmitase (EC 2.6.1.1, *Got 1*, *Got 2*, *Got 3*), isocitrate dehydrogenase (EC 1.1.1.42, *Idh*), leucine aminopeptidase (EC 3.4.11.1, *Lap 1*, *Lap 2*), menadione reductase (EC 1.6.99.2, *Men*), malate dehydrogenase (EC 1.1.1.37, *Mdh 1*, *Mdh 3*), 6-phospholuconate dehydrogenase (EC 1.1.1.44, *6Pdh 1*, *6Pgd2*), phosphoglucoisomerase (EC 5.3.1.9, *Pgi*), phosphoglucomutase (EC 2.7.5.1, *Pgm 1*, *Pgm 2*), shikimate dehydrogenase (EC 1.1.1.25, *Shdh*) and sorbitol dehydrogenase (EC 1.1.1.14, *Srdh*).

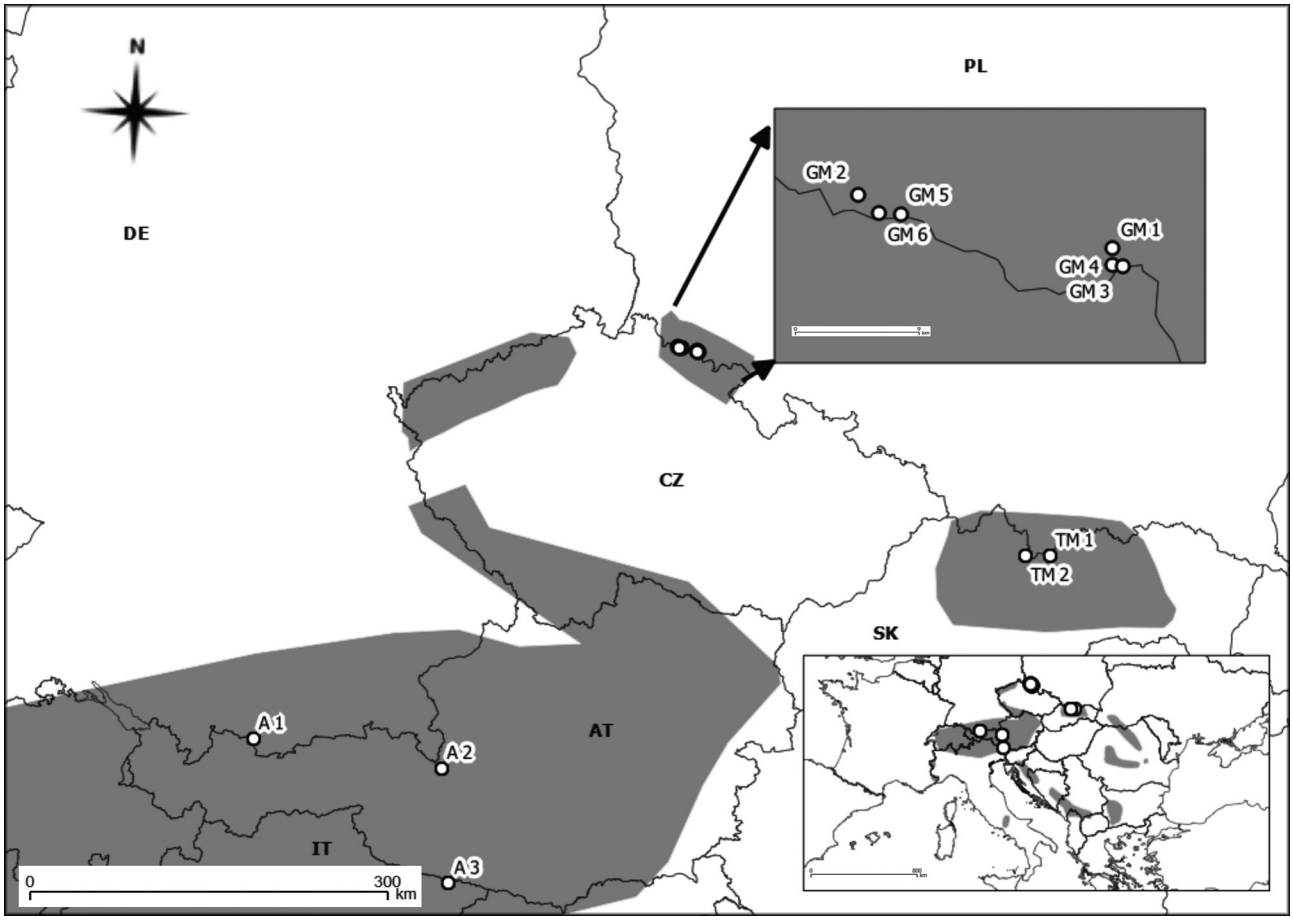

Fig. 1. Distribution of *Pinus mugo* (shaded area, after Jalas and Suominen (1973), simplified) and the sites sampled in the study; acronyms as in Table 1

The electrophoresis of *Fle*, *Gdh*, *Got*, *Lap*, *Pgi*, *Pgm* and *Srdh* was conducted in the first buffer system, and *Adh*, *Mdh*, *Men*, *6Pgd* and *Shda* in the second. Each gel was cut after electrophoresis and every slice was coloured to the activity of another enzyme using a standardised set of painting mixtures (Cheliak and Pitel 1984). The separation of isozymes on starch gels and genetic interpretation of the results were performed as described by Odrzykoski (2002). Alleles at each locus were numbered according to their electrophoretic migration, and the most anodally migrating band was named 1, the next 2, and so on.

cpDNA

Details on amplification of 10 cpSSR loci in a single multiplex PCR reaction are given elsewhere (Dzialuk et al. 2009, 2012).

Phenotypic characteristics

The needles and cones of every individual within a sample were characterised separately. Specimen variation was determined on the basis of ten needles, each from a separate brachyblast, and on the basis of ten cones. In total, morphological differentiation of *P. mugo* was analysed on the basis of 1910, 900 and 660 needles and cones from the Giant Mts., Alps and Tatras, respectively. Some of the data had been utilised earlier to describe the variation in local populations and/or taxonomical comparisons (Boratyńska and Bobowicz 2001; Boratyńska et al. 2003, 2005) and in the Giant Mts. separately (Sobierajska and Boratyńska 2008; Sobierajska et al. 2010).

The 17 needle and 15 cone characteristics were examined (Table 2). The needle traits were selected from published papers on *P. mugo* agg. taxonomy (Boratyńska and Bobowicz 2001; Boratyńska and Boratyński 2007), the cone traits from papers concerning *P. sylvestris* (Staszkiewicz 1968) and *P. mugo* agg. variation and taxonomy (Marcysiak and Boratyński 2007).

Statistical data analysis

Genetic diversity

On the basis of the estimated allele frequencies in isozyme loci, the mean number of alleles per locus (A), effective number of alleles per locus (A_e), per-

Table 2. Average values (mean), coefficient of variation (CV), discrimination power (λ – partial λ value. P – significance of λ) and significance level (** – P ≥0.01; * – P≥0.05) of differences between analysed traits of needles and cones of *Pinus mugo* from the Alps, Tatra and Giant Mts. evaluated by Tukey's *T*-test and/or Kruskal-Wallis test (for explanation see text)

