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ORIGINAL ARTICLE

Nuclear microsatellite markers reveal the low genetic structure of *Pinus mugo* Turra (dwarf mountain pine) populations in Europe

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Abstract Twenty-one populations (555 individuals) covering the entire native range of *Pinus mugo* Turra (dwarf mountain pine) were investigated for genetic variation scored at 13 nuclear microsatellite markers (nSSRs). The main objective of the present study was to determine the genetic structure across the present distribution of the species and locate populations of different genetic compositions. Most of the genetic variation was observed within the populations (95%). The assignment of populations based on Bayesian clustering methods revealed that the Sudeten populations of *P. mugo* form a separate genetic cluster. These stands have likely been established through the founder effects of Alpine migrants. The distribution and level of SSR polymorphisms, along with no evidence of isolation by distance or phylogeographic structure, indicate that the present populations of *P. mugo* have diverged relatively recently and originate from a larger glacial distribution of the species. One peripheral stand from Italy had the lowest values of most calculated genetic variation indices. This stand could therefore be more susceptible to genetic drift and a negative impact of predicted environmental changes. We discuss our findings with respect to previously published results on the genetic and morphological variation of *P. mugo* and with consideration for the conservation genetics of the species.

Keywords Adaptation · Conservation genetics · Genetic diversity · Phylogeography · Population differentiation · Postglacial recolonization

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Introduction

Polymorphisms observed in the genomes of various populations originate from demographic and evolutionary processes, such as gene flow, drift, mutations, recombination and selection (Nosil and Feder 2013). Across environmental landscapes, individuals experience various selective pressures that may result in differences observed among their genomes due to local adaptation (Schoville et al. 2012). Nonetheless, the genetic signatures of adaptive genetic variation (Lowry 2010) are different from neutral genetic variation resulting from population history, including long-term isolation and population range shifts.

The complexity of population history makes it challenging to elucidate the precise forces responsible for the genomic patterns of the polymorphisms observed in present populations. Studying the population genetics of forest tree species is a particularly formidable task, as most tree genomes are large. Forest tree species also have high genetic and phenotypic variation, large population sizes, low levels of linkage disequilibrium and long generation times. Moreover, forest tree research primarily focuses on a few species of high economic importance, and species that have an ecological value are generally ignored (Neale and Kremer 2011). Thus, for many species, the location of

Pleistocene refugia and recolonization routes are difficult to trace back.

Undoubtedly, the population genetics of trees has recently gained widespread attention. Tree species have become useful models to study evolutionary processes in wild populations. Genomic approaches are constantly developed, and considerable effort has been placed into identifying genetic markers that will facilitate evolutionary studies and tree breeding. It is crucial to assess the genetic variation of present tree populations not only to determine which evolutionary factors have influenced populations during their evolution but also to preserve genetic resources, particularly in the face of ongoing environmental changes.

Pinus mugo Turra (dwarf mountain pine) is a European forest tree species that plays a key role in alpine areas, preventing avalanches and soil erosion. This species belongs to the *P. mugo* complex, a European group of closely related pine species that has a uniform genetic background but variable ecology, morphology and geographic distribution. In addition, the complex constitutes a good candidate for genetic studies of adaptive variation and speciation (Żukowska and Wachowiak 2016). *P. mugo* forms shrubs up to a few metres in height that inhabit the subalpine belt of mountain ranges in Europe (Critchfield and Little 1971). In the Alps, its range overlaps with another member of the *P. mugo* complex, *Pinus uncinata* (Ramond) Domin (mountain pine), a single-stemmed tree common in the Western Alps and the Pyrenees (Monteleone et al. 2006).

Studies on *P. mugo* have primarily concentrated on the morphology of its cones and needles (e.g. Boratyńska and Boratyński 2007; Boratyńska et al. 2004, 2014, 2015). Populations from the Eastern and Southern Carpathians differ morphologically from the stands in other locations. Furthermore, some anatomical traits of the needles exhibit adaptive and ecological variation. However, little is known about the genetic background underlying the phenotypic differentiation of *P. mugo* and the postglacial history of this species. Historical demographic processes in *P. mugo* cannot be inferred from palynological records, as the pollen of its trees greatly resembles the pollen of *Pinus sylvestris* L. and *Pinus nigra* Arn. (Willis et al. 1998). Macrofossil data are also insufficient to distinguish these species (García-Amorena et al. 2007). Mitochondrial DNA, which is maternally inherited in conifers, has a relatively high mutation rate, which is sufficient to study recent divergence events (Sinclair et al. 1998). Unfortunately, variations in mtDNA genetic markers originally developed for *P. sylvestris* (Soranzo et al. 2000) are also too weak to make significant inferences regarding postglacial history of *P. mugo* (Wachowiak et al. 2013). A few studies using chloroplast microsatellite markers and/or isozymes have demonstrated the high genetic diversity accompanied by low interpopulation differentiation of *P. mugo* (Lewandowski et al. 2000; Slavov and Zhelev 2004; Heuertz et al. 2010; Sannikov et al. 2011; Dzialuk et al. 2012; Boratyńska et al. 2014). Higher differentiation among mountain ranges than within these areas was also shown (Dzialuk et al. 2012; Boratyńska et al. 2014). However, these studies were restricted to narrow areas of the present range of *P. mugo*.

