This is an Accepted Manuscript of an article published in Systematics and Biodiversity on December 12, 2016, available online: https://doi.org/10.1080/14772000.2016.1257518

Taxonomic and geographic variation of the *Pinus mugo* complex on chloroplast microsatellite markers

Runing title: Phylogeography of mountain and peat-bog pines

Abstract

The high mountain plants of Central and Southern Europe survived the glacial periods in the same mountain ridges, but at lower altitudes and possibly covering larger areas than during interglacials. This implies the high level of species differentiation between isolated mountain ridges. *Pinus mugo* complex, which includes *P. mugo* s.s. (Alps, Sudetes, Carpathians, Dynaric Alps and Rhodopes), *P. uncinata* (Pyrenees and Alps) and *P. uliginosa* (Sudetes and neighbouring mountain ridges) is a good group to examine such a scenario. We screened 44 populations across the geographic range of the complex, using ten cpSSR markers to study (1) taxonomic relations among *P. mugo* s.s., *P. uncinata* and *P. uliginosa* and (2) genetic and phylogeographic structure in *P. mugo* s.s. and *P. uncinata*. Allelic combinations of 87 size variants produced a total of 757 haplotypes. Haplotypic diversity was high and similar in every species (0.997, 0.986 and 0.991, respectively). The highest divergence between haplotypes was observed in *P. uliginosa* (D_{sh}^2 =10.29). The AMOVA revealed that most of the overall genetic variation is explained by the within-population component (F_{ST}=0.121, R_{ST}=0.206) and by the geography (F_{CT}=0.056, R_{CT}=0.083). The differentiation between *P. mugo* s.s., *P. uncinata* and *P. uliginosa* is explained by about 5% (P<0.001) of the total variation. Vicariant gene pools for the complex were identified in the Pyrenees, the Alps with the Tatra Mts., the Sudetes, and the East and South Carpathians along with

the Balkan mountains. The phylogeographic structure was observed in *P. mugo* s.l., *P. mugo* s.s. and *P. uncinata.* Results support the separate taxonomic status of *P. uncinata* and *P. mugo* s.s. and possible hybrid origin of *P. uliginosa*.

Keywords: biogeography, Coniferae, genetic structure, mountain plants, phylogeography, Pinaceae, systematics.

Introduction

The Quaternary, mainly Pleistocene, climate oscillations with cold periods had influenced the gene pools of organisms in temperate and boreal zones of Europe (Hewitt, 2004; Hewitt & Godfrey, 1996). In response to Quaternary expansions of the glacial ice sheets, the temperate tree species were generally confined throughout glacial periods to low-latitude refugia. Comparative analyses have revealed the existence of three major refugia located in the Mediterranean peninsulas of Iberia, Italy and the Balkans. During interglacials, the species migrated from these areas (Hewitt, 2004; Petit, 2003). As a consequence of successive founder events during the recolonization process, genetic diversity should be a gradually declining function of distance from refugia (Petit, 2003). This pattern of genetic diversity known as 'southern richness versus northern purity' has been observed in many tree species, which colonized European lowlands (Hewitt & Godfrey, 1996; Magri et al., 2006). The influence of Pleistocene migrations is well recognized for coniferous forest trees, such as *Pinus sylvestris* L., *Abies alba* Mill., *Picea abies* (L.) H.Karst. (Cheddadi et al., 2013; Dering, Misiorny, Lewandowski, & Korczyk, 2011; Gömöry, Paule, Krajmerová, Romšáková, & Longauer, 2012; Liepelt et al., 2009; Pyhäjärvi, Salmela, & Savolainen, 2007). These migrations are responsible for a partly common geographical pattern of allozyme loci differentiation in Central Europe (Gömöry, Longauer, Paule, Krajmerová, & Schmidtová, 2010). However, numerous temperate trees also maintained a substantial amount of diversity from the north to the southern European glacial refugia. Such contrasting patterns of diversity are inferred as footprints of cryptic refugia at high latitudes (e.g. in the area around the Carpathians) or may be an effect of a 'suture' zone

where the divergent colonizing lineages from different refugia met and merged (Petit, 2003; Provan, & Bennett, 2008; Temunović et al., 2012).

The high mountain plants (alpine, and subalpine sensu Ellenberg & Leuschner, 2010; Zarzycki et al., 2002) followed another pattern of Pleistocene migration. Their relatively low thermal demands permitted them to survive the glacial periods of Pleistocene in the same mountain ridges, but at lower altitudes and possibly covering larger areas, than during interglacial periods (de Beaulieu et al., 2001; Fauvart et al., 2012; Jankovská, 2008; Obidowicz, 1996; Ronikier, 2011). The genetic differentiation of the subalpine conifer species of central European mountains is recognized for *Pinus cembra* L. (Höhn et al., 2009; Lendvay, Höhn, Brodbeck, Mîndrescu, & Gugerli, 2014; Dzialuk et al., 2014; Wojnicka-Półtorak, Celiński, Chudzińska, Prus-Głowacki, & Niemtur, 2015) and *Larix decidua* Mill. (Pluess, 2011). Meanwhile, the genetic structure, phylogeography and glacial history of the *P. mugo* Turra complex are merely partly resolved.

The *Pinus mugo* complex (*P. mugo* s.l.) includes *P. mugo* s.s., *P. uncinata* Ramond, *P. uliginosa* G.E.Naumann ex Wimmer and *P. rotundata* Link/*P. pseudopumilio* (Willk.) Beck (Businsky & Kirschner, 2006). The dwarf mountain pine – *P. mugo* s.s. (*P. mugo* subsp. *mugo* sensu Christensen, 1987) is a large, dwarf shrub which occurs in the subalpine zone of the Alps, Sudetes, Carpathians and mountains of the Balkan Peninsula (Jalas & Suominen, 1973; Ozenda, 1988; Tsaryk, Didukh, Tasenkevich, Waldon, & Boratyński, 2006). The mountain pine – *P. uncinata* (*P. mugo* subsp. *uncinata* sensu Christensen, 1987) is a monocormic tree known from the Pyrenees, western Alps, Massif Central, Sierra de Cebollera and Sierra de Gúdar (Ozenda, 1988; Villar, Sesé, Franco, & Ferrández, 1997). The peat-bog pines – *P.*

uliginosa and *P. rotundata* have intermediate character between *P. mugo* and *P. uncinata* (Fig. 1). They are trees with one-two(-three) stems and occur mainly on the peat-bogs in and around the Sudetes and in the northern Alps (for more details see: (Boratyńska, Boratyński, Lewandowski, 2003; Businsky & Kirschner, 2006; Christensen, 1987; Heuertz et al., 2010; Lewandowski, Boratyński, & Mejnartowicz, 2000). The names *P. uliginosa* and *P. rotundata* are sometimes treated as synonyms (Hamerník & Musil, 2007; Heuertz et al., 2010). The systematic status of the taxa of the *P. mugo* complex is still disputed (Boratyńska et al., 2003; Businsky & Kirschner, 2006; Hamerník & Musil, 2007; Heuertz et al., 2010). However, we treat them as the *P. mugo* complex in this study, but using the names *P. uncinata*, *P. mugo* s.s. and *P. uliginosa* to indicate a particular taxon.

To date, only Pyrenean, western- and central Alpine, West Carpathian, Sudetan and a few peripheral populations of the *P. mugo* complex have been used in comparative studies of genetic diversity (Celiński, Zbránková, Wojnicka-Półtorak, & Chudzińska, 2015; Dzialuk et al., 2009; Dzialuk, Boratyński, Boratyńska, & Burczyk, 2012; Heuertz et al., 2010; Wachowiak, Boratyńska, & Cavers, 2013; Wachowiak, Palmé, & Savolainen, 2011). Moreover, in the East- and South-Carpathians, as well as in the Balkans, the genetic diversity and differentiation were assessed separately (Sannikov, Petrova, Schweingruber, Egorov, & Parpan, 2011; Slavov & Zhelev, 2004).