Character	Code	Alps A			Carpathians TM			Giant Mts. GM			Discrimination between regions		Student's and/or Kruskal–Wallis test		
		Min/max	mean	V	Min/max	mean	V	Min/max	mean	V	λ	P	A/TM	A/GM	TM/GM
Needle characteristics															
Needle length [mm]	NL	30/70	46.38	13.3	26/62	44.75	11.3	22/64	41.83	14.0	0.954	0.000	–	**	**
Number of stomata rows on convex (abaxial) side	RSC	5/15	9.28	12.6	5/15	9.91	13.2	5/16	9.21	11.5	0.993	0.285	**	**	**
Number of stomata rows on flat (adaxial) side	RSF	4/11	6.77	13.5	4/13	7.45	13.5	3/12	6.71	12.0	0.981	0.025	**	**	**
Number of stomata on convex side	STC	12.3/25.33	18.93	7.8	13/26.67	18.79	7.9	13/27.67	19.07	6.6	0.971	0.003	–	–	–
Number of stomata on flat side	STF	12.7/24.67	18.70	7.6	12/25.33	18.53	7.9	12.67/25.67	18.94	6.7	0.953	0.000	–	*	**
Number of resin canals	RC	0/8	4.43	21.2	0/8	4.28	21.7	0/8	4.17	18.1	0.989	0.129	–	**	*
Width of needle cross–section [μm]	NW	1093/1934	1481	7.7	786/1913	1509	6.5	850/2104	1427	6.3	0.974	0.006	–	**	**
Height of needle cross–section [μm]	NH	680/1190	874	7.4	638/1063	873	5.6	507/1084	838	5.5	0.981	0.024	–	**	**
Distance between vascular bundles [μm]	VBD	9/206	98	24.7	17/210	103	24.6	4/214	87	31.5	0.994	0.311	–	**	**
Height of epidermis with hypodermis layer [μm]	EH	10/24	15	5.7	10/24	14	7.5	6/34	16	7.7	0.881	0.000	**	**	**
Width of epidermis cell layer [μm]	EW	30/56	42	7.6	27/80	38	6.0	26/81	42	8.0	0.901	0.000	**	–	**
Marcet's coefficient (=VBD×NW/NH)	MC	14/389	167	26.3	27/413	180	25.7	7/472	150	32.3	0.994	0.338	–	**	**
Stomatal rows ratio (=RSC/RSF)		0.75/2.80	1.40	9.1	0.78/2.25	1.35	7.3	0.75/3.33	1.40	9.4	0.989	0.120	**	–	**
Needle thickness/width ratio (=NH/NW)		0.47/0.73	0.59	3.7	0.43/0.97	0.58	3.2	0.40/0.88	0.59	3.7	0.984	0.042	**	–	**
Epidermis width/epidermis with hypodermis thickness ratio (=EW/EH)		0.24/0.55	0.36	6.9	0.24/0.66	0.37	8.6	0.14/0.80	0.37	8.8	0.984	0.047	–	**	–
Sclerenchyma cells between vascular bundles:	PSV														
– fibre-like cells [%]	PSVF	0/8	0.7	270.2	0/6	0.0	0.0	0/20	0.5	301.0	0.974	0.006	–	–	–
– semi–fibrous cells [%]	PSVSF	0/97	7.7	228.7	0/50	1.0	119.5	0/50	3.4	249.5	0.935	0.000	**	–	–
– intermediate [%]	PSVI	0/92	24.0	112.2	1/100	22.4	217.1	0/97	25.1	100.4	0.998	0.689	*	–	**
– cells with thin walls and large lumens [%]	PSVT	0/100	61.2	46.4	10/100	67.3	27.7	0/100	62.7	100.8	0.957	0.000	**	–	**
Sclerenchyma cells around resin canal:	PSR														
– fibre-like cells [%]	PSRF	0/58	8.2	272.4	0/32	6.3	275.8	0/66	3.4	275.1	0.976	0.008	–	**	–
– intermediate cells [%]	PSRI	0/100	40.9	63.6	0/74	25.4	112.1	0/98	31.9	93.2	0.998	0.689	**	**	**
– cells with thin walls and large lumina [%]	PSRT	0/100	45.2	63.2	4/100	62.3	29.0	0/100	56.5	45.4	0.939	0.000	**	**	**
Cone characteristics															
Cone length [mm]	CL	16/49	30.34	14.4	19/46	32.43	15.7	15/58	31.18	13.9	0.999	0.839	**	–	*
Cone maximal diameter [mm]	CD	11/26	18.82	10.3	14/47	19.77	11.2	11/32	19.07	10.8	0.995	0.414	**	–	**
Cone scale number	CSN	45/131	77.77	15.8	53/107	76.34	14.6	47/128	86.13	11.2	0.982	0.036	–	**	**
Length of scale apophysis [mm]	AL	2.9/9.6	5.57	17.3	3.9/9.0	5.78	15.8	2.8/9.3	5.92	11.9	0.965	0.001	**	**	–
Width of scale apophysis [mm]	AW	4.1/10.7	7.35	10.9	5.7/12.5	7.98	13.6	2.5/10.3	7.23	10.7	0.973	0.006	**	–	**
Thickness of scale apophysis [mm]	AT	0.7/5.6	2.41	23.7	1.6/4.7	2.80	15.3	0.8/4.8	2.18	17.5	0.954	0.000	**	**	**
Distance between umbo and scale apex [mm]	AU	1.6/6.2	3.28	16.5	2.3/5.3	3.36	13.5	1.3/5.9	3.04	13.8	0.928	0.000	*	**	**
Cone diameter at midpoint between maximal diameter and cone apex [mm]	CDM	4/25	13.54	17.6	11/21	14.67	13.3	7/25	14.36	18.5	0.997	0.564	**	*	*
Cone protuberant measurement [mm]	CPM	18/66	43.59	12.6	30/79	44.40	13.3	19/68	44.06	11.4	0.986	0.082	–	–	–
Cone concave measurement [mm]	CCM	14/58	36.79	12.8	28/55	40.33	13.3	20/60	38.74	12.0	0.979	0.023	**	**	**
Cone length/maximal diameter (=CL/CD)		0.73/2.54	1.61	10.4	0.70/2.12	1.65	10.8	1.00/3.11	1.64	9.8	0.992	0.261	–	–	–
Cone length/scale number (=CL/CSN)		0.23/0.70	0.40	15.9	0.25/0.64	0.43	17.8	0.20/0.72	0.37	13.2	0.962	0.000	*	**	**
Cone scale apophysis length/width (=AL/AW)		0.43/1.62	0.77	17.1	0.48/1.20	0.73	13.3	0.43/2.18	0.83	12.0	0.994	0.337	–	**	**
Cone scale apophysis length/thickness (=AL/AT)		0.95/7.43	2.51	31.0	1.08/4.72	2.13	26.2	1.26/8.13	2.84	21.3	0.961	0.000	–	**	**
Cone asymmetry (=CPM/CCM)		0.84/2.47	1.20	6.5	0.98/1.93	1.10	5.0	0.45/2.00	1.14	4.8	0.998	0.757	**	**	**

centage of polymorphic loci (*P*, 95% criterion), expected heterozygosity (H_e), observed heterozygosity (H_o) and fixation index (F_{IS}) were calculated for each population and geographic region using GenAlEx 6.5 software (Peakall and Smouse 2012) and GDA software (Lewis and Zaykin 2001).

For chloroplast microsatellites, the data from Dzialuk et al. (2012) were reanalysed. The least squares method (Idury and Cardon 1997) was used for binning of allele lengths, then the haplotypes were identified by allele combinations of polymorphic SSRs. The variation within populations was measured by estimating the total number of haplotypes (A_h), number of private haplotypes (P_h), frequency of the most common haplotype in a particular population (C_h), the effective number of haplotypes (N_e), haplotypic richness (H_r, Mousadik and Petit 1996), unbiased haplotype diversity (H_e) and the mean genetic distance between individuals within populations (D^2_{sh}, Goldstein et al. 1995, applied to cpSSRs by Morgante et al. 1998).

The statistical significance of differences in the genetic parameters between geographic regions was evaluated by the Kruskal-Wallis test using the program PAST 2.17 (Hammer et al. 2001). Spatial patterns of genetic variability were visualised by Pearson's correlation analysis between intra-population parameters of genetic variation and geographic data for each population (latitude, longitude and altitude). Additional, genetic diversity parameters were regressed on climatic data retrieved from DIVA-GIS database (Hijmans et al. 2012).

Morphological comparisons

The Shapiro-Wilk's test was used to assess the symmetry and unimodality of the data The homoscedasticity of the data was checked using the Brown-Forsythe test as implemented by STATISTICA (StatSoft) to assess the possibility of using parametric statistical tests (Zar 1999; Sokal and Rohlf 2003). The arithmetic means and standard deviations were calculated for each population and region. Prior to the analyses, all data were standardized using STATISTICA (StatSoft) procedures to avoid possible influences from the various types of traits used. The level of diversity of particular characteristics was compared using the Student's t-test (Boratyńska et al. 2005).

Relationships between traits were checked using the Pearson's correlation coefficient and discrimination analysis, which also identified the power of each trait to discriminate between regions (Sokal and Rohlf 2003; Tabachnik and Fidell 2007). The possible dependence of phenotypic traits on the geographic data and climatic conditions of each population was verified via regression analysis.