The aim of the present study was to assess the level and distribution of neutral genetic variation across the native range of *P. mugo*. We used a set of 13 nuclear simple sequence repeat (nSSR) markers to examine genetic relationships within and among the studied populations and evaluate their phylogeographic structure. We also assessed whether populations occupying different mountain ranges have similar or different genetic compositions, i.e. whether they share a recent history or whether they are genetically isolated. To our knowledge, the genetic variation of *P. mugo* has not yet been addressed using nSSR markers. Furthermore, the present study encompasses all parts of the present geographic range of *P. mugo*. The results are discussed in terms of previous genetic research and their influence on the conservation genetics of *P. mugo*.

Materials and methods

Plant material and DNA extraction

We analysed 21 populations of *P. mugo*, with 24–30 individuals per population, yielding a total of 555 trees studied (Fig. 1; Table 1). The populations cover the native range and putative glacial refugia of the species. The collected samples were dried at 37 °C and ground in a MM 400 Mixer Mill (Retsch, Germany) prior to DNA extraction. Genomic DNA was extracted from 50–100 mg of needle tissue, according to Dumolin et al. (1995). The final incubation was conducted using RNase A at 60 °C for 30 min. The DNA concentration was measured using a BioPhotometer (Eppendorf AG, Germany) and adjusted to 15 ng/µl.

Molecular analysis

We selected 13 nSSRs that provided repeatable, unambiguous bands of expected sizes with sufficient polymorphisms from the set of 22 nSSRs initially screened on our plant material (Online Resource 1). The selected loci were amplified in three multiplex PCRs, each in a total volume of 10 µl, using the Qiagen Multiplex PCR Kit (Qiagen,

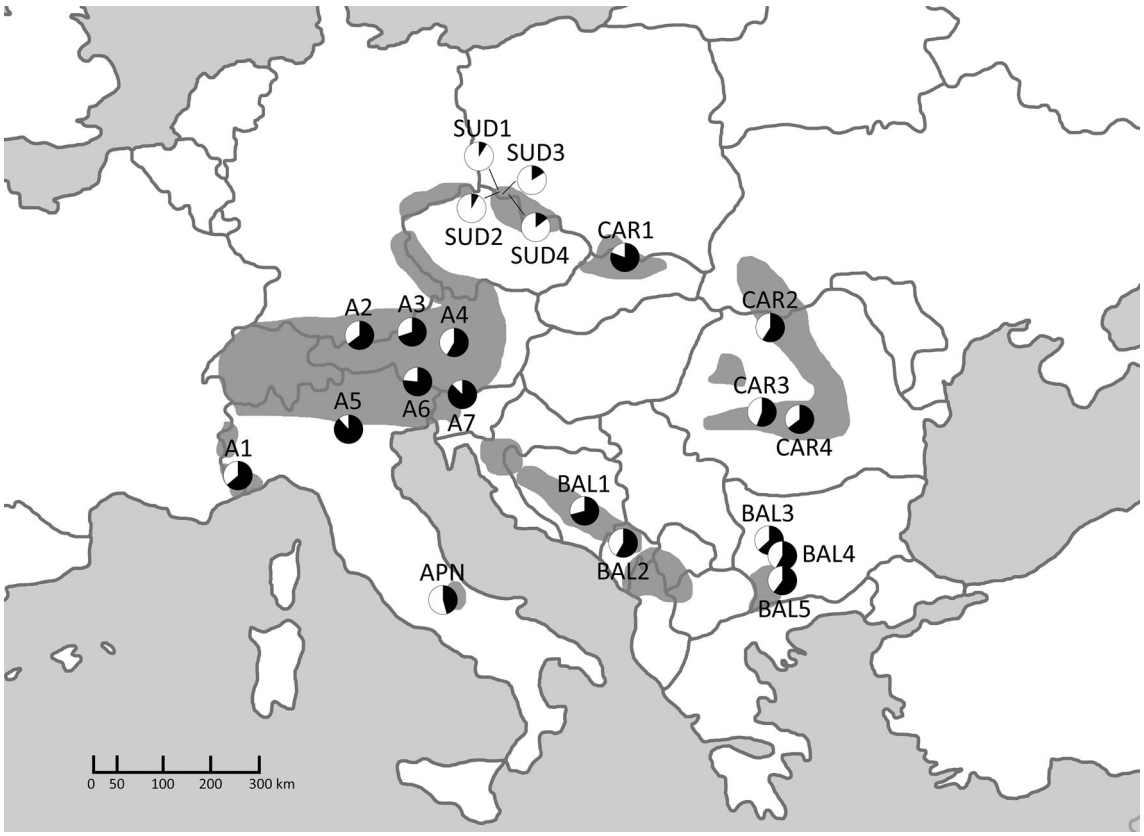

Fig. 1 Map showing the genetic structure and approximate geographic location of the *Pinus mugo* populations analysed in the present study. Bayesian clustering revealed two genetic clusters (I: SUD1–4; and II: A1–7, CAR1–4, APN, BAL1–5). *Pie charts* show the probability of belonging to one of the two genetic clusters. The range of the species is shaded in *grey* (modified after Critchfield and Little (1971))