The high level of genetic differentiation between distinct mountain ranges and lower level of differences among populations within the same mountain range was observed in the *P. mugo* complex using DNA markers. The differentiation of *P. uncinata* in the south-western (Dzialuk et al., 2009) and of *P. uncinata*, *P. mugo* s.s. and *P. rotundata/P. ×pseudopumilio* in the western part of the geographic range of the

complex confirmed the geographic pattern of genetic variation (Heuertz et al., 2010). Also, differences among Sudetan, Carpathian and Alpine populations of *P. mugo* s.s followed this trend (Boratyńska et al., 2014; Dzialuk et al., 2012). No clear taxonomic and/or geographic structure of the complex was found using twelve nuclear loci, but there was a geographic structure for *nad1* and *nad2* mitochondrial DNA regions (Wachowiak et al., 2013).

As shown by Heuertz et al. (2010), based on data from three chloroplast microsatellites (cpSSR), in western Europe two main distinct vicariant gene pools are present, the Pyrenean and Alpine, with several smaller clusters of peripheral populations of the *P. mugo* complex. This suggests that Pyrenean and Alpine populations were recolonized respectively by *P. uncinata* and *P. uncinata*/*P. mugo* s.s. from independent multiple glacial refugia located within these mountain chains. Although Heuertz et al. (2010) did not include populations from the Balkans, they predicted a third refugium for *P. mugo* s.s. within the Balkan Peninsula. Unfortunately, the genetic data could not be compared with isopollen maps because pollen grains of *P. mugo* complex species are very similar to other *Diploxylon* pines and have not been distinguished during palynological investigations (e.g. de Beaulieu et al., 2001; Fauvart et al., 2012; Jankovská, 2008; Obidowicz, 1996). Only scarce pre-Holocene macrofossils data are available, mostly from the low areas of the mountain ridges (de Beaulieu et al., 2001; Fauvart et al., 2012; Obidowicz, 1996).

In this study, we aimed to understand genetic variation and structure among populations of *P. mugo* complex across most of the taxa range. More specifically, our objectives were (1) to identify the genetically homogenous groups of populations (2) to investigate the molecular distinctiveness and the taxonomic relationships between *P.*

mugo s.s., *P. uncinata* and *P. uliginosa* (3) to assess whether observed patterns of differentiation are better explained by geographical distance or taxonomy and (4) to describe the potential glacial history of the mountain pines in Europe. We hypothesised that genetic differentiation will confirm the distinctiveness between *P. mugo* s.s and *P. uncinata,* and an intermediate between these two taxa character of *P. uliginosa* (Christensen, 1987). We also expect the genetic differentiation and diversity of the whole geographic range of *P. mugo* s.l. to be influenced by the natural physical barriers. In Europe, the Mediterranean Sea in the south and the east–west orientation of the main mountain ranges of the Alps and the Pyrenees acted as a barrier preventing the lowland and sub-mountain species from spreading during cold and warm periods (Taberlet, Fumagalli, Wust-Saucy, & Cosson, 1998). At the same time, the mountain ridges function as refugia for the high-mountain and arctic high-mountain plants.

Material and methods

Population sampling and cpSSR genotyping

The taxa of the *P. mugo* complex are common at and above the treeline in mountain areas. Sampling was conducted in accordance with national and international guidelines, and the collection of 5 needles does not represent a threat for the sampled individuals. We obtained authorization from the Polish Ministry of the Environment for the populations M TM 1, M TM 2, M GM 4, M GM 5, M GM 6, UL BAT, UL TOP, UL WEG, and no permission was needed for the other sites. Forty-four natural populations were sampled within the entire geographic range of the species, except for isolated localities in the north-western Alps, Jura and Vosges. Needles were collected from 1331 adult, cone bearing individuals of *P. mugo* s.s., *P. uncinata* and *P. uliginosa*, distributed

in 31, 10 and 3 natural populations, respectively (Table 1). The taxa were identified in the field using ecological and morphological characteristics: *P. mugo* s.s. as a high-altitude, prostrate shrub with ±relatively small, ±symmetric cones and thin cone scales; *P. uncinata* as a high-altitude, monocormic tree, bearing large, asymmetric cones with hooked, shovel-like umbos on the scales; *P. uliginosa* as a montane peat-bog tree of 1-2(-3) stems and small, asymmetric cones with prominent but not shovel-like umbos and relatively thick scales (Fig. 1). To avoid the collection of clones, individuals of *P. mugo* s.s sampled within each population were spaced at least 40 m apart (Boratyńska, Marcysiak, & Boratyński, 2005). Data from 18 populations used in previous studies (Dzialuk et al., 2009; Dzialuk et al., 2012) were reanalysed.

Total DNA was extracted following the protocol of (Doyle & Doyle, 1990).

Chloroplast DNA variation was analysed in single multiplex PCR reaction of ten pairs of microsatellite primers originally characterized in *P. sylvestris*: Pt26081, Pt36480, Pt45002, Pt71936, Pt15169, Pt30204 (Vendramin, Lelli, Rossi, & Morgante, 1996) and PCP1289, PCP41131, PCP87314, PCP102652 (Provan et al., 1998). The DNA amplification was carried out in 10 µl volumes using a PTC-200 thermocycler (MJ Research). The PCR started with the denaturation phase at 94°C for 5 min. We then performed 30 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min (10 min for the last one). The reactions consisted of 30 ng of template DNA, 1x Qiagen PCR buffer, 4.0 mM $MgCl_2$, 0.2 mM each of dNTP, 25–250 nM each of forward and revers primers (Table 2), 5 µg/µl of BSA and 0.25 U of Taq Polymerase (Qiagen).

The PCR products were sized by using the capillary sequencer ABI PRISM 310 (Applied Biosystems) and the genotypes were scored using the GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems).

Genetic diversity

For each tree, the haplotype was defined as the unique combination of alleles across the ten microsatellite loci. Haplotype variations were calculated at the population and taxa levels by estimating: number of haplotypes (A_h), effective number of haplotypes (N_e), number of private haplotypes (P_h) and unbiased haplotype diversity (H_e) using GenAlEx 6.501 (Peakall & Smouse, 2006). In order to correct for the unequal sample sizes within populations a rarefaction analysis was performed with HP-Rare 1.0 (Kalinowski, 2005). Within-population genetic distance between haplotypes (D_{sh}^2) (Vendramin, Anzidei, Madaghiele, & Bucci, 1998), as defined by Goldstein, Linares, Cavalli-Sforza, & Feldman (1995), was also computed. This distance is based on the differences among the number of repeat units at the microsatellite regions (stepwise mutation model, SMM) considering the chloroplast DNA as a single locus. Moreover, haplotypic richness was estimated by 'proportion distinguishable' (PD), which is the ratio of haplotypes relative to the total number of individuals analyzed in the population (Ellstrand & Roose, 1987). For descriptive purposes, correlations between characteristics of genetic diversity and altitude as well as longitude/latitude were quantified using the Spearman rank correlation coefficient (r_S). Finally, to compare the genetic diversity parameters between taxa, we used the non-parametric Kruskal-Wallis test. All the analyses were performed with PAST 2.17 software (Hammer, Harper, & Ryan, 2001).

Genetic and taxonomic structure

Population genetic structure was examined using analysis of molecular variance (AMOVA) in Arlequin 3.5.1.2 (Excoffier, & Lischer, 2010). The analyses were based on the number of different alleles (F_{ST}-like, based on unordered alleles) and on the sum of the squared differences (R_{ST}-like, based on ordered alleles). Hierarchical AMOVA was conducted to partition components of genetic variation among taxa (*P. mugo* s.s.*, P. uncinata, P. uliginosa*) and regions (Sierra Cebolera; Sierra de Gúdar; Pyrenees; Massif Central; Alps; Apennines; Sudetes; Dinaric Alps; Balkan Mts.; West Carpathians; East and South Carpathians). The taxa and geographical regions were treated as groups resulting in three hierarchical levels of differentiation: among taxa/geographical regions (fixation indexes F_{CT} and R_{CT}), among populations within taxa/geographical regions (fixation indexes F_{SC} and R_{SC}) and within populations (fixation indexes F_{ST} and R_{ST}). To better understand the relationships among taxa, comparison of F_{ST} values and AMOVA between pair of taxa were performed. The significance of the results was tested using 20,000 random permutations of the data. PCoA was also used to investigate the molecular distinctiveness of the three taxa.