Differentiation and grouping of populations

We estimated genetic structure among populations using the widely accepted Nei's G_{ST} statistic. Additionally, the phylogeographic structure of chloroplast haplotypes was assessed by the permutation test of N_{ST} and G_{ST} values for significant differentiation. G_{ST} is solely based on allele frequencies, while N_{ST} takes into account similarities or relatedness among haplotypes. A N_{ST} higher than the estimated G_{ST} suggests that allele size mutations contributed to population differentiation; thus alleles within populations are more related than alleles in the overall sample. The program Permut & CpSSR v. 2.0 (Pons and Petit 1996) was applied to compare G_{ST} vs. N_{ST} values using 10,000 random permutations. Because Jost (2008) has shown that, when using highly polymorphic markers, G_{ST} does not provide a straightforward assessment of how different populations are, we also computed the *D* estimator. The significance of *D* estimates was evaluated using bootstrap resampling in GenAlEx 6.5 (Peakall and Smouse 2006, 2012) and SPADE (Chao and Shen 2010).

The hypothesis that dwarf pines from the same geographic region (mountain range) are more closely similar genetically and morphologically was tested using three approaches. First, their genetic differentiation was quantified using a hierarchical analysis of molecular variance (AMOVA). Total genetic variation was partitioned into (1) among regions (Tatra Mts., Giant Mts., Alps), (2) among populations within regions, and (3) within populations. The significance was tested by resampling with 1,000 randomizations using the program Arlequin ver. 3.5.1.2 (Excoffier and Lischer 2010). The distribution of variation of every phenotypic trait between regions, populations and individuals was tested by ANOVA.

Second, to determine if genetic differences between populations corresponded to geographic distribution patterns, the genetic distance (*D*) of Nei (1972) for isozymes and cpSSRs and Euclidean distance (D_{EU}) for morphological traits were used to construct unrooted trees using the NEIGHBOR and DRAWTREE options in the PHYLIP package v 3.68 (Felsenstein 1995). The neighbour joining (NJ) method was used for building trees, because Kalinowski (2009) has recently shown the algorithm describes genetic relationships between populations that have an isolation-by-distance structure more faithfully than UPGMA. To analyze how well a tree fit the genetic data the tree was calculated from, the R^2 parameter was calculated using the TreeFit program (Kalinowski 2009). If R^2 is near 1.0, the tree represents a good summary of the genetic relationships shown in the distance matrix. Values less than 0.90 suggest the tree should not be used to describe population struc-

ture. The statistical confidence in the topology of the trees was also measured by bootstrapping 10,000 NJ trees in PowerMarker v.3.25 software (Liu and Muse 2005). The CONSENSE software from the PHYLIP v 3.68 package (Felsenstein 1995) was used to construct consensus trees.

Third, to confirm the spatial pattern of genetic grouping, a principal coordinate analysis (PCoA) was performed and the ordination of the populations on the first two principal coordinates was plotted using GenAlEx 6.5 software (Peakall and Smouse 2012). To test whether population differentiation was caused by isolation by distance (IBD, Wright 1943), we conducted a Mantel test by regressing the genetic differentiation between populations ($F_{ST}/(1-F_{ST})$ for isozymes and cpSSRs ($D_{EU}/(1-D_{EU})$ for traits) versus the log geographic distance. The test was carried out on 9,999 permutations of the data with GenAlEx 6.5 software (Peakall and Smouse 2012).

Additionally, the significance of differences between mathematical means of morphological traits for the three regions was verified using the t-Student's for non-biased and the Kruskal-Wallis tests for biased data (Sokal and Rohlf 2003).

Results

Genetic diversity within populations

Estimates of genetic diversity within populations are shown in Table 3. Among 19 isozyme loci, Pgi 1 was monomorphic in the whole sampled populations and thus excluded from further statistical analyses. Additionally, Got 1, Mdh 1, Pgi 2, Pgm 1 were monomorphic in at least one population. The most highly polymorphic was Adh 2, with 7 alleles, and Gdh, Got 2, Shdh 1, each with 6 alleles. On average, all loci had at least 3 alleles. Averaged across all populations, the percentage of polymorphic loci (95% criterion) was 86%, with the minimum percentage in GM 1 (79%) and the maximum in TM 1 and A 1 (92%). At the regional level, the mean percentage of polymorphic loci was slightly higher in the Tatras Mts. region (90%) than in populations from the Giant Mts. (85%) and the Alps (88%) (Table 3). Among 98 alleles identified in tested populations of *P. mugo*, 12 were identified as private (3 in the Tatras Mts., 3 in the Giant Mts. and 6 in the Alps) and 15 as region private (6 in the Giant Mts., 3 in the Tatras Mts. and 6 in the Alps).

Across populations, allele number per locus ranged between 2.7 and 3.0. On average, the number of alleles differed significantly between regions (Kruskal-Wallis test: $\chi^2=6.2$, $p=0.035$), being the highest in the Alps. A similar pattern was observed for effective number of alleles ($\chi^2=6.4$, $p=0.039$).

Table 3. Estimates of genetic diversity for eleven *P. mugo* populations and means for three geographic regions (bold) based on twenty-four isozyme loci and nine chloroplast microsatellites

Region/Pop	isozymes							cpSSR							
	N	A	A_E	P_{95}(%)	H_o	H_e	F_{IS}	N	A_h	P_h	C_h	N_e	H_r	H_e	D^2_{sh}
GM_1	31	2.71	1.47	79	0.276	0.270	−0.018	32	22	4	0.09	17.66	20.05	0.974	6.47
GM_2	30	2.71	1.54	83	0.310	0.303	−0.028	31	21	3	0.10	17.47	19.58	0.974	4.70
GM_3	29	2.79	1.45	88	0.299	0.269	−0.083**	32	19	5	0.09	16.00	17.42	0.968	8.03
GM_4	30	2.83	1.54	88	0.311	0.310	0.017	31	21	7	0.16	14.78	19.52	0.963	6.14
GM_5	30	2.67	1.47	83	0.318	0.271	−0.131***	33	20	1	0.15	13.78	17.80	0.956	9.28
GM_6	30	2.79	1.52	88	0.300	0.288	−0.025	32	21	7	0.16	15.06	19.11	0.964	6.58
Giant Mts.	**30**	**2.75**	**1.50**	**85**	**0.302**	**0.285**	**−0.044****	**32**	**21**	**6**	**0.16**	**15.79**	**18.91**	**0.967**	**6.87**
TM_1	29	3.04	1.59	92	0.313	0.310	0.013	33	23	11	0.15	16.75	20.51	0.970	7.05
TM_2	30	2.75	1.53	88	0.297	0.305	0.033	33	25	15	0.15	17.29	22.08	0.972	6.56
Tatra Mts.	**30**	**2.90**	**1.56**	**90**	**0.305**	**0.307**	**0.023**	**33**	**24**	**20**	**0.15**	**17.02**	**21.30**	**0.971**	**6.81**
A_1	23	3.04	1.64	92	0.368	0.338	−0.073	30	28	26	0.07	26.47	27.00	0.990	7.66
A_2	29	2.92	1.61	88	0.336	0.328	−0.009	30	26	21	0.07	23.68	25.00	0.991	5.38
A_3	30	2.92	1.55	83	0.299	0.299	0.008	30	23	16	0.10	19.57	22.00	0.982	6.56
Alps	**27**	**2.96**	**1.60**	**88**	**0.334**	**0.322**	**−0.023**	**30**	**26**	**21**	**0.10**	**23.24**	**24.67**	**0.987**	**6.54**
Total	29.2	2.83	1.54	86	0.311	0.299	−0.026*	31.5	22.6	11	0.12	18.05	20.92	0.973	6.77

isozymes: *N*, mean number of individuals analysed per locus; *A*, mean number of alleles per locus; A_E, effective number of alleles per locus; *P*, percentage of polymorphic loci (95 % criterion); H_o, observed heterozygosity; H_e, mean unbiased estimate of expected heterozygosity; F_{IS}, fixation index; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

cpSSR: A_h, number of haplotypes, P_h, number of private haplotypes; C_h, frequency of the most common haplotype in a particular population; N_e, effective number of haplotypes; H_r, haplotypic richness; H_e, Nei's index of genetic diversity estimated without bias; D^2_{sh}, mean genetic distance between individuals within populations

The highest observed average heterozygosity (H_o) for a population was found in A 1 (H_o=0.368) and the lowest in GM 1 (H_o=0.276). Although populations from the Alps had higher values of average observed heterozygosity, no statistical differences were found for populations within regions (Kruskal-Wallis test: χ^2=1.8, p=0.411). A similar pattern was observed for expected heterozygosity (H_e; Kruskal-Wallis test: χ^2=4.5, p=0.104).