Germany) in Applied Biosystems Veriti and 2720 thermal cyclers (Life Technologies, USA). The PCRs contained 3 µl of template DNA, 1× Qiagen Multiplex PCR Master Mix, 0.5× Q-Solution and 0.05–0.1 µM each of forward and reverse primer. A specific fluorescent dye was attached to the forward primer in each primer pair. The standard amplification procedure for each of the three multiplexes started with initial denaturation at 95 °C for 15 min and varied as follows: (1) psyl2, psyl16, psyl18, psyl25, psyl36, psyl42, psyl44 and psyl57 were amplified in Multiplex I with 38 cycles (30-s denaturation at 94 °C, 90-s annealing at 55 °C and 90-s extension at 72 °C); (2) ptTX2146 and SPAG 7.14 were amplified in Multiplex II with 35 cycles (30-s denaturation at 94 °C, 90-s annealing at 56 °C and 90-s extension at 72 °C); and (3) ptTX3025, ptTX4001 and ptTX4011 were amplified in Multiplex III with 10 cycles of touchdown (30-s denaturation at 94 °C, 40-s annealing at 65 °C $\Delta\downarrow$ 1 °C and 60-s extension at 72 °C), followed by 32 cycles (30-s denaturation at 94 °C, 60-s annealing at 55 °C and 60-s extension at 72 °C). The final extension at 72 °C lasted 15 min for Multiplex I and II and 7 min for Multiplex III. The PCR products and the GeneScan 500 LIZ Size Standard (Life Technologies, USA) were separated on a capillary sequencer using the Applied Biosystems 3130 Genetic Analyser (Life Technologies, USA). The alleles were determined based on their sizes using the GeneMapper Software ver. 4.0 (Life Technologies, USA). The raw data were manually assessed and converted into discrete allele sizes using the automated binning software Tandem (Matschiner and Salzburger 2009).

Tests for genetic equilibrium at the loci

The score test (*U* test for heterozygote deficit, Raymond and Rousset 1995), implemented in GENEPOP ver. 4.3 (Rousset 2008), was used to assess deviations from Hardy–Weinberg equilibrium (HWE) for each *P. mugo* population. Additionally, the multiple sample score test was used for each locus across all populations (Raymond and Rousset 1995). Whenever significant deficiencies of heterozygotes were observed, we suspected the presence of relatively high frequency of null alleles. As null alleles may significantly overestimate population differentiation as a result of the presence of false homozygotes (Chapuis

Table 1 List of the *Pinus mugo* populations analysed in the present study

Acronym	Region	Location	Longitude (E)	Latitude (N)	Altitude (mamsl)	No. of individuals
SUD1	S	Poland, Sudetes, Czarny Kocioł Jagniątkowski	15°35′30″	50°47′05″	1350	30
SUD2	S	Poland, Sudetes, Śląskie Kamienie	15°36′10″	50°46′40″	1420	30
SUD3	S	Poland, Sudetes, Plateau under Śnieżka Mt.	15°47′41″	50°44′44″	1410	30
SUD4	S	Poland, Sudetes, side of Śnieżka Mt. above Kocioł Łomniczki	15°47′50″	50°44′40″	1400	30
A1	WA	Italy, Alpes-Maritimes, Col de Tende	7°22′30″	44°08′00″	2000	25
A2	EA	Germany, Ammergau Alps, NW side of Kreuzspitze Mt.	10°55′12″	47°31′30″	1870	30
A3	EA	Austria, Berchtesgaden Alps, SW side of Hochkönig Mt.	13°05′00″	47°26′00″	1500	25
A4	EA	Austria, Austrian Central Alps, S side of Negleck Mt. above Sölkpass	14°04′50″	47°16′21″	1900	25
A5	EA	Italy, Italian Alps, Monte Altissimo di Nago	10°56′08″	45°48′32″	2025	25
A6	EA	Italy, Carnic Alps, Passo di Pramollo	13°15′35″	46°32′45″	1530	25
A7	EA	Slovenia, Kamnik-Savinja Alps, Kamniška Bistrica	14°32′00″	46°21′26″	1600	26
CAR1	WC	Poland, Tatra Mts., N side of Grześ-Wołowiec Ridge	19°45′50″	49°13′07″	1620	25
CAR2	EC	Romania, Rodna Mts., S side of Pasul Prislop	24°48′00″	47°34′03″	1720	25
CAR3	SC	Romania, Făgăraş Mts., NE side of Negoiu above Cascadă Bâlea	24°32′19″	45°36′30″	2025	25
CAR4	SC	Romania, Bucegi Mts., Buşteni	25°27′06″	45°25′55″	2070	25
APN	Ap	Italy, Apennines, La Maiella	13°58′30″	41°46′20″	2200	25
BAL1	DA	Bosnia and Herzegovina, Dinaric Alps, NE side below the top of Bjelašnica Mt.	18°13′08″	43°45′00″	2120	25
BAL2	DA	Montenegro, Dinaric Alps, E side of Meded near Strug	19°05′27″	43°09′33″	2100	30
BAL3	SB	Bulgaria, Vitosha Mts., Chrna Mt. above Aleko	23°16′08″	42°34′01″	1900	24
BAL4	SB	Bulgaria, Rila Mts., Ribni Ezera above Rilski Manastir	23°26′24″	42°05′20″	2100	25
BAL5	SB	Bulgaria, Pirin Mts., Vikhren Mt. above Bansco	23°25′22″	41°46′07″	2000	25