To discern whether populations are differentiated due to isolation by distance (Wright, 1965), a Mantel test with 9,999 random permutations was applied to analyze the association between genetic [$F_{ST}/(1-F_{ST})$] and geographic distances for each sampled population using GenAlEx 6.501 (Peakall, & Smouse, 2006). The test was conducted for *P. mugo* s.l., as well as for *P. mugo* s.s., *P. uncinata* and *P. uliginosa*, separately. The geographic distances between populations have been retrieved from geographic coordinates (Table 1), using GenAlEx 6.501 (Peakall, & Smouse, 2006).

Phylogeographic patterns

A Bayesian approach was used to further explore the population structures using both a non-spatial and a spatial genetic mixture analysis implemented in BAPS 5.3 software (Corander, Waldmann, & Sillanpää, 2003). Genetically homogeneous groups of populations were inferred based on a linked loci model because the chloroplast genome does not genetically recombine and may be viewed as a single locus or circular haploid chromosome with non-recombining loci (Vendramin et al., 1998). In order to test the reproducibility of the results, for the maximum number of clusters (K) ranging from K = 1 up to K = 44, we ran the model ten times. The most likely partition of populations into K clusters was identified as the one with the highest log likelihood.

To identify clusters of populations sharing similar cpSSR compositions, based on the allelic frequencies matrix and the assumption of a stepwise mutation model (SMM), we computed 1,000 pairwise shared allele distance D_{SA} matrices (Chakraborty, & Jin, 1993) in PowerMarker (Liu, & Muse, 2005). A consensus tree was made using the programs CONSENSE and DRAWTREE of the package PHYLIP version 3.68 (Felsenstein, 1989). In the preliminary tests, the proportion of variation in the genetic distance matrix that is explained by the tree (R^2) was calculated using TreeFit software (Kalinowski, 2009). If R^2 is near 1.0, the tree represents a good summary of the genetic relationships shown in the distance matrix. The neighbour joining (NJ) method was used for tree building, because the algorithm was considered to be preferable to the unweighted pair group method with arithmetic mean (UPGMA). To validate and further define naturally occurring genetic clusters, GenAlEx 6.501 software (Peakall, &

Smouse, 2006) was used to calculate principal coordinate analysis (PCoA) on the pairwise genetic distance matrix for D_A (Nei, Tajima, & Tateno, 1983).

The presence of phylogeographical structure was tested by comparing the observed R_{ST} value with its value after permuting allele sizes within loci (pR_{ST}) using SPAGeDi 1.3 (Hardy & Vekemans, 2002). This analysis indicates whether shifts in allele size resulting from stepwise mutations contribute to population differentiation (Hardy, Charbonnel, Fréville, & Heuertz, 2003). We applied the permutation tests (20,000 permutations) on the whole dataset as well as on each taxon (*P. mugo* s.s., *P. uncinata* and *P. uliginosa*) and geographical region (Alps, Sudetes, E and S Carpathians, W Carpathians, Balkan Mts.) separately, with the expectation that mutations contributed unevenly to the taxon/region history. If R_{ST} is significantly higher than pR_{ST}, then allele size mutations contributed to population differentiation and can be interpreted as phylogeographical structure in which closely related plastid haplotypes are located in nearby populations (Hardy, & Vekemans, 2002).

Results

Genetic diversity and structure

The 10 microsatellite loci yielded a total of 87 size variants (alleles), including 6-15 alleles per locus among 1331 individuals of the *P. mugo* complex. The most common alleles and the allele sizes ranges were roughly the same in all taxa. We observed only small size shifts at the loci Pt36480 and Pt 71936 in *P. uliginosa* as well as at loci PCP41131 and PCP102652 in *P. uncinata*. However, the highest differences in fragment lengths were observed at the locus PCP1289, where the maximum length alleles were 106, 110 and 103 base pairs (bp) for *P. mugo* s.s., *P. uncinata* and *P.*

uligionosa, respectively (Table 2). Allelic combinations produced a total of 757 haplotypes, from which about 74% were exclusive to a given population (private haplotypes). The most abundant haplotype occurred only in 25 individuals of *P. mugo* s.l.

About 95% of the total haplotypes were taxon specific (*P. mugo* s.s. = 74.2%; *P. uncinata* = 18.4% and *P. uliginosa* = 7.3%). Only 4.7% of the haplotypes were shared between taxa (*P. mugo* s.s. and *P. uncinata* = 2.5%, *P. mugo* s.s. and *P. uliginosa* = 1.2%, *P. uncinata* and *P. uliginosa* = 0.7%, *P. mugo* s.s., *P. uncinata* and *P. uliginosa* = 0.3%). As indicated by A_h, % PD, P_h and N_e (Table 3), the highest values of haplotype diversity were observed in *P. mugo* s.s.: M A1, M A3, M A6, M DYN1, M DYN2 in the Alps; M CAR4, M CAR6, M CAR7, M LT in the Carpathians; M RM1 and M VIT in the Balkan Mts. as well as in *P. uncinata* (U MAS2, Massif Central) and *P. uliginosa* (UL WEG, Sudetes). The lowest values of haplotype diversity were noted in M A10 (Alps), M RM2 (Balkan Mts.), U GUD (Cordillera Iberica) and UL BAT (Sudetes).

The within-population diversity parameters showed that the mean haplotypic richness (23.61, 22.50 and 22.64) and the haplotypic diversity (0.975, 0.963 and 0.973) were high and similar in *P. mugo* s.s., *P. uncinata* and *P. uliginosa*, respectively. We did not find a direct connection between geographic position of population (longi-/ lati-/ altitude) and level of diversity, neither within the *P. mugo* complex, nor in particular taxa. The highest divergence between haplotypes D_{sh}^2 was observed in UL TOP, U MAS2 and U CEB. The genetic differentiation between populations (pairwise F_{ST}) was high and mostly significant. However, the differences in genetic diversity parameters between the taxa of the *P. mugo* complex were not significant (P>0.05, Kruskal-Wallis test, Table 3).

The Mantel permutation test proved a significant correlation between genetic and geographic distance ($R^2 = 0.205$, $P<0.0001$), suggesting a pattern of isolation by distance (IBD) in the *P. mugo* complex. When the taxa were analysed separately, the test was statistically significant for *P. mugo* s.s. ($R^2 = 0.084$, $P<0.0001$) but not for remaining taxa (Fig. 2).

The non-hierarchical AMOVA based on unordered alleles (F_{ST}) and ordered alleles (R_{ST}) (Table 4) revealed higher genetic diversity within populations (87.94% and 79.41%, respectively) in comparison with differentiation among populations of the *P. mugo* complex (12.06% and 20.59%, $P<0.001$ in both cases). This pattern of genetic structure in *P. mugo* s.l. was confirmed by the geographical grouping AMOVA (F_{CT}=0.056, R_{CT}=0.083, $P<0.001$).

Taxonomic relations

Hierarchical AMOVA indicated that barely 5% of genetic variance was accounted for by the species grouping (F_{CT}= 0.051, R_{CT}= 0.050, $P<0.001$), while most of the diversity was still located within populations (Table 3). In the PCoA the first two axes explained 66.8% and 33.20% of the variance and revealed distinct separation between the taxa (Fig. 4.4). The most differentiated pair of taxa was *P. mugo* s.s. and *P. uncinata* (F_{CT} = 0.064 and R_{CT} = 0.063). Much lower differences were attributable to differences among *P. uncinata* and *P. uliginosa* (F_{CT} = 0.035 and R_{CT} = 0.046, Table 5). Meanwhile, a non-significant proportion of variation was among *P. mugo* s.s. and *P. uliginosa* (Table 5). High F_{ST} values between each pair of three pine taxa were estimated between *P. mugo* s.s. and *P. uncinata* (F_{ST}=0.699) as well as *P. uncinata* and *P. uliginosa* (F_{ST}=0.558).