Although the mean fixation index (F_{IS}= −0.026) indicated a significant deficiency of homozygotes (p<0.05) in the whole sample, an excess of heterozygotes was statistically significant only in populations from the Giant Mts. (p<0.01). Unlike other regions, the Tatras Mts. showed a deficiency of heterozygotes (but it was not significant) (Table 3). No statistical differences in fixation indices were found between populations (Kruskal-Wallis test: χ^2=3.9, p=0.144).

Among ten chloroplast microsatellite loci, nine were polymorphic. Prior to data analysis, the monomorphic locus (PCP 102652) was discarded. With respect to cpSSRs, we identified a total of 51 alleles (variances) with an average of 3.7 alleles per population and marker. The most highly polymorphic locus was Pt71936 with 8 followed by Pt45002 and PCP41131, each with 7 alleles. The variants were combined in 163 different haplotypes out of 4,233,600 mathematically possible combinations. From these, no haplotype was common among all populations, 121 were private (observed in a single population) and 148 were region private (observed in a single geographic region). The most common haplotype was detected in all populations from the Giant Mts. and in one population from the Tatra Mts.

At the regional level, slightly smaller numbers of haplotypes, private haplotypes and haplotypic richness were observed in the Giant Mts. (21, 6 and 18.91, respectively) than in the Tatra Mts. (24, 20 and 21.30, respectively) or the Alps (26, 21 and 24.67, respectively). The differences between regions were statistically significant for these parameters (Kruskal-Wallis test: χ^2=7.7, p=0.020; χ^2=8.2, p=0.016; χ^2=7.8, p=0.020, respectively). Similarly, a higher probability of randomly sampling two identical haplotypes (N_e) was observed in the Giant Mts. than in the Tatras Mts. or the Alps (Kruskal-Wallis test: χ^2=6.2, p=0.044). As a result of the low haplotype frequencies, very high within population diversity values were found (H_e=0.973), with the highest values in the Alps (Kruskal-Wallis test: χ^2=6.2, p=0.043). No statistical differences in genetic distance between individuals within populations were found between populations (Kruskal-Wallis test: χ^2=0.2, p=0.918), with mean D^2_{sh}=6.77.

The Pearson correlation analysis revealed that the genetic diversity within *P. mugo* populations in Central Europe is significantly positively correlated with altitude but decreases with increasing latitude (Fig. 2). When the mean number of alleles per isozyme locus (*A)* and haplotypic richness (H_R) for chloroplast DNA were correlated with the geographic variables, a positive correlation was observed versus altitude (R^2=0.560; p=0.008 and R^2=0.655; p=0.003, respectively), while the correlation with latitude was negative (R^2=0.497; p=0.015 and R^2=0.665; p=0.002, respectively). No correlations were found between the isozyme or chloroplast genetic diversity parameters versus longitude (p=0.483 and p=0.252,

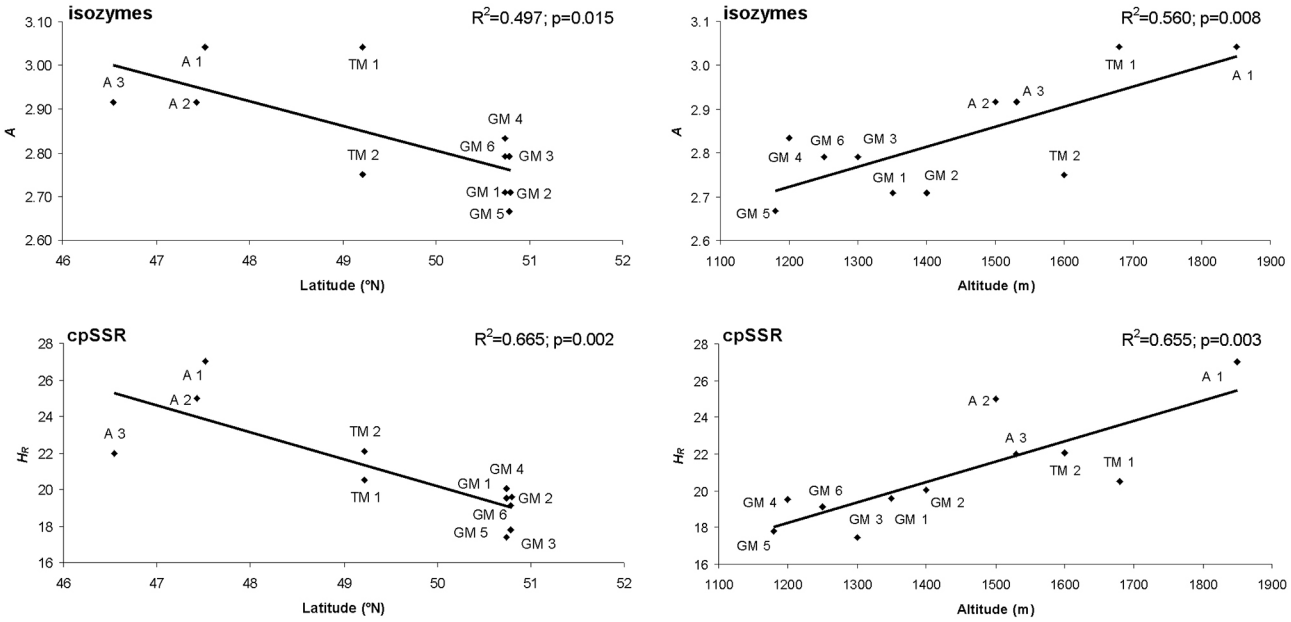

Fig. 2. Pearson's correlation analysis between mean number of alleles per isozyme locus (*A*) and cpSSR haplotype richness (H_R) of *P. mugo* populations versus geographic data (latitude and altitude)

respectively). Therefore, higher intra-population diversity was observed in the Alps, with lower values in the Tatras and Giant Mts. The regression analyses revealed no effect of most climatic conditions on genetic diversity parameters. Marginally significant positive relationships between annual precipitation, precipitation in the wettest month, precipitation in the wettest quarter and the mean number of alleles per isozyme locus (R^2=0.312, p=0.074; R^2=0.314, p=0.073; R^2=0.331, p=0.064, respectively), as well as precipitation in the wettest month and cpSSR haplotype richness (R^2=0.325, p=0.067) were detected. Only haplotypic richness increased significantly with increasing annual precipitation and precipitation in the wettest quarter (R^2=0.365, p=0.049; R^2=0.369, p=0.048, respectively).

Morphological variation

The most of data examined had a normal or very close to normal frequency distribution, which enabled us to use multivariate statistical methods for further analyses. Only PSV and PSR data were excluded from further analyses because of biased frequencies.

The average values of both needle and cone traits differed between the three geographic groups of populations at very low yet in most cases statistically significant levels. In the discrimination analysis, 10 characters discriminated between groups at a statistically significant level, with p≤0.05. Eight out of 15 cone characteristics in the same test discriminated at a significant level between the Alps, Tatras and Giant Mts. (Table 2). Student's t-test showed that all characteristics, except for STC from needles and CPM and the CL/CD ratio of cones, differentiated at a significant level between at least one pair of groups.

The variation in particular characteristics of needles and cones from the Alps, Tatras and Giant Mts. was found to be at similar levels. The CV values of the needle traits were statistically the same in the Alps and Tatras. The different CV values between the Alps and Giant Mts. have NH, WBD and MC (t=3.470, p=0.008, t=4.255, p=0.003 and t=4.175, p=0.003, respectively), and between the Tatras and Giant Mts. WBD and MC (t=3.967, p=0.005 and t=4.753, p=0.002, respectively). The CV values of the cone characteristics were also similar, with significant differences in CM between populations from the Alps and Tatras and between the Alps and Giant Mts. (t=−10.61, p=0.001, t=−3.735, p=0.006, respectively). However, significant differences in CV at p≤0.05 were detected for CL, CDM, CPM CCM and the AL/AW ratio between Tatras and Giant Mts. populations, and for AL, CDM, and AL/AW, AL/AT and CPM/CCM ratios between Alpine and Giant Mts. populations.

The values of morphological characteristics correlated with each other. Positive, statistically significant connections were detected among groups of dimensional characteristics of needles and among dimensional characteristics of cones. The correlation between the cone and needle characters was generally weak; however, a few significant dependencies were found, as for example AT to NW and NH ($r_{Pearson}$=0.23 and 0.27, respectively, p≤0.05 in both cases).