SUD, S, Sudetes; A, Alps; WA, Western Alps; EA, Eastern Alps; CAR, Carpathians; WC, Western Carpathians; EC, Eastern Carpathians; SC, Southern Carpathians; APN, Ap, Apennines; BAL, Balkans; DA, Dinaric Alps; SB, southern Bulgaria

and Estoup 2007), we assessed whether our data set contained null alleles using Micro-Checker software ver. 2.2.3 (Van Oosterhout et al. 2004). In a subsequent analysis, we calculated the inbreeding coefficients for each population using INEst software ver. 2.0 (Chybicki 2015). The calculations were performed using a Bayesian approach, 500,000 Markov chain Monte Carlo (MCMC) iterations with the thinning parameter set at 500 and burning set to 50,000 cycles. We selected the full model, considering the presence of both null alleles and inbreeding and potential genotyping failures (F_{ISnull}). To verify the significance of inbreeding, we repeated the analysis using a random mating model (i.e. assuming that $F = 0$; F_{IS}) and performed the Bayesian procedure of model comparison. Finally, we determined which model (full model = F_{ISnull} vs. random mating model = F_{IS}) fitted better to the data by computing deviance information criterion (DIC) for each option. In addition, the exact test for genotypic disequilibrium between all pairs of loci in each population was performed using GENEPOP ver. 4.3 with the following settings: MCMC dememorization number set at 10,000, 100 batches and 5000 iterations per batch.

Genetic diversity and differentiation

Within-population genetic diversity was assessed with the following parameters: the mean number of alleles (A_{N}), mean effective number of alleles (A_{E}), number of private alleles (i.e. unique to a single population; A_{P}), and observed and unbiased expected heterozygosity (H_{O} and $\mathrm{uH_E}$, respectively), using GenAlEx software ver. 6.5 (Peakall and Smouse 2006). The mean rarefied allelic richness across all loci (A_{R24}), based on a minimum sample size of 24 diploid individuals, was calculated with FSTAT ver. 2.9.3.2 (Goudet 2001). The statistical significance of the differences in the values of the calculated genetic indices between the Sudeten versus the other stands (groups defined by the Bayesian clustering; see 'Results' section) was calculated using Student's *t* test implemented in JMP Pro ver. 12.1.0 software.

To estimate the proportion of the overall genetic variation resulting from differentiation among the *P. mugo* populations, we computed unbiased global and pairwise F_{ST} using FreeNA software (Chapuis and Estoup 2007). FreeNA considers the potential bias reflecting the presence

of null alleles using the Excluding Null Alleles (ENA) correction method (F_{STnull}). A total of 10,000 replicates were set to calculate the bootstrap 95% confidence interval (95% CI) for the global and pairwise F_{ST} and F_{STnull}.

Population structure

The genetic structure of the *P. mugo* populations was determined using the Bayesian clustering approach implemented in STRUCTURE ver. 2.3 (Pritchard et al. 2000). This software uses multilocus genotype data to assign individuals to genetically divergent clusters. The settings in STRUCTURE were as follows: a burn-in period of 50,000 followed by 500,000 iterations using the correlated allele frequency model and admixture ancestry model. A total of 20 independent runs were set for each potential number of clusters ($K = 1$–21). The output from STRUCTURE was visualized in STRUCTURE HARVESTER (Earl and von Holdt 2011), and the optimum value of K was determined according to the Evanno method (Evanno et al. 2005). Multiple runs for the optimum K value were aligned using CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg 2007). Additionally, we conducted the hierarchical analysis of molecular variance (AMOVA) to assess the level of differentiation among the following groups: (a) all populations; (b) groups of populations divided into mountain regions according to their geographic locations; and (c) groups defined by the Bayesian clustering. The analysis was performed using both F_{ST} and R_{ST} distance type in GenAlEx ver. 6.5.

Isolation by distance and phylogeographic structure

A Mantel test was performed to determine whether differentiation among the *P. mugo* populations results from isolation by distance (IBD). Specifically, we tested the correlation between the matrices of pairwise geographic distances (logarithmic scale) and pairwise population F_{STnull} with 1000 permutations as implemented in GenAlEx ver. 6.5.

The potential existence of phylogeographic structure was assessed with the permutation test according to Hardy et al. (2003). To that end, we computed and compared pairwise R_{ST} and permuted R_{ST} (pR_{ST}) with 10,000 permutations using the SPAGeDI software ver. 1.5 (Hardy and Vekemans 2002). *R*-statistics is an analogue to *F*-statistics, but it is allele-size-based, as it considers not only the identity of the alleles (as *F*-statistics) but also the differences among their sizes. Thus, R_{ST} reflects interpopulation differences resulting from genetic drift and mutation processes according to the stepwise mutation model (SMM). When R_{ST} is significantly higher than pR_{ST}, allele sizes contribute to the observed genetic differentiation, indicating the existence of phylogeographic structure (Hardy et al. 2003).