The lowest value was found between *P. mugo* s.s. and *P. uliginosa* (F_{ST}=0.029). All F_{ST} values were significantly different from zero ($P < 0.001$, Table 6).

Phylogeographical structure

Bayesian analysis of population structure gave similar results using either a spatial or a non-spatial model for genetic mixture analysis. BAPS showed an optimal partition of the 44 populations into nine or eight genetically distinct groups, respectively (Fig. 3). All the East and South Carpathian populations shared a unique gene-pool and were assigned to cluster 4, which also included populations from the Balkan Mts., except for M RM2. Populations from the Alps were assigned to two different groups: cluster 2 or cluster 3. Moreover, despite being geographically isolated, both the Tatra and most of the Sudetes populations were assigned to the Alpine cluster 3. The westernmost alpine population of *P. mugo* s.s. (M A11) was grouped with the Apennines (MAPP) in cluster 5 and the easternmost alpine population (M A10) was included into the East Carpathian cluster 4. Similarly, some signs of close association between Alpine cluster 3 and Carpathian cluster 4 were observed in the West Carpathians and the Sudetes. Moreover, the southernmost Dynaric population (M DYN2) was assigned to the Apennines cluster 5 in non-spatial analysis. Most populations of the *P. uncinata* were included in cluster 6. The remaining population of this taxon was grouped in cluster 7 (U GUD) and cluster 8 (U CEB, A MAS2). Each population of *P. uliginosa* was assigned to a different cluster (Fig. 3).

The population structure revealed by the NJ dendrogram using shared allele distance D_{SA} and the PCoA graph appeared similar to that inferred from the Bayesian analysis. According to these results, populations of the *P. mugo* complex clustered into

four main groups, the Pyrenean, the Alpine and West Carpathian, the Sudetan, and the East and South Carpathian. Only three marginal populations (U GUD, U CEB and U MAS2) were separated from the *P. uncinata* cluster, and all populations of *P. uliginosa* were mixed in one group with marginal populations of *P. mugo* s.l. (Fig. 4).

A permutation test revealed that R_{ST} (0.206) was significantly higher than the mean permuted R_{ST} (0.126; P=0.0012), indicating the geographical association of closely related plastid haplotypes in the *P. mugo* complex (Table 7). However, this pattern was consistent only for populations of *P. mugo* s.s. (R_{ST}=0.178 > pR_{ST}=0.090; P<0.001) and for *P. uncinata* (R_{ST}=0.214 > pR_{ST}=0.089; P<0.001), but not for *P. uliginosa* (R_{ST}=0.178 > pR_{ST}=0.132, P=0.243). Moreover, analyses within mountain chains indicated that SMM contributed significantly to regional differentiation in *P. mugo* s.s. only in the Alps and East Carpathians. No significant differences were found between observed and permuted R_{ST} in the Sudetes, Tatras and Balkan Mts. (Table 7).

Discussion

Genetic diversity and differentiation

Due to lack of polymorphic, maternally inherited mitochondrial DNA markers, and only three nuclear microsatellite markers (nSSR) available so far in the *P. mugo* complex (Celiński, Pawlaczyk, Wojnicka-Półtorak, Chudzińska, & Prus-Głowacki, 2013), cpSSRs are still markers of the choice in the population genetic study. Their non-recombinant nature, paternal inheritance and transmission by both pollen and seeds (Neale, & Sederoff, 1989), make them a useful tool in the study of genetic variation, differentiation and phylogenetic relationships in natural populations of pines (Powell, Morgante, McDevitt, Vendramin, & Rafalski, 1995).

Heuertz et al. (2010) identified 100 haplotypes in 786 individuals from 29 populations of the *P. mugo* s.l. in West Europe using 3 cpSSRs. In conclusion, they recommended improving the detection power of the DNA markers to avoid weak cpSSR data differentiation. Thus, we used ten microsatellite loci, which number is high compared with that typically used in these types of studies. Moreover, this approach avoids a downward bias in estimates of genetic differentiation of microsatellites via size homoplasy, which occurs when different copies of a locus are identical in state but not by descent (Estoup, Jarne, & Cornuet, 2002). The risk of homoplasy is high in the *P. mugo* complex (Provan, Powell, & Hollingsworth, 2001), because population divergence times are great (Heuertz et al., 2010; Wachowiak et al., 2011) and the stepwise mutations contribute to population differentiation (Dzialuk et al., 2009; Heuertz et al., 2010).

Using 10 chloroplast microsatellites we found 757 haplotypes across 1331 individuals in 44 populations, thus the level of genetic diversity found within the *P. mugo* complex taxa is high and comparable with other pine species studied with cpSSRs in Europe and the Mediterranean (e.g. Afzal-Rafii, & Dodd, 2007; Bucci et al., 2007; Gómez, Vendramin, González-Martínez, & Alía, 2005; Kurt, González-Martínez, Alía, & Isik, 2011; Ribeiro, Plomion, Petit, Vendramin, & Szmidt, 2001; Robledo-Arnuncio, Collada, Alía, & Gil, 2005). Interestingly, most populations with a D_{sh}^2 value higher than 10 (UL TOP, U MAS2, U CEB) were sampled in the isolated localities of the *P. mugo* complex surrounded by extensive *P. sylvestris* stands with hybridization zones (Dzialuk et al., 2009; Jasińska et al., 2010). Introgression from *P. sylvestris* into the *P. mugo* complex was suggested by Heuertz et al. (2010), but a reverse direction of that process has been confirmed by Jasińska et al. (2010). In the Topieliska Nature Reserve

(UL TOP) the gene pool of *P. uliginosa* might be affected by co-occurring *P. mugo* and *P. sylvestris* (Wachowiak & Prus-Głowacki, 2009). Thus, hybridization among mountain pine taxa seems still to be the key future research topic in the *P. mugo* complex (Wachowiak, Cavers, & Żukowska, 2015; Wachowiak, Żukowska, Wójkiewicz, Cavers, & Litkowiec, 2016) and introgression should be further distinguished with suitable DNA markers.

Despite adaptation to local conditions resulting sometimes in a strong differentiation in quantitative traits, pines show generally only low genetic differentiation at neutral markers (Karhu et al., 1996; Savolainen, Pyhäjärvi, & Knürr, 2007), mainly due to effective pollen-mediated gene flow (Muona & Harju, 1989). *Pinus mugo* s.l. shows only weak genetic differentiation in West Europe (F_{ST}=0.076, N_{ST}=0.263, see Heuertz et al., 2010). Meanwhile, the level of genetic differentiation in our study (F_{ST}=0.121) is one of the highest revealed between populations of *P. mugo* s.l. to date. It may be explained by the fact that this attempt was the nearest thing to a range-wide study, based on a relatively high number of cpSSR markers.

Taxonomic differentiation

As stated by Heuertz et al. (2010), the genetic variation of *P. mugo* agg. in the western part of the geographic range is structured according to geography but not morphology and taxonomy. Our results indicated that geography and taxonomy are relatively similar determinants of genetic structure at cpSSRs (Table 4). These somewhat contradictory results could be explained by differences in sampling. Heuertz et al. (2010) collected materials from 29 populations in the western part of the complex' geographic range, where *P. uncinata*, *P. mugo* s.s. and *P. rotundata*/*P. ×pseudopumilio* were represented

by 17, 4 and 8 populations, respectively. The *P. rotundata*/*P. ×pseudopumilio* are treated as synonyms of *P. uliginosa* (Christensen, 1987). In fact, Heuertz et al.'s (2010) sampling area corresponded mostly to the range-wide sampling for *P. uncinata* and relatively few westernmost populations of other taxa. Additionally, *P. rotundata/P. ×pseudopumilio* potentially represented hybrids between *P. uncinata* and *P. mugo* (Christensen, 1987), which could blur the difference between the last taxa. Populations of *P. mugo* s.s sampled from the central and eastern parts of their geographic range allow us to find both geographic and taxonomic differentiation within *P. mugo* s.l. Moreover, the use of 10 cpSSRs may also have played an important role, providing genetic support for morphological taxa of mountain pines in our study. We have well distinguished *P. uncinata*, *P. mugo* s.s. and to some degree also *P. uliginosa* (Figs 3 and 4), as likewise in the biometric study (Boratyńska, Jasińska & Boratyński, 2015, Fig. 2). The differences between taxa in *P. mugo* complex have been reported during last decades using allozymes (Lewandowski et al., 2000; Prus-Głowacki, Bajus, & Ratyńska, 1998), DNA markers (Danusevičius et al., 2013) and chemosystematic markers (Bonikowski, Celiński, Wojnicka-Półtorak, & Maliński, 2015; Celiński, Bonikowski, Wojnicka-Półtorak, Chudzińska, & Maliński, 2015).