The regression analysis revealed that none of the needle phenotypic characteristics correlated either to geographic coordinates or to the altitude of the analysed *P. mugo* populations, but some traits revealed a significant dependence on climatic factors. EH and EW positively correlated with the minimal temperature of the coldest month and mean temperature of the coldest quarter (R^2=0.76, p=0.005, R^2=0.63, p=0.0034, and R^2=0.63, p=0.003, R^2=0.60, p=0.005, respectively). The same needle traits negatively correlated with the mean precipitation in the wettest month (R^2=0.56, p=0.008) and wettest quarter (R^2=0.53, p=0.011).

Most cone traits were dependent neither on geographic position nor climatic conditions. Only AW negatively correlated with minimal temperatures in the coldest month and quarter (R^2=0.44, p=0.027 and R^2=0.39, p=0.042, respectively). AU correlated positively to altitude and to precipitation in the wettest month (R^2=0.52, p=0.012, R^2=0.40, p=0.038, respectively), and cone asymmetry (CPM/CCM) negatively to geographic longitude and positively to maximum temperature of the warmest month, as well as to mean temperature of the driest quarter (R^2=0.58, p=0.007, R^2=0.46, p=0.023 and R^2=0.56, p=0.008, respectively).

Differentiation between populations

In the overall dataset, the estimated coefficient of genetic differentiation among populations (G_{ST}) was low but significant for isozymes (G_{ST} =0.027, p<0.001) and cpSSRs (G_{ST} =0.017, p<0.001). Comparisons of cpSSR's G_{ST} vs. N_{ST} (N_{ST} =0.022, p<0.001) indicated insignificant differences (p>0.05) for haplotype differentiation measures, suggesting a lack of phylogeographic structure. Jost's *D* was significant but much higher for chloroplast microsatellites than for isozymes (D_{iso} =0.013, p<0.001; D_{cpSSR} =0.620, p<0.001).

A hierarchical AMOVA using isozymes revealed that variation among regions accounted for 4%, while among populations within regions and within populations for 3% and 93% of the total variation, respectively. However, chloroplast SSRs confirmed the presence of a much more pronounced and significant differentiation among regions (Φ_{RT} =0.121; p=0.001) (Table 4). A hierarchical ANOVA on particular traits

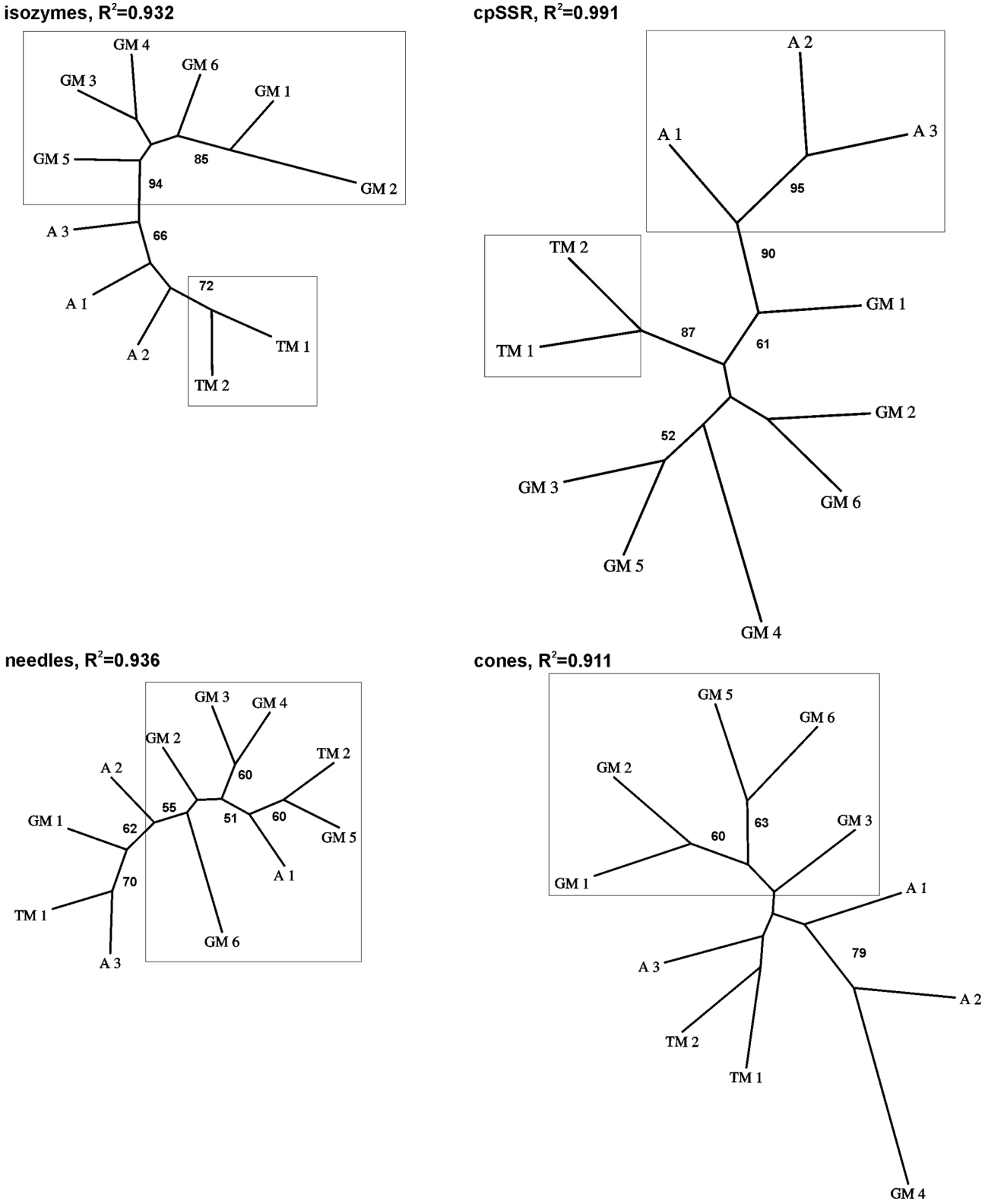

Fig. 3. Consensus of 10,000 NJ trees inferred from comparative analysis of eleven *P. mugo* populations. Branches are labeled with bootstrap support above 50%

of needles and cones detected the major portion of variation located among individuals and then among populations (Table 5). Between the Alps, Tatras and Giant Mts. only small amounts of variation were found among the needle traits, significant only for EH, and among cone traits for AU and CL/CSN and CPM/CCM ratios. The distribution of variance for these characteristics revealed a higher proportion between regions than between populations within a region (Table 5).

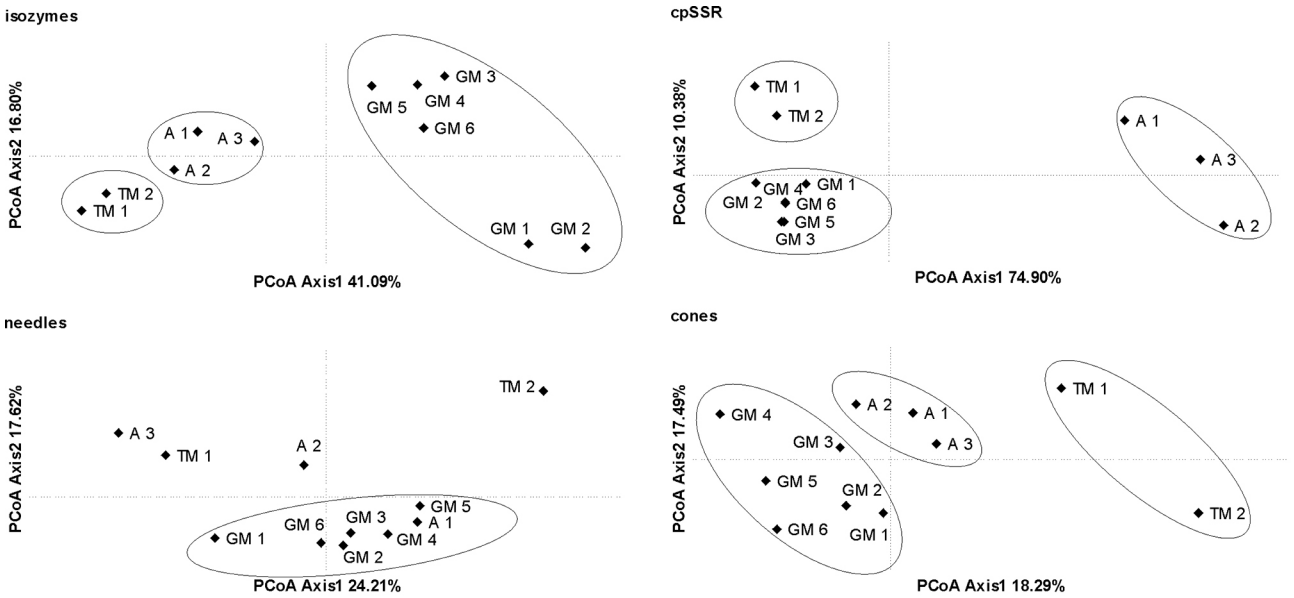

Fig. 4. Principal Coordinates Analysis (PCoA) calculated from the genetic (isozymes and cpSSR) and phenotypic (needle and cone) characteristics of the 11 P. mugo populations; population acronyms as in Table 1