Considering potential population size fluctuations, we assessed whether the *P. mugo* stands analysed in the present study experienced a genetic bottleneck. Such populations tend to lose rare alleles and experience a reduction in heterozygosity. However, the allelic diversity decreases faster than heterozygosity; thus, the observed heterozygosity is greater than that expected from the observed number of alleles (Luikart and Cornuet 1998). We selected a one-tailed Wilcoxon's sign-rank test to examine departures from HWE using the Bottleneck ver. 1.2.02 software (Cornuet and Luikart 1996). This test is considered the most powerful approach to examine heterozygosity excess (Peery et al. 2012). The analysis was conducted under both the pure SMM model and the two-phase model (TPM), enabling single-step and multi-step mutations. TPM is considered the most suitable for most microsatellite markers, as these markers do not mutate under strict SMM (Di Rienzo et al. 1994). The TPM option was set to incorporate 70% of the single-step mutations and 30% of the multi-step changes.

Results

Polymorphism at microsatellite loci

All 13 microsatellite loci included in the final data set were polymorphic. The degree of genetic diversity calculated in the present study was highly dependent on the set of loci, as the 'psyl' markers were less polymorphic than the 'ptTX' and 'SPAG' loci. In total, we detected 133 alleles, yielding a mean number of $\sim$10 alleles per locus. We detected 15 alleles that are specific to particular *P. mugo* stands, with a frequency varying from 0.017 to 0.067. These alleles were identified in populations from different mountain regions for seven nSSRs (Online Resource 2).

The mean number of alleles per population (A_N) was equal to 4.98 and did not significantly vary across all populations of *P. mugo*, except for A5 ($A_N = 3.76$). In addition, the frequency of many alleles was low; thus, the number of alleles that had a major influence on the level of genetic variation (effective number of alleles, A_E) was lower than A_N (mean $A_E = 2.64$). The values of allelic richness (A_{R24}) were only slightly lower than A_N (mean $A_{R24} = 4.89$). Therefore, the variation in the number of individuals sampled from a particular population did not affect the degree of A_N (Table 2).

The results obtained using Micro-Checker indicated that null alleles were present in 10 loci with frequencies

Table 2 Descriptive statistics of genetic variation within the *Pinus mugo* populations using 13 nSSRs

Population	N	A_N	A_E	A_{R24}	A_P	H_O	uH_E	Deviation from HWE	F_{ISnull}
SUD1	30	4.61	2.60	4.39	1	0.37	0.43	**	0.07
SUD2	30	4.30	2.21	4.11	0	0.33	0.35	ns	0.03
SUD3	30	5.00	2.58	4.73	0	0.37	0.43	**	0.10
SUD4	30	4.46	2.74	4.33	1	0.41	0.41	ns	0.02
A1	25	4.15	2.34	4.12	0	0.38	0.45	**	0.12
A2	30	5.46	2.95	5.26	1	0.52	0.51	ns	0.02
A3	25	5.46	2.91	5.41	1	0.41	0.47	**	0.08
A4	25	5.00	2.35	4.95	2	0.39	0.42	**	0.05
A5	25	3.76	2.10	3.74	0	0.38	0.43	**	0.09
A6	25	4.38	2.63	4.35	0	0.41	0.45	**	0.06
A7	26	5.15	2.88	5.08	0	0.43	0.53	**	0.03
CAR1	25	5.46	2.78	5.42	3	0.39	0.47	**	0.11
CAR2	25	5.53	2.88	5.48	1	0.40	0.43	**	0.08
CAR3	25	5.38	3.06	5.35	1	0.44	0.49	*	0.04
CAR4	25	5.53	2.84	5.47	0	0.43	0.47	*	0.05
APN	25	4.69	2.47	4.64	0	0.38	0.44	**	0.10
BAL1	25	5.92	2.77	5.86	0	0.47	0.49	ns	0.02
BAL2	30	5.07	2.53	4.78	3	0.45	0.45	*	0.03
BAL3	24	5.15	2.44	5.15	0	0.40	0.42	ns	0.04
BAL4	25	5.00	2.73	4.95	0	0.40	0.44	ns	0.01
BAL5	25	5.23	2.73	5.17	1	0.39	0.45	**	0.12
Mean	26.42	4.98	2.64	4.89	0.71	0.40	0.44		0.06

Acronyms as in Table 1

N, no. of individuals; A_N, mean no. of alleles; A_E, mean effective no. of alleles; A_{R24}, mean rarefied allelic richness based on a minimum sample size of 24 individuals; A_P, no. of private alleles; H_O, observed heterozygosity; uH_E, unbiased expected heterozygosity; F_{ISnull}, average inbreeding coefficient with null alleles correction; ns, not significant

* $p < 0.01$; ** $p < 0.001$

between ~0.03 for psyl42 to ~0.14 for ptTX4011. However, the levels of observed heterozygosity (H_O) for particular populations (range 0.33–0.52, average 0.40) were only slightly lower than the values of unbiased expected heterozygosity (uH_E, equivalent to gene diversity) (range 0.35–0.53, average 0.44). The values of inbreeding coefficient with null alleles correction (F_{ISnull}) were generally low and significantly different from zero only in some populations (Table 2). We concluded that inbreeding did not have a relevant influence on genetic diversity, and deviations from HWE at some loci primarily reflected the presence of null alleles. The frequency of null alleles in our data set was lower than the threshold value of 0.19 (Chapuis et al. 2008), above which the value of H_E is significantly underestimated due to null alleles. Therefore, all 13 loci were used in further analyses.