NJ tree and PCoA (Fig. 4) support the suggestion that *P. uncinata* evolved in the western, and *P. mugo* s.s. in the eastern regions, and hybridized giving *P. uliginosa*/*P. rotundata* in the northern parts of the Alps, Sudetes and West Carpathians (Christensen, 1987). Particular populations of that taxon have a relict character, are isolated from each other and evolved in separation, which could have influenced morphological differences among them, detected on the allozyme markers (e.g. Lewandowski, Samoćko, Boratyńska, & Boratyński, 2002; Wachowiak & Prus-Głowacki, 2009) and in biometric

studies (e.g. Boratyńska et al., 2003, 2015; Boratyńska & Lewandowska, 2009; Staszkiewicz, & Tyszkiewicz 1976). The differences between populations of *P. uliginosa* may be enhanced by different demographic events, founder effects, selection pressure on the local, isolated localities, and also by hybridization with *P. mugo* s.s. or even with *P. sylvestris* (Boratyński, Boratyńska, Lewandowski, Gołąb, & Kiciński, 2003; Heuertz et al., 2010; Lewandowski et al., 2002; Wachowiak et al., 2011).

Geographic pattern of diversity and differentiation

The long distance between marginal East (M CAR7) and West (UL CEB) populations of *P. mugo* s.l., ca 2.5 times longer than between northern- (UL WEG) and southern-most (M APP) populations implies larger genetic distances between the East-Carpathian and Pyrenean populations, and such differences have been detected. Also, the significant correlation between genetic and geographic distances (Fig. 2) suggests isolation by distance (IBD). And in fact, the results of the Bayesian analyses, the cluster analysis of shared allele distance D_{SA} (Chakraborty & Jin, 1993) and PCoA of Nei's unbiased genetic distances revealed a subdivision of populations of *P. mugo* s.l. into at least four core geographic groups, the two detected earlier by Heuertz et al. (2010) and two others, the East-Carpathian and Sudetan, with several clusters of the marginal populations (Figs 3 and 4). This grouping is similar to that detected on the morphological characteristics where, however, Sudetan populations fell into Alpine-West Carpathian populations, but forming among them a compact cluster (compare Fig. 3 with Boratyńska et al., 2015, Fig. 5). It is worth noting that the IBD concerned only *P. mugo* s.l. and, on a much lower level, *P. mugo* s.s., but not the other taxa (Fig. 2). These confirm the distinct character of the Pyrenean group, which contains *P. uncinata* and

differences between Alpine – West Carpathian and East Carpathian populations of *P. mugo* s.s. The differences between Alpine and East Carpathian populations of *P. mugo* s.l. were also detected using allozymes (Sannikov et al., 2011), morphological cone characteristics (Staszkiewicz, & Tyszkiewicz, 1976) and cone and needle characteristics (Boratyńska et al., 2005; 2015).

The high level of genetic distinctiveness of the East- and South-Carpathian when compared to all the other populations of *P. mugo* s.s. could be explained by long lasting, spatial isolation among them during Pleistocene (Latałowa, Tobolski, & Nalepka, 2004; Magyari et al., 2012). Long-lasting isolation is a reason for independent genetic processes, exposition of particular populations to different environmental pressures and distinct demographic events, such as have been recognized in populations of several high-mountain plant taxa (e.g. Ronikier, 2011; Stachurska-Swakoń, Cieślak, & Ronikier, 2013).

We also detected some intermingling of populations from different regions (Figs 3 and 4). The presence of populations of different origin within the Alpine-West Carpathian group of populations could indicate the possible gene flow between the East Alps, Sudetes, Carpathians, Dinaric Alps and Balkan populations, as proposed in Fig. 5. This could have taken place during glacial periods, when the vertical geographic range of *P. mugo* s.l. was reduced, but covered a broader area, compared to that of the present day (e.g. Benito Garzón, Sánchez de Dios, & Sáinz Ollero, 2007; Burga, 1988; de Beaulieu et al., 2001; Jankovská, 2008; Magyari et al., 2012; Wohlfarth et al., 2001).

The Balkan populations, which have been suggested by Heuertz et al. (2010) as constituting a possible different gene pool, appeared genetically heterogeneous, with influence from the East and South Carpathian and Alpine gene pools (Figs 3 and 4).

Again, this is similar to that described in reference to morphological characters, by Boratyńska et al. (2015). Inversely, the influence of a Balkan to Alpine gene pool of *Abies alba* has been detected by Liepelt et al. (2009), but *A. alba* is a montane, not sub-alpine species.

The migration history of *P. mugo* s.l. could be better understood when information on its possible micro-refugia detected on the palynological data be considered. The pre-Holocene findings of the species were reported from the Bohemian Carpathians and Sudetes (Jankovská, 2008), around the Tatras (Obidowicz, 1996) and from north-western and southern Romania (Magyari et al., 2012; Wohlfarth et al., 2001). The populations of *P. mugo* from these regions also revealed specific mitochondrial haplotypes (Wachowiak et al., 2013).

Our results generally did not support the hypothesis that population differentiation within particular massifs was at a lower level than between different mountain ridges (Table 4). The results of AMOVA indicate a small (about 5.5%) but statistically significant percentage of variation among the 11 main regions of occurrence, but a slightly higher (about 7.1%) and also significant percentage of variation among populations within regions. This only partly confirms our hypothesis regarding the long-lasting isolation of populations in particular mountain massifs, but also indicates the high level of differences within the largest of them. Such a pattern of differentiation could be explained by the extensive populations of the species, which produced great amounts of pollen, transported over relatively large distances (Burczyk, & Chalupka, 1997; Muona, & Harju, 1989; Smouse, Dyer, Westfall, & Sork, 2001; Wachowiak et al., 2011). The high level of differences between populations within mountain ridges concerned first of all the Alps and Carpathians, which have several

separate massifs high enough for a subalpine vegetation belt to be developed. Recently, using SNPs located in genes encoding proteins, Mosca et al. (2012) found a clear separation between eastern and western Alpine populations of *P. mugo*, which was not observed in studies based on neutral markers (Heuertz et al., 2010; Monteleone, Ferrazzini, & Belletti, 2006; Wachowiak et al., 2013). She also found that genetic variation of *P. cembra*, *Larix decidua* and *Abies alba* is structured according to geographic regions of the Alps, which corresponds with the general phytogeographic pattern of plant differentiation in the Alps (Thiel-Egenter et al., 2011).

Comparisons of R_{ST} and pR_{ST} revealed a strong signal for phylogeographic structure in *P. mugo* s.s. and *P. uncinata*, but not in *P. uliginosa,* indicating a different glacial history of the taxa. Moreover, a signature of stepwise mutational events in differentiation between Alpine and East and South Carpathian populations of *P. mugo* s.s. suggest a relatively stable demographic history that would be consistent with populations having persisted *in situ* at least during the last full glacial stage (Afzal-Rafii, & Dodd, 2007). Thus, our data provide support for the glacial refugia of *P. mugo* s.s. in the Alps and the Carpathians. Conversely, in the Sudetes, the West Carpathians and the Balkan Mts. populations of *P. mugo* s.s., as well as in populations of *P. uliginosa*, a stepwise mutation process did not contribute to genetic differentiation, so the mutation rate is negligible compared to migration or drift. Therefore, these populations might suffer from decolonization processes following glacial maxima.