The pattern of population grouping is visualized in consensus NJ trees (Fig. 3). The R^2 values were high (above 0.9) for all dendrograms, indicating accurately depicted genetic relationships between populations. In general, NJ analyses clustered all populations together within regions (mountain systems). Isozymes and cpSSRs revealed pronounced separation between regions, and this geographic grouping was supported by high bootstrap values (Fig. 3). The Giant Mts. region was more strongly differentiated compared with the others on all dendrograms; however, this tendency was less evident using morphological characteristics of needles and cones. There were exceptions to this geographic trend, e.g. the appearance in the Giant Mts. group of single populations from the Tatras and Alps (needle traits) or the grouping of the GM 4 population within the Alpine cluster (cone traits). Additionally, dendrograms based on morphological characteristics had lower bootstrap supports than those based on isozymes or chloroplast microsatellites. The dendrograms also revealed the Tatras region was more similar to the Alpine, forming a single mixed group, nevertheless distinct from the Giant Mts., as shown by morphological traits.

The PCoA confirmed the geographic pattern of the groupings. The first two axes of the PCoA using isozymes, cpSSR, needle and cone traits accounted for 57.89%, 85.28%, 41.82% and 35.78% of the total variance, respectively. The PCoA based on isozymes, cpSSR and cone traits clearly separated the 11 *Pinus mugo* populations into three groups, similar to that observed in the NJ analyses. The grouping of populations using needle characters was less evident. In spite of this, six populations from the Giant Mts. clustered together in a large group with a single Alpine population (Fig. 4).

A significant IBD pattern was observed when genetic differentiation was correlated with the logarithm of pairwise spatial distances among populations based on isozymes (R^2=0.129; p=0.008), cpSSRs (R^2=0.407; p=0.002) and needle traits (R^2=0.151; p=0.032) (Fig. 5). This correlation was significant only when all populations were analysed, regardless of region. Within regions (the Giant Mts. and Alps) there was no correlation between genetic differentiation and geographic separation. No IBD was found either using cone traits (R^2=0.030; p=0.214).

Table 4. Analysis of molecular variance (AMOVA) based on isozyme and cpSSR data assuming a geographic population structuring based on isolation in three regions: Giant Mts., Tatra Mts. and Alps.

Markers	Source of variance	*df*	Variance component	Variation (%)	Φ statistic	p
isozymes	Among regions	2	0.316	4	0.041	0.001
	Among populations within regions	8	0.244	3	0.033	0.001
	Within populations	310	7.122	93	0.073	0.001
cpSSR	Among regions	2	0.350	12	0.121	0.001
	Among populations within regions	8	0.016	1	0.006	0.093
	Within populations	336	2.540	87	0.126	0.001

Table 5. Analysis of variance (ANOVA) based on phenotypic needle and cone data assuming a geographical population structuring among the regions Giant Mts., Tatra Mts. and Alps.

Trait	Source of variance	*df*	Variance component	Variation (%)	F Ratio	p
		Needle traits				
NL	Among regions	2	3.623	7	2.2296	0.1642
	Among populations within regions	9	8.917	17	9.8476	<.0001
	Among individuals	391	32.020	62	48.6020	0.0000
RSC	Among regions	2	0.018	1	1.1385	0.3629
	Among populations within regions	9	0.388	14	10.7650	<.0001
	Among individuals	391	1.170	42	10.8240	0.0000
RSF	Among regions	2	0.068	4	1.6279	0.2496
	Among populations within regions	9	0.340	18	15.6940	<.0001
	Among individuals	391	0.665	34	8.7439	<.0001
STC	Among regions	2	−0.076	−2	0.2023	0.8206
	Among populations within regions	9	0.260	7	5.0881	<.0001
	Among individuals	391	1.900	51	12.8080	0.0000
STF	Among regions	2	−0.0100	−3	0.2934	0.7527
	Among populations within regions	9	0.414	11	7.6927	<.0001
	Among individuals	391	1.843	49	12.2460	0.0000
RC	Among regions	2	−0.022	−2	0.4587	0.6464
	Among populations within regions	9	0.119	10	6.5141	<.0001
	Among individuals	391	0.653	55	15.6930	0.0000
NW	Among regions	2	1120.627	6	1.9703	0.1956
	Among populations within regions	9	3582.564	19	12.9460	<.0001
	Among individuals	391	9255.427	50	20.5600	0.0000
NH	Among regions	2	388.062	7	2.2224	0.1648
	Among populations within regions	9	981.975	18	12.5010	<.0001
	Among individuals	391	2626.010	48	19.2700	0.0000
VBD	Among regions	2	16.972	2	1.4998	0.2751
	Among populations within regions	9	94.584	8	5.6584	<.0001
	Among individuals	391	616.808	54	15.7410	0.0000
EH	Among regions	2	0.751	13	12.323	0.0028
	Among populations within regions	9	0.187	3	6.1424	<.0001
	Among individuals	391	0.745	13	2.7248	<.0001
EW	Among regions	2	2.702	10	3.5355	0.0741
	Among populations within regions	9	3.264	12	11.5690	<.0001
	Among individuals	391	8.829	34	8.4663	<.0001
MC	Among regions	2	15.413	0	1.11240	0.3708
	Among populations within regions	9	396.655	10	6.9910	<.0001
	Among individuals	391	2003.946	52	14.9380	0.0000
RSC/RSF	Among regions	2	0.0006	1	3.2894	0.0877
	Among populations within regions	9	0.0005	1	2.1442	0.0252
	Among individuals	391	0.0087	12	2.5033	<.0001
NH/NW	Among regions	2	−0.0001	−3	0.1849	0.8343
	Among populations within regions	9	0.0002	12	13.3770	<.0001
	Among individuals	391	0.0004	25	4.6939	<.0001
EW/EH	Among regions	2	0.000001	0	1.0847	0.3793
	Among populations within regions	9	0.00015	4	6.2866	<.0001
	Among individuals	391	0.00065	18	3.2437	<.0001
		Cone traits				
CL	Among regions	2	−0.0535	−0	0.9074	0.4365
	Among populations within regions	9	1.5050	5	3.7612	0.0002
	Among individuals	362	15.7820	57	14.8010	0.0000
CD	Among regions	2	−0.0178	−0	0.8922	0.4423
	Among populations within regions	9	0.472	8	5.0576	<.0001
	Among individuals	362	3.356	55	14.2310	0.0000