Evidence of linkage disequilibrium between some pairs of loci was detected in populations A5 (psyl57 vs. ptTX2146), A7 (psyl2 vs. psyl25, psyl2 vs. psyl36 and psyl25 vs. psyl36) and BAL2 (psyl16 vs. psyl57, psyl57 vs. SPAG 7.14 and ptTX4001 vs. ptTX4011) ($p < 0.001$).

Genetic variation within populations

On average, we observed 4.98 different alleles per locus for each population. The mean value of A_{R24} was 4.89. The population from Monte Baldo in Italy (A5) exhibited the lowest values of genetic diversity of all tested *P. mugo* stands for most parameters ($A_N = 3.76, A_E = 2.10, A_{R24} = 3.74$, no private alleles). H_O varied from 0.33 for SUD2 to 0.52 for A2 (mean $H_O = 0.40$). This value was slightly lower than uH_E, with values between 0.35 for SUD2 and 0.53 for A7 (mean $uH_E = 0.44$) (Table 2). The values of H_O and H_E differed across loci from 0.07 and 0.10 for psyl18 (for H_O and H_E, respectively) to 0.79 and 0.89 for SPAG 7.14 (data not shown). In addition, the differences between the Sudeten and the remaining populations were statistically significant for A_N, A_{R24}, H_O and uH_E (Student's t test; $p < 0.05$). We observed a weak inbreeding across the *P. mugo* populations (F_{ISnull} ranging from 0.01 to 0.12, average 0.06), which was significantly different from zero only in a subset of the populations, namely in SUD3, A1, A5, CAR1, CAR2, APN and BAL5 (Table 2). In other samples, 95% CI overlapped zero.

Differentiation among populations

Most of the genetic diversity was observed within the studied populations. The global level of genetic differentiation among the *P. mugo* stands calculated using FreeNA software (F_{ST}) was low and equal to 0.051 (95% CI 0.034–0.069). The value of F_{STnull}, i.e. using the Excluding Null Alleles correction method, was 0.052 (95% CI 0.037–0.070). Almost all pairwise population values of F_{STnull} were significantly greater than zero and varied from 0.008 between A3 and CAR2 to 0.156 between SUD2 and A7 (Online Resource 3). The F_{ST}/F_{STnull} values per locus ranged between 0.012/0.023 for ptTX3025 and 0.116/0.114 for ptTX4011 (data not shown).

Population structure

Based on the results of the Bayesian clustering, the *P. mugo* populations were clearly separated into two clusters using STRUCTURE according to the method described by Evanno et al. (2005). The four Sudeten populations (SUD1-4) were assigned to the first genetic cluster, whereas all the remaining stands from the other mountain ranges formed the second cluster (Figs. 1, 2). The AMOVA results using the F_{ST} distance indicated that 5% of the variance was among the studied *P. mugo* populations. The remaining 95% reflected the differentiation observed within populations. When divided into mountain regions (see Table 1), the analysis showed that only 1% of the genetic variance reflected differences among the mountain regions, whereas 4% of the genetic variance among the *P. mugo* populations reflected differences within the regions. Furthermore, when we separated the populations into two groups detected in the Bayesian clustering (SUD vs. the other stands), the variance was 3% between the groups and 4% among the populations within the groups (all results significant at $p < 0.001$) (Table 3). When we performed the analysis on the R_{ST} distance, only 3% of variance reflected the differentiation among populations ($p < 0.001$). These results were not significant when the analysis was performed on the mountain regions or groups obtained using Bayesian clustering.

Isolation by distance and phylogeographic structure

The Mantel test yielded no significant correlation ($R = 0.070$; $p = 0.153$). Similarly, the results of the permutation test did not support the existence of phylogeographic structure among the *P. mugo* populations analysed in the present study ($R_{ST} = 0.037 < pR_{ST} = 0.043$; $p = 0.649$). Therefore, neither the SMM nor IBD could explain the genetic differentiation observed in our data set. Finally, no signs of bottlenecks were observed either under the SMM or TPM model in any population.