Conclusion

The phylogeographical structure in the *P. mugo* complex, and separately in *P. mugo* s.s. and *P. uncinata*, has been detected. Four gene pools could be distinguished in the *P.*

mugo complex, the Pyrenean including *P. uncinata*, the Alpine – West Carpathian, Sudetan and East and South Carpathian all iuncluding *P. mugo* s.s. The most of the Balkan mountain populations are placed in the East and South Carpathian group of populations. Within these four gene pools, the groups of populations from the isolated mountain massifs revealed further differentiation inside *P. uncinata* (3 clusters) and *P. mugo* s.s. (6 clusters). The populations of *P. uliginosa* fall into *P. mugo* s.s. except of one, which formed separate cluster. The revealed differentiation and genetic distances between *P. uncinata*, *P. mugo* s.s. and the *P. uliginosa* supports their separate taxonomic status, but the relation of the latter taxon is closer to *P. mugo* s.s. than to *P. uncinata*.

Acknowledgements

We would like to thank prof. Yakiv P. Didukh from NASU Botanical Institute in Kiev, prof. Roman Gout from UNFTU Department of Forestry in Lviv, Dr. Josep M. Montserrat from the Botanical Institute of Barcelona, Dr. to C. Farkas from the Brasov Forest Faculty, Romania, the authorities of Karkonosze and Tatra National Parks in Poland for their valuable help during field work, help and assistance in plant material collection and Ewa Sztupecka for technical assistance in the laboratory work. This work was supported by the Polish Ministry of Science under Grants No. 2P04C 018 29, 6P04G 06016, 2P06L 04628 and NN303 360535 and by Institute of Dendrology of Polish Academy of Sciences under Statutory Activity. The collection of plant material was made possible thanks to the cooperation between CSIC (Spanish Research Council) and PAS (Polish Academy of Sciences), NASU (National Academy of Sciences of

Ukraine) and PAS, KWU (Kazimierz Wielki University) and UNFTU (Ukrainian National Forest-Technical University).

Data Archiving Statement

cpSSR data has been deposited in TreeGenes database (the TGDR Accession number: TGDR016).

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Figure captions

Fig. 1 The taxa of *Pinus mugo* complex (*P. mugo* s.l.) investigated: a) dwarf mountain pine - *P. mugo* Turra = *P. mugo* s.s. [*P. mugo* subsp. *mugo* (Turra) Christensen] in the Sudetes, b) mountain pine - *P. uncinata* Ramond [*P. mugo* subsp. *uncinata* (Ramond.) Christensen] in the Pyrenees, c) peat-bog pine - *P. uliginosa* Naumann in the Sudetes (photo by A. Boratyński)

Fig. 2 Relationship between geographic distance and the pairwise F_{ST} (isolation by distance analysis) in the *P. mugo* s.l. and particular taxa of the *P. mugo* complex

Fig. 3 Results of spatial (big dots) and non-spatial Bayesian cluster analysis (inside dots), BAPS, showing genetically homogenous groups of populations of the *Pinus mugo* complex. Colours indicate nine (K = 9) and eight clusters (K = 8), respectively. Geographic range of *Pinus mugo* s.l. taxa [20, simplified]. Acronyms as in Table 1 (first letters denote: M – *P. mugo* s.s., U – *P. uncinata*, UL – *P. uliginosa*).

Fig. 4 Genetic relatedness among populations of *P. mugo* s.l using: 1 – shared allele distance D_{SA} and cluster analysis via the neighbor joining method, with relative strength of the nodes determined using bootstrapping analysis, the numbers indicate strength of node more than 50%, R^2 describes how well a tree fit the genetic data; 2 – geographical location of the clusters; 3 – the principal coordinate analysis (PCoA) based on Nei's genetic distances between populations; 4 – the principal coordinate analysis (PCoA) based on Nei's genetic distances between *P. mugo* s.s., *P. uncinata* and *P. uliginosa*.

Fig. 5 Putative gene exchange between populations of the *P. mugo* complex during glacial periods based on data from microsatellite markers in chloroplast DNA

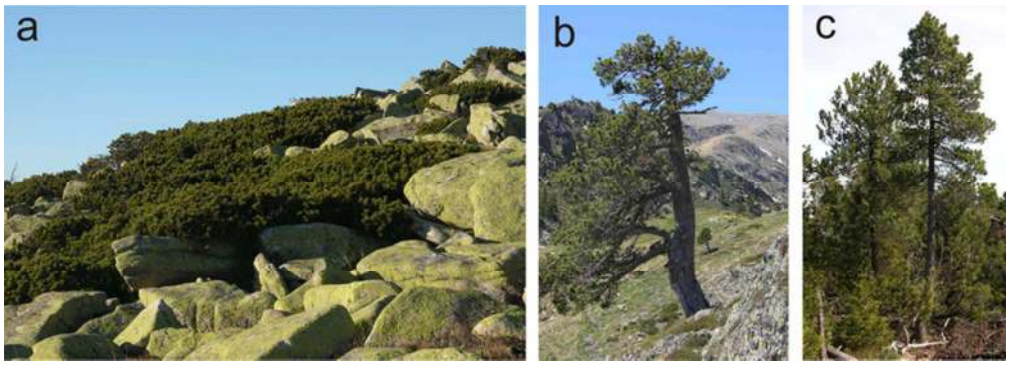

Fig. 1

71x25mm (300 x 300 DPI)

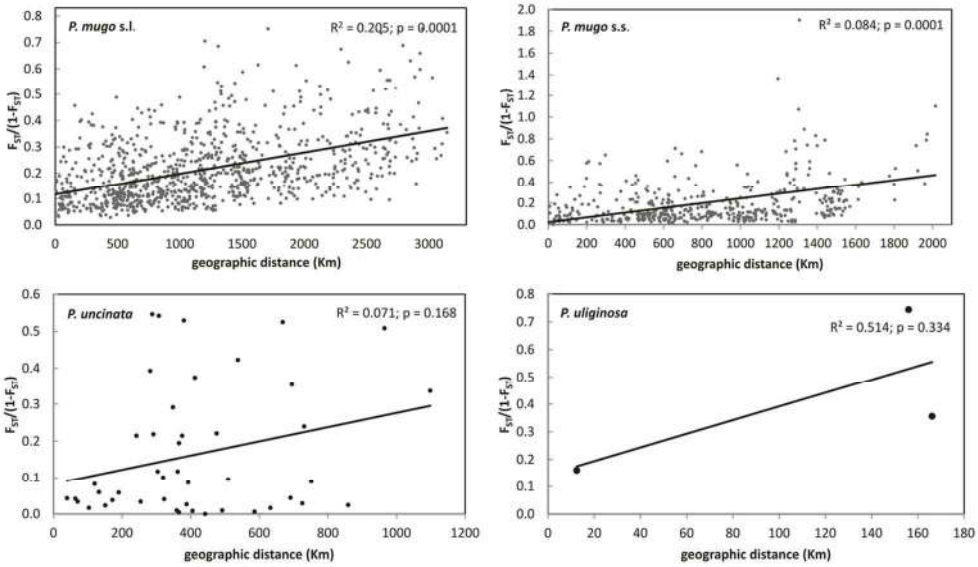

Fig. 2

113x66mm (300 x 300 DPI)

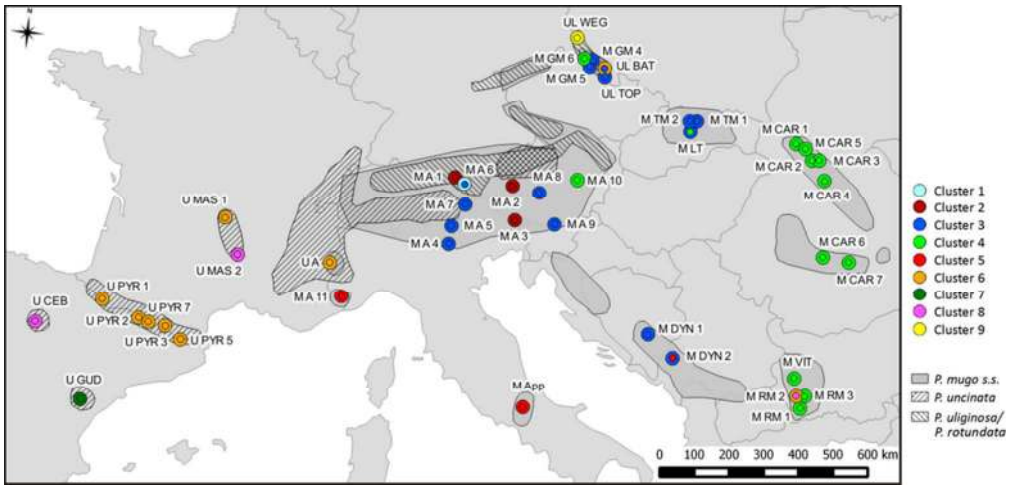

Fig. 3

83x40mm (300 x 300 DPI)