CSN	Among regions	2	22.573	12	3.8530	0.0613
	Among populations within regions	9	25.762	14	10.7660	<.0001
	Among individuals	362	75.529	41	12.8820	0.0000
AL	Among regions	2	0.027	3	1.6887	0.2376
	Among populations within regions	9	0.120	12	8.3625	<.0001
	Among individuals	362	0.463	45	11.4320	0.0000
AW	Among regions	2	0.104	9	3.2485	0.0861
	Among populations within regions	9	0.151	15	10.5060	<.0001
	Among individuals	362	0.447	40	10.9330	0.0000
AT	Among regions	2	0.026	8	1.9998	0.1909
	Among populations within regions	9	0.091	29	24.6550	<.0001
	Among individuals	362	0.110	35	12.5640	0.0000
AU	Among regions	2	0.032	9	5.2508	0.0298
	Among populations within regions	9	0.021	6	4.3926	<.0001
	Among individuals	362	0.172	46	11.5430	0.0000
CDM	Among regions	2	−0.821	−10	0.3168	0.7363
	Among populations within regions	9	4.234	50	43.9110	<.0001
	Among individuals	362	2.813	33	12.2860	0.0000
CPM	Among regions	2	−0.481	−1	0.1916	0.8288
	Among populations within regions	9	1.317	3	2.6819	0.0050
	Among individuals	362	22.581	57	14.0980	0.0000
CCM	Among regions	2	1.444	4	3.6336	0.0677
	Among populations within regions	9	1.305	4	3.0639	0.0015
	Among individuals	362	17.973	50	11.9350	0.0000
CL/CD	Among regions	2	−0.001	−3	0.1889	0.8310
	Among populations within regions	9	0.004	9	6.5339	<.0001
	Among individuals	362	0.021	48	10.6960	0.0000
CL/CSN	Among regions	2	0.0006	13	7.2883	0.0125
	Among populations within regions	9	0.0003	6	4.4299	<.0001
	Among individuals	362	0.0024	48	14.3720	0.0000
AL/AW	Among regions	2	0.0017	7	3.4416	0.0767
	Among populations within regions	9	0.0021	9	8.0621	<.0001
	Among individuals	362	0.0082	36	7.9026	<.0001*
AL/AT	Among regions	2	−0.0089	−1	0.8778	0.4484
	Among populations within regions	9	0.2552	38	37.6100	<.0001*
	Among individuals	362	0.1912	29	8.6874	<.0001*
CPM/CCM	Among regions	2	0.0011	7	5.7533	0.0234*
	Among populations within regions	9	0.0007	4	8.3508	<.0001*
	Among individuals	362	0.0015	10	2.1045	<.0001*

Discussion

Genetic diversity

Genetic variation within most outcrossing forest tree species is high in comparison to other organisms (Hamrick and Godt 1996). The level of genetic diversity found in isozymes in the studied populations of *P. mugo* does not differ radically from that reported for another gymnosperm species characterised by a broad geographic rangeand is also similar to, but slightly higher than, levels observed earlier in the dwarf mountain pine and closely related taxa from the *P. mugo* agg. (Prus-Głowacki et al.1998; Lewandowski et al. 2000; Odrzykoski 2002; Slavov and Zhelev 2004). The higher level of genetic diversity in our study may result from analysing a higher number of populations representing the three distinct centres of the species' geographic range, that were probably isolated for a long period during of Pleistocene (Latałowa et al. 2004; Jankovská 2008; Jankovská and Pokorný 2008).

The non-conformity with Hardy-Weinberg proportions suggests nonrandom mating and indicates a nonequlibrium population genetic structure. However, while significant departures with excess heterozygotes were observed for the whole dataset (F_{IS}=−0.026). Contrary to our results, a great deficiency in heterozygotes was observed earlier for 15 Bulgarian populations of *P. mugo* (F_{IS} =0.252) and two from the Tatras Mts. (F_{IS} =0.283) (Slavov and Zhelev 2004). The latter is partially consistent with our results, because we found a statistically insignificant excess of homozygotes in these mountains.

The level of diversity revealed in cpSSR loci has been discussed in a separate paper (Dzialuk et al.

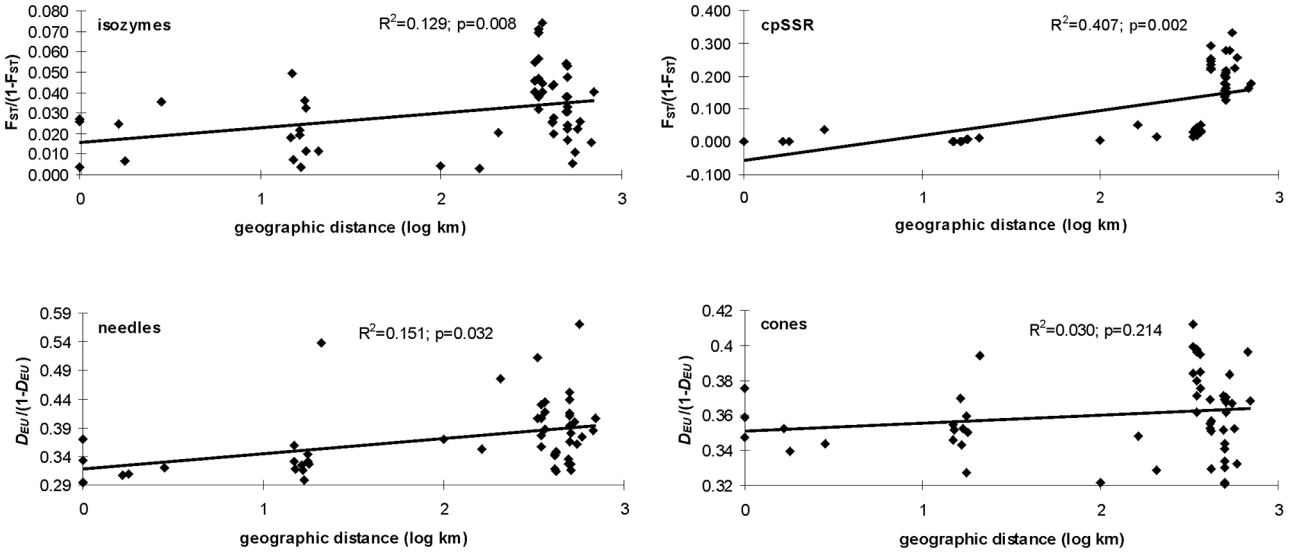

Fig. 5. Test of isolation by distance in *P. mugo* populations. Regression lines of linearized genetic distance vs. natural logarithm of geographic distance (km) are shown. Pearson's correlation coefficients and p values after Mantel test with 9,999 random cycles are indicated.

2012). It also appeared similar to that reported for *P. uncinata* (Dzialuk et al. 2009), *P. mugo* complex (Heuertz et al. 2010) and *P. mugo* s.s. (Sannikov et al. 2011), in each case based on material sampled in their natural localities. A study on material collected from populations of *P. mugo* agg. introduced in Lithuania also showed a high level of diversity interpreted as origin from different mother regions (Danusevičius et al. 2013).

In this study we found greater diversity of *P. mugo* in the Alps than in other mountains in Central Europe. Similarly to our observations, Sannikov et al. (2011) found greater genetic diversity of *P. mugo* in the Alps (A=2.9; H_e=0.305; H_o=0.310) compared to the Carpathians (A=2.3; H_e=0.197; H_o=0.187). Greater genetic diversity in the Alps compared to the Apennines was also observed for *Abies alba* Mill. (Piovani et al. 2010), but the reverse relation was found for *Pinus cembra* L. (Höhn et al. 2009). The greater genetic diversity of *P. mugo* found in the Alps could result from 1) differentiation of the *P. mugo* populations during Pleistocene cold periods and independent, long lasting genetic processes in the isolated populations, which then came together in the Holocene (Thiel-Egenter et al. 2011), or 2) hybridisation between *P. mugo* and *P. uncinata* (Christensen 1987; Lewandowski et al. 2000).

The distribution of genetic within-population diversity that we found in *P. mugo* in Central Europe represents the classical postglacial colonization theory of ''southern richness to northern purity'' (Hewitt 2000), where glacial refugia harbour high levels of genetic diversity and recolonizing populations are usually composed of subsets of the genetic diversity present in the refugial source population (Comes and Kadereit 1998; Taberlet et al. 1998). Sequential founder effects, bottlenecks and long term isolation of populations within geographically separate refugia may lead to genetic differentiation due to drift (Provan and Bennett 2008). Unfortunately, the locations of glacial populations of *P. mugo* complex are poorly known.

Our finding is also in line with the "leading edge" concept, as concern the level of within-population genetic variation (Hampe and Petit 2005).

Morphological variation

Values of variation coefficients of morphological and anatomical characters of needles and cones for samples representing the Giant Mts., Tatras and Alps did not differ at a statistically significant level. Samples of *P. mugo* from the Giant Mts., the northernmost localities of the species (Jalas and Suominen 1973; Boratyński 1994), have similar levels of morphological variation as those from the central part of its geographic range (Staszkiewicz and Tyszkiewicz 1976; Boratyńska et al. 2005). Generally, cone characters are more variable than those of needles (Boratyńska et al. 2005), when the frequencies of sclerenchyma cells between vascular bundles and around resin canals are excluded. These data are strongly biased and extremely variable (Boratyńska and Boratyński 2007), which is also expressed in our data (Table 2).