Discussion

Distinct genetic composition of *Pinus mugo* in the Sudetes

The present study revealed that *P. mugo* in the Sudetes differs from other populations of this species. Notably, in the past, large areas of *P. mugo* in the Sudetes were replaced with pastures from the fifteenth century until the end of the nineteenth century. *P. mugo* naturally recovered, at least partially, particularly on the abrupt slopes of the glacial cirques, but this species may have been planted in certain places, such as the Czech Republic (Sobierajska et al. 2010). Therefore, the origin of some of the present Sudeten populations of *P. mugo* is uncertain. Nevertheless, morphological studies showed that populations on the plateaux do not differ from those inhabiting the glacial cirques (Sobierajska and Boratyńska 2008; Sobierajska et al. 2010). Thus, even if some parts of the Polish Sudetes were artificially reforested, this restoration was conducted most likely with the seeds of local origin (Sobierajska and Boratyńska 2008; Sobierajska et al. 2010).

Dzialuk et al. (2012) suggested that *P. mugo* survived the Last Glacial Maximum in different refugia, namely in the Alps, Sudetes and Carpathians. In morphological surveys, however, the Sudeten populations are only slightly different compared with the other mountain regions (Boratyńska and Boratyński 2007; Boratyńska et al. 2015). We hypothesize that the Polish Sudeten populations of *P. mugo*

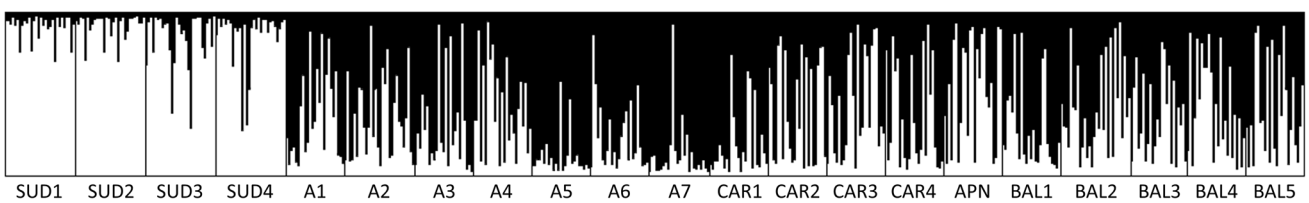

Fig. 2 Bar plot showing STRUCTURE results for $K = 2$. *Vertical bars* represent *Pinus mugo* individuals. The *bars* are partitioned into two colour segments corresponding to the probability of belonging to one of the two genetic clusters

Table 3 Analysis of molecular variance (AMOVA) using the F_{ST} distance among the following groups of the *Pinus mugo* populations ($p < 0.001$)

Source of variation	*df*	Variance component	Variance (%)	Φ statistics
(a)				
Among populations	20	0.165	5	0.053
Within populations	1089	2.947	95	
(b)				
Among regions	8	0.045	1	0.014
Among populations within regions	12	0.126	4	0.041
Within populations	1089	2.947	95	0.055
(c)				
Among groups	1	0.095	3	0.030
Among populations within groups	19	0.131	4	0.043
Within populations	1089	2.947	93	0.071

(a) All populations; (b) populations divided into mountain regions (see Table 1); and (c) groups defined using Bayesian clustering

are younger than the populations occupying other mountain ranges. According to the 'leading edge' model of colonization, these species may have been established via the founder effects (Hampe and Petit 2005) of Alpine migrants. The slightly lower genetic diversity (i.e. the values of A_N, A_{R24}, H_O and uH_E) of the Sudeten populations compared with the average supports this idea. Orsini et al. (2013) argued that patterns of genetic variation should be interpreted with caution, as neutral genetic variation may be determined not only via dispersal limitation but also through colonization history and local adaptation. In the present study, we assume isolation based on a colonization (IBC) scenario, under which populations are expected to differentiate at neutral loci, but this differentiation does not correlate with geographic or ecological distance (Orsini et al. 2013). The differentiation between the Sudeten populations and other stands and lack of IBD signal is consistent with this scenario. The standing genetic variation present in the founding population of *P. mugo* was likely sufficient to facilitate rapid genetic adaptation to the local environment in the Sudetes. As a consequence, the newly established populations may have rapidly grown in size. Even if we assume a free gene exchange between the Sudetes and Alps, a simple numerical advantage of the first migrants led to the pattern of genetic differentiation. The rate of gene flow between the Sudetes and Alps has been too slow, or simply, there has not been enough time to eliminate this long-lasting founder effect.

Differentiation among populations and mountain regions

We observed that populations of *P. mugo* are differentiated only to a small extent ($F_{STnull} = 0.052$) and that variation is primarily distributed within the populations. The classical value of F_{ST} (without the correction of null alleles) was equal to 0.051. Therefore, the occurrence of null alleles did not affect the level of F_{ST}. Indeed, high within-population diversity accompanied with little interpopulation differentiation at neutral markers is expected for highly outcrossing species such as conifers (Hamrick and Godt 1996).

The Mantel test performed on our data set was insignificant. Thus, IBD was not a mechanism shaping the genetic structure of *P. mugo* populations. The global genetic differentiation that considered allele sizes (R_{ST}) was lower than the F_{ST} value. The results of the permutation test were also insignificant ($R_{ST} = 0.037 < pR_{ST} = 0.043$; $p = 0.649$). This finding indicates the absence of phylogeographic structure and that SMM did not play a significant role in shaping the genetic structure observed in the data set obtained in the present study.