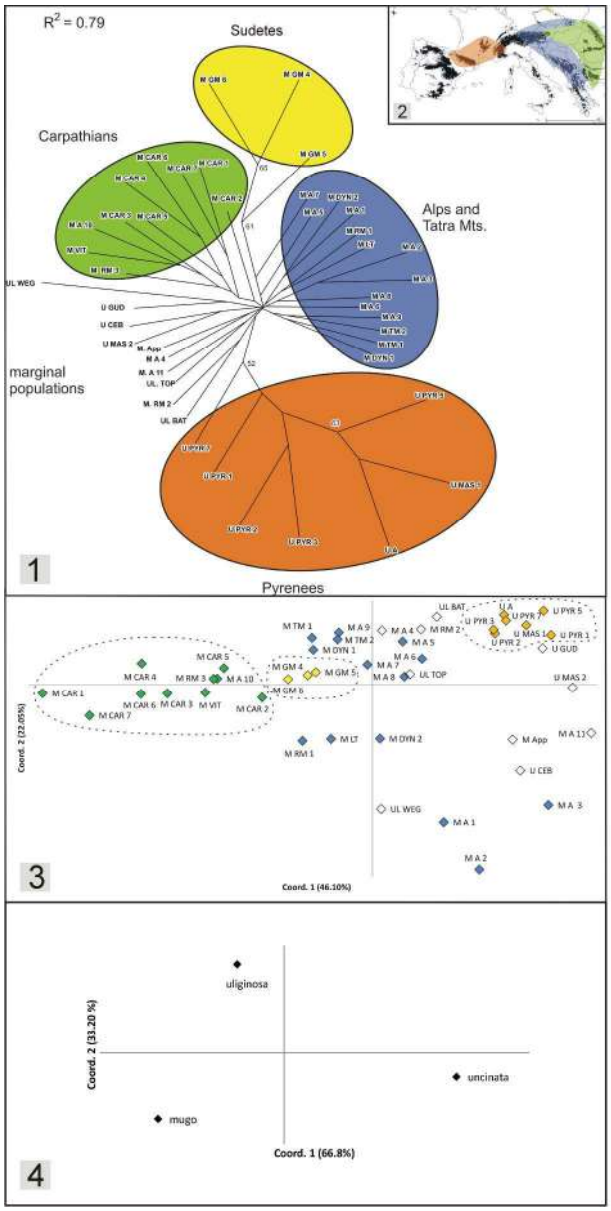

Fig. 4

227x448mm (300 x 300 DPI)

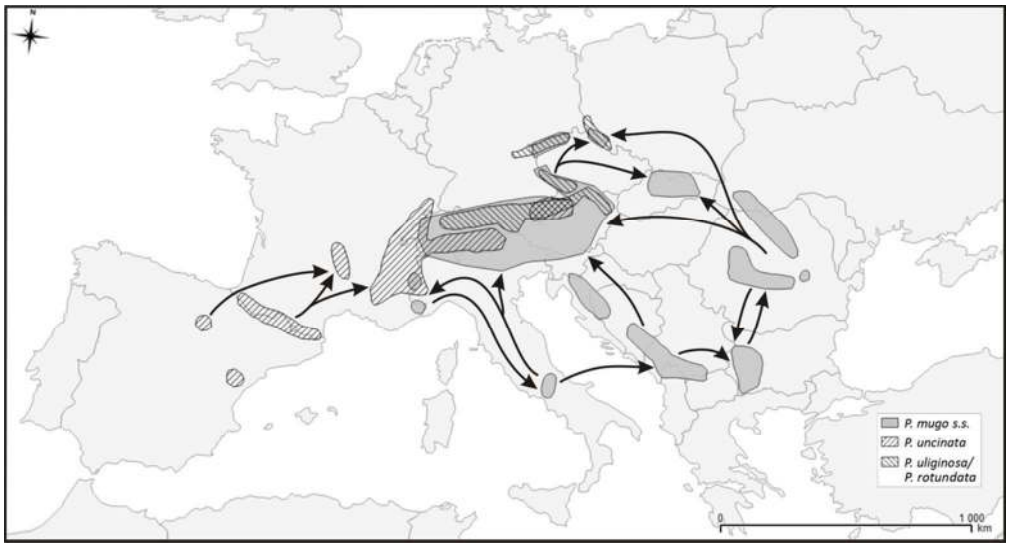

Fig. 5

93x50mm (300 x 300 DPI)

Table 1 Geographical characteristics of the *P. mugo* s.s., *P. uncinata* and *P. uliginosa* populations