The phenotypic characteristics of trees is an outcome of the interaction between the genetic constitution of the species and the environmental conditions. Nevertheless, in our data only a few among the analysed set of phenotypic traits of needles and cones revealed relations to geographic position and/or climate conditions in place of origin. This can result from not too high differentiation of the site and

climate conditions in the subalpine vegetation layer of the Alps, Tatras and Giant Mts. (Table 1), where *P. mugo* plant community is formed (Ozenda 1988; Jirásek 1996; Poldini et al. 2004; Tsaryk et al. 2006). Only the epidermal cells were higher and somewhat broader (EH and EW) on the sites with higher minimal temperatures during winter and lower precipitation during the summer season. The thicker epidermis and hypodermis may be an adaptation to winter and early spring frosts, which can desiccate the needles (Wieser and Tausz 2007), and/or to higher insolation and higher UV-radiation in the regions with less precipitation during the summer (Wieser 2007).

The only detected cone asymmetry (CPM/CCM) positive correlation to geographic longitude could have resulted from contact and possible hybridization between *P. mugo* with *P. uncinata* in the westernmost localities we sampled in the present study. Nevertheless, the cones were also more asymmetric in localities with higher temperatures in the hot and dry periods of the year, which is difficult to explain. It should be stressed that most of the analysed needle and cone traits were resistant to influences of climatic conditions.

The generally weak correlation of the analysed phenotypic traits to environmental factors could allow us to expect their genetic conditioning and, consequently, similar pattern of diversity, as was found using genetic markers. In reality, analyses of the morphological characters of cones and the morphological and anatomical characters of needles confirmed closer relations among populations within the three regions than between them (Fig. 3). In spite of the differences between the Alpine, Carpathian and Giant Mts. populations detected by Student's test and discrimination analysis (Table 2), only a few particular traits were responsible for a significant portion of the variance between these regions revealed by ANOVA (Table 5). A possible adaptation of morphological traits of needles and cones to the local environmental conditions of particular populations, although not fully confirmed in the present study, can be responsible for intermingled conglomerations on the NJ unrooted trees (Fig. 3) and PCoA scatter-plots (Fig. 4) constructed based on the phenotypic characteristics.

Differentiation and genetic structure

The analysis of genetic differentiation in *P. mugo* revealed slight differences among geographic regions in Central Europe (Dzialuk et al. 2012) but, as we expected, the recently introduced Jost *D* estimator of population structure was significantly higher in this study for the more polymorphic chloroplast markers (D_{cpSSR} =0.620) than for isozymes (D_{iso} =0.013). Taking into account the very large number of private haplotypes in our study (above 74%), the very low value of $G_{ST\ cpSSR}$=0.017 seems to be a very biased estimator. Because traditional measures of differentiation (such as G_{ST}) can approach zero even if populations are completely differentiated, there is ongoing discussion about the new estimators of genetic population differentiation (Jost 2008; Gerlach et al. 2010).

In this study we used different types of genetic markers. Compared to nuclear markers, chloroplast DNA can better detect genetic structure because of its uniparental inheritance, nearly neutral evolution, low evolutionary rate, zero recombination and smaller effective population size than the nuclear genome (Provan et al. 1999; Wicke et al. 2011; Wachowiak et al. 2013). During the last few years, molecular data on cpDNA have been applied extensively in studies on the genetic diversity, population structure and phylogeography of *P. mugo* complex (Heuertz et al. 2010; Dzialuk et al. 2009, 2012; Danusevičius et al. 2013).

The weak taxonomic differentiation with clear phylogeographic structure in *P. mugo* s.l., identified using three cpSSRs by Heuertz et al. (2010), was only partially confirmed in our study. In fact, we found low but significant differentiation among mountain ranges by analysis of molecular variance (AMOVA), with 4 and 12% variation at the isozyme and chloroplast markers, respectively. Similarly, our estimates of the differentiation parameters were relatively low ($G_{ST\ izo}$ =0.027 and $G_{ST\ cpSSR}$=0.017), as one would expect in species with extensive gene flow, a complex demographic history (Heuertz et al. 2010) and nonequlibrium genetic structure (Slavov and Zhelev 2004). Comparable or greater differentiation was observed in natural populations of *P. mugo* s.l (G_{ST} =0.070, F_{ST}=0.076, Heuertz et al. 2010), in *P. mugo* in the Carpathians and Alps (F_{ST}=0.069 and F_{ST}=0.036, respectively, Sannikov et al. 2011) in *P. mugo* in Bulgaria (F_{ST}=0.041, Slavov and Zhelev 2004). However, in contrast to Heuertz et al. (2010), we didn't find phylogeographic structure in *P. mugo* populations; this may be an effect of the greater number of cpSSR loci used (nine versus three) and the smaller geographic area of our study and/or the taxonomy (*P. mugo* s.s. versus *P. mugo* s.l.).

It is well known that genetic variation is structured according to geography in *P. mugo* s.l. (Heuertz et al. 2010; Sannikov et al. 2011). More specifically, as we reported on cpSSR data (Dzialuk et al. 2012), vicariant gene pools for *P. mugo* s.s. lie in the Alps, Tatras Mts. and Giant Mts. Distinct vicariant gene pools for *P. mugo* complex can be expected on major mountain chains in the species range, similar to other conifers (Vendramin et al. 1999; Afzal-Rafii and Dodd 2007; Höhn et al. 2009). Although the absence of geographic structure of *P. mugo* was observed recently by Danusevičius et al. (2013) in plantations and by Wa-

chowiak et al. (2013) in *P. mugo* complex, we found isolation by distance structure and clear geographic structure in the autochthonous populations. The results obtained using isozymes, DNA markers and morphological characters differed between populations of *P. mugo* sampled in the Alps, Tatras and Giant Mts. The cpSSR markers showed a closer connection between populations sampled in the Tatras Mts. and Giant Mts., while isozymes between the Tatras Mts. and Alps (Figs. 2 and 3). A close relation between the Alps and Tatras Mts. can be an effect of long distance pollen transportation by anticyclonic circulations in the Giant Mts. during May–June (Kwiatkowski and Hołdys 1985). The winds from the south and southwest, especially the dynamic foens, are able to transport *P. mugo* pollen from the Alps (Sjögren et al. 2008). As the chloroplast DNA in the species of the Pinaceae family is paternally inherited (Mogensen 1996), the connection between populations of *P. mugo* from the Alps and the Giant Mts. may be evidence of such long distance pollen transport. This kind of influence, however, could have been much greater during cold periods in the Pleistocene, when the *P. mugo* geographic range covered a much larger area than at present (Jankovská 2001; Latałowa et al. 2004; Jankovská 2008; Jankovská and Pokorný 2008) and the distances between the Alps and Giant Mts. centres of the species were shorter.

A closer connection between populations of *P. mugo* from the Alps and Tatras was detected by analysis of isozymes (Fig. 2), which are generally considered to be neutral (Kimura and Ohta 1974) and, therefore, suitable indicators to describe historical processes. In view of this, our result can be interpreted as a possible exchange of genes via small, cryptic refugia between the Tatras and Alps during the cold periods of the Pleistocene (Jankovská 2008; Jankovská and Pokorný 2008). Macrofossils of the species have been reported from the end of the Late Glacial Maximum (LGM) and early Holocene from altitudes of about 600 m in the West Carpathians (Obidowicz 1996; Rybníček and Rybníčková 2002). Unfortunately, the pollen of *P. mugo* has not been distinguished from that of *P. sylvestris*, making direct interpretation of palynological reports impossible (Burga 1988; Latałowa et al. 2004; Jankovská 2008). However, very high percentages of *Pinus* pollen, determined as "*sylvestris*" or "*diploxylon*" type, have frequently been interpreted as the presence of *P. mugo*, especially in records from the LGM and early Holocene in the mountainous regions (Latałowa et al. 2004). This suggests a broader area of distribution of *P. mugo* during the cold periods of the Pleistocene, similar to what was proposed for *P. uncinata* within the Iberian Peninsula (Ramil-Rego et al. 1998; Benito Garzón et al. 2007; Dzialuk et al. 2009).

In conclusion we can state that our study shows clear geographic structure despite low differentiation of *P. mugo* populations in Central Europe. The distribution of genetic diversity suggests northward movement of the species according to the postglacial colonization theory of "southern richness to northern purity". However, this should be viewed with caution and needs to be further confirmed, because of small number of geographic regions in the species range investigated. Meanwhile, paleobotanical evidence provides unambiguous support for glacial refugia of *P. mugo* s.l. in the Alps and Czech Republic.

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