Previous work on *P. mugo* yielded similar or higher values of interpopulation genetic differentiation ($F_{ST} = 0.041$ for isoenzymes, Slavov and Zhelev 2004; $F_{ST} = 0.076$ and $G_{ST} = 0.070$ for cpSSRs, Heuertz et al. 2010; $F_{ST} = 0.069$ in the Carpathians and $F_{ST} = 0.033$ in the Alps for isoenzymes, Sannikov et al. 2011; $F_{ST} = 0.082$ and $R_{ST} = 0.137$ for cpSSRs, Dzialuk et al. 2012; $G_{ST} = 0.020$ for isoenzymes and $G_{ST} = 0.017$ for cpSSRs, Boratyńska et al. 2014). Moreover, Dzialuk et al. (2012) observed a high level of differentiation among the mountain ranges (the Sudetes, Alps and Carpathians) using cpSSRs (11.86% for F_{ST} and 19.64% for R_{ST}). These authors suggested that this finding reflected ancient fragmentation and the long isolation of *P. mugo* stands. The genetic differentiation among the mountain regions calculated in the present study was considerably lower (1% for F_{ST} and insignificant for R_{ST}), as the variation primarily reflected differentiation within the populations (95% for F_{ST} and insignificant for R_{ST}) and among the populations within the regions (4% for F_{ST} and insignificant for R_{ST}).

Higher degrees of genetic differentiation may reflect the fact that the mentioned study was based on different markers (cpSSRs) and conducted on a much smaller area of the *P. mugo* range compared with the present study.

Morphological studies of *P. mugo* showed that populations from the Eastern and Southern Carpathians differ from the other stands of *P. mugo* (Boratyńska et al. 2004, 2015). This result indicates that these populations may have originated from a different Pleistocene refugium or reflect the adaptive significance of needle features. The results of the present study rule out the first option. The weak genetic differentiation, lack of IBD signal and the lack of phylogeographic structure in the present study suggest the relatively recent fragmentation of a historically larger glacial *P. mugo* range. As the species alternately spread and retreated during cold and warm periods of the Pleistocene (Willis et al. 2000; Latałowa et al. 2004), populations from distinct mountain ranges may have come into contact, thereby mixing the initial gene pools. The isolation of particular stands in the most elevated mountains likely started only 8000–9000 years ago (Boratyńska et al. 2004). Hence, there is a weak genetic differentiation of *P. mugo*. Previous genetic studies indeed identified a distinct origin of populations of conifers inhabiting major mountain ranges (Vendramin et al. 1999; Afzal-Rafii and Dodd 2007), but other studies did not reveal the strong genetic divergence of mountain isolates (Robledo-Arnuncio et al. 2005), similar to the results of the present study.

Implications for conservation genetics of *Pinus mugo*

In our study, most parts of the analysed range of *P. mugo* (the Alps, Carpathians, Apennines and Balkans) showed no evidence of genetic differentiation. Consequently, based on the results of the nSSRs analysis, these populations should be treated as a single conservation unit. However, populations from the Sudetes, which are located in the northern limits of the natural range of *P. mugo*, showed distinct genetic structure, so they should be considered as a separate genetic group. Additionally, in the present study, one of the Italian populations (A5) had the lowest values for most calculated genetic variation indices, even lower than the most isolated stand from the Apennines (APN). This population inhabits the south-western-most area of the *P. mugo* range. We even observed that four genotypes were repeated twice in this stand. Nevertheless, no evidence of a recent genetic bottleneck was observed in this population or in any other studied location. Nevertheless, considering its evidently lower genetic diversity, A5 may be more prone to the negative effect of genetic drift. Maintaining high within-population genetic diversity is particularly important to adapt to a changing environment (Markert et al. 2010). *P. mugo* is an important component of alpine landscapes, as its trees prevent avalanches and soil erosion. Climate warming is likely to cause migration of *P. mugo* to higher altitudes, similarly to the retreats observed during warmer interglacials (Heuertz et al. 2010). As the response of various species to environmental changes will most likely depend on the reaction of populations located at range margins (Hampe and Petit 2005), it is important to obtain comprehensive genetic information, particularly for peripheral populations of *P. mugo*, to properly formulate management strategies for the conservation of genetic resources of this species.

Conclusions

It appears that present populations of *P. mugo* originate from a larger distribution of the species. They have most likely been isolated for a relatively short period of time, not long enough to cause pronounced genetic differentiation among them. Therefore, the location of the ancient refugial populations of *P. mugo* remains poorly recognized. The data obtained in the present study confirm the potential northward movement of *P. mugo* during the postglacial recolonization of Europe. Specifically, the Sudeten populations may have been established through the founder effects of migrants from the Alps. We also identified a single peripheral population of *P. mugo* that should be given particular attention regarding the preservation of the genetic resources of the species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Information on Electronic Supplementary Material

Online Resource 1. Characteristics of the 22 nSSR markers initially tested.
Online Resource 2. Private alleles detected in the analysed *Pinus mugo* populations.
Online Resource 3. Pairwise matrix of F_{ST} Excluding Null Alleles (F_{STnull}) among the *Pinus mugo* populations.

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