Taxon	Label	Region	Locality	Latitude /Longitude [º]	Elevation [m]	Data source
mugo s.s	M A 1	E Alps, Bavarian Alpen (Germany)	Ammergauer Kreuzspitze	47.53 N/10.92 E	1800	Dzialuk et al. 2012; Boratyńska et al., 2014
	M A 2	E Alps, Salzburgische Alpen (Austria)	Hochkonig	47.43 N/13.08 E	1800	Dzialuk et al. 2012; Boratyńska et al., 2014
	M A 3	E Alps, Karnische Alpen (Italy)	Passo di Pramollo	46.55 N/13.26 E	1530	Dzialuk et al. 2012; Boratyńska et al., 2014
	M A 4	E Alps, Prealpi Venete (Italy)	Monte Baldo,	45.81 N/10.94 E	1750	
	M A 5	Alpi Orientale, Gruppo di Brenta (Italy)	Lago di Tovel	46.27 N/10.97 E	1200	
	M A 6	E Alps, Karwendel Gebirge (Austria)	Scharnitz, above Isar	47.38 N/11.30 E	1400	
	M A 7	West Alps, Sarntaler Alpen (Italy)	Passo di Penser Joch	46.85 N/11.40 E	1900	
	M A 8	E Alps, Schlaudminger Tauern (Austria)	Negleck above Solkpass	47.31 N/14.10 E	1900	
	M A 9	E Alps, Savinj Alpi (Slovenia)	Kamniška Bistrica	46.51 N/14.74 E	1600	
	M A 10	E Alps, Steirische Kalkalpen (Austria)	Turntaler-Kg	47.69 N/15.49 E	1600	
	M A 11	W Alps, Maritime Alps (Italy)	Coll de Tende	44.13 N/7.38 E	2000	
	M APP	Appenines (Italy)	Abruzzi, La Maiella	41.77 N/13.98 E	2200	
	M CAR 1	E Carpathians, Gorgany (Ukraine)	Kanch Mt near Synevir	48.55 N/23.83 E	1550	
	M CAR 2	E Carpathians, Chornokhora (Ukraine)	Breskulec	48.11 N/24.58 E	1650	
	M CAR 3	E Carpathians, Chornokhora (Ukraine)	Pozhyzhevska	48.09 N/24.66 E	1700	
	M CAR 4	E Carpathians, Rodna (Romania)	Borsa, Prislop,	47.57 N/24.80 E	1750	
	M CAR 5	E Carpathians, Gorgan (Ukraine)	Gorgany Nature Reserve	48.41 N/24.17 E	1300	
	M CAR 6	S Carpathians, Fagaraş (Romania)	Mt Negoiu	45.61 N/24.54 E	2050	
	M CAR 7	S Carpathians, Bucegi (Romania)	Piatra Arsa	45.43 N/25.45 E	2050	
	M DYN 1	Dynaric Alps (Bosnia and Herzegovina)	Belashnica Mt.	43.75 N/18.22 E	2100	
	M DYN 2	Dynaric Alps (Montenegro)	Durmitor Mts.	43.16 N/19.09 E	2100	
	M GM 4	Sudetes (Poland)	Giant Mts, Mały Staw	50.75 N/15.79 E	1150	Dzialuk et al. 2012; Boratyńska et al., 2014
	M GM 5	Sudetes (Poland)	Giant Mts, Czarny Kocioł	50.79 N/15.59 E	1200	Dzialuk et al. 2012; Boratyńska et al., 2014
	M GM 6	Sudetes (Poland)	Śnieżne Kotły	50.78 N/15.57 E	1250	Dzialuk et al. 2012; Boratyńska et al., 2014
	M LT	W Carpathians (Slovakia)	Lover Tatra, Lysa	48.98 N/19.59 E	1500	
	M RM 1	Pirin (Bulgaria)	Vikhren, above Bansko	41.77 N/23.42 E	2000	
	M RM 2	Rila (Bulgaria)	Ribni Ezera	42.09 N/23.44 E	1950	
	M RM 3	Rila (Bulgaria)	Chemerna above Semkovo)	42.07 N/23.50 E	2000	
	M TM 1	W Carpathians, Tatra (Poland)	Dolina Pięciu Stawów	49.22 N/20.05 E	1710	Dzialuk et al. 2012; Boratyńska et al., 2014
	M TM 2	W Carpathians, Tatra (Poland)	Grześ – Wołowiec	49.22 N/19.76 E	1670	Dzialuk et al. 2012; Boratyńska et al., 2014
	M VIT	Vitosha (Bulgaria)	Aleko Mt	42.57 N/23.27 E	1500	
uncinata	U A	W Alps (Italy)	Claviere	44.96 N/6.79 E	1800	Dzialuk et al. 2009
	U CEB	Cordillera Iberica (Spain)	Sierra Cebollera	42.00 N/2.64 W	2050	Dzialuk et al. 2009
	U GUD	Cordillera Iberica (Spain)	Sierra de Gúdar	40.39 N/0.61 W	2000	Dzialuk et al. 2009
	U MAS 1	Massif Central (France)	Col de la Croix de Morand,	45.60 N/2.86 E	1350	Dzialuk et al. 2009
	U MAS 2	Massif Central (France)	Margaride Mts, Bouviers	44.76 N/3.53 E	1400	Dzialuk et al. 2009
	U PYR 1	W Pyrenees (Spain)	Belagua	42.93 N/0.67 W	1700	Dzialuk et al. 2009
	U PYR 2	Central Pyrenees (Spain)	Benasque, Maladeta	42.69 N/0.66 E	2050	Dzialuk et al. 2009
	U PYR 3	Central Pyrenees (Andorra)	Vall de Ransol	42.63 N/1.60 E	2000	Dzialuk et al. 2009
	U PYR 5	E Pyrenees (Spain)	Vall de Núria	42.38 N/2.18 E	2150	Dzialuk et al. 2009
	U PYR 7	Central Pyrenees (Spain)	Port de la Bonaigua	42.63 N/1.02 E	2100	Dzialuk et al. 2009
uliginosa	UL BAT	Sudetes (Poland)	Stołowe Mts, Batorów Moor	50.46 N/16.38 E	750	
	UL TOP	Sudetes (Poland)	Bystrzyckie Mts, Topieliska	50.35 N/16.41 E	800	
	UL WEG	Silesian Lowland (Poland)	Węgliniec Nature Reserve	51.29 N/15.23 E	190	

Table 2 Description of the loci studied

Locus	Dye[a]	Primer conc. (nM)	Taxon[b]	Allele number	Range		Size[c]	
					min.	max.	allele	freq.
PCP87314	FAM	30	*mugo* s.s.	7	109	115	113	0.335
			uncinata	5	109	113	112	0.393
			uliginosa	6	109	114	111	0.264
PCP41131	FAM	200	*mugo* s.s.	7	138	144	142	0.557
			uncinata	8	138	148	142	0.617
			uliginosa	4	140	143	142	0.560
Pt26081	VIC	25	*mugo* s.s.	6	102	107	105	0.598
			uncinata	4	103	106	106	0.387
			uliginosa	4	103	106	105	0.440
Pt15169	VIC	60	*mugo* s.s.	7	119	125	122	0.599
			uncinata	5	120	124	122	0.563
			uliginosa	5	120	124	122	0.516
Pt36480	VIC	250	*mugo* s.s.	4	139	142	141	0.552
			uncinata	3	140	142	141	0.940
			uliginosa	6	140	148	141	0.538
Pt45002	VIC	60	*mugo* s.s.	11	159	169	164	0.517
			uncinata	7	161	168	164	0.733
			uliginosa	7	160	166	164	0.670
PCP102652	NED	40	*mugo* s.s.	6	108	115	112	0.986
			uncinata	3	111	113	112	0.993
			uliginosa	3	108	112	112	0.967
Pt71936	NED	75	*mugo* s.s.	12	138	151	148	0.347
			uncinata	11	136	150	148	0.233
			uliginosa	6	143	148	147	0.429
PCP1289	PET	25	*mugo* s.s.	6	101	106	102	0.679
			uncinata	8	101	110	102	0.737
			uliginosa	3	101	103	102	0.593
Pt30204	PET	100	*mugo* s.s.	9	138	147	143	0.480
			uncinata	8	141	148	143	0.520
			uliginosa	5	141	145	142	0.330

[a] forward primer

[b] number of individuals were 940 for *P. mugo* s.s., 300 for *P. uncinata* and 91 for *P.uliginosa*

[c] the size indicates the most common allele

Table 3 Haplotypic diversity and differentiation statistics estimated across populations of *Pinus mugo* s.l.

Location	Population	n	A_h	%PD	H_r	P_h	N_e	H_e	F_{ST}	$\#F_{ST}$	D^2_{sh}
P. mugo s.s.		**940**	**566**	**60.2**	**24.11**		**267.92**	**0.997**			**8.43**
Alps	M A 1	30	27	90.0	26.62	23	25.00	0.993	0.142	42	6.66
	M A 10	33	17	51.5	16.57	5	10.57	0.934	0.108	36	6.16
	M A 11	30	24	80.0	23.64	19	12.50	0.952	0.162	42	6.47
	M A 2	30	25	83.3	24.67	22	21.43	0.986	0.211	42	4.73
	M A 3	30	27	90.0	26.60	20	23.68	0.991	0.134	42	6.44
	M A 4	30	20	66.7	19.78	13	14.06	0.961	0.116	41	5.97
	M A 5	30	21	70.0	20.76	11	16.07	0.970	0.067	31	7.08
	M A 6	30	27	90.0	26.62	24	25.00	0.993	0.126	42	5.39
	M A 7	29	18	62.1	17.90	9	14.25	0.963	0.073	36	7.76
	M A 8	33	19	57.6	18.53	9	14.14	0.958	0.081	40	6.27
	M A 9	26	23	88.5	23.00	12	17.79	0.982	0.090	39	9.43
Dynaric Alps	M DYN 1	30	27	90.0	26.62	13	25.00	0.993	0.095	40	7.80
	M DYN 2	30	28	93.3	27.59	18	26.47	0.995	0.099	42	4.92
E Carpathians	M CAR 1	30	24	80.0	23.68	18	19.57	0.982	0.193	40	4.90
	M CAR 2	27	20	74.1	19.99	16	13.76	0.963	0.108	42	9.95
	M CAR 3	31	25	80.6	24.52	14	21.36	0.985	0.113	35	7.34
	M CAR 4	32	29	90.6	28.12	16	25.60	0.992	0.130	36	4.07
	M CAR 5	29	25	86.2	24.79	17	19.56	0.983	0.095	37	5.82
	M CAR 6	30	29	96.7	28.56	20	28.13	0.998	0.116	36	7.58
	M CAR 7	27	25	92.6	24.98	18	22.09	0.992	0.145	37	7.27
W Carpathians	M LT	30	28	93.3	27.59	20	26.47	0.995	0.079	42	10.10
	M TM 1	33	26	78.8	25.10	8	20.55	0.981	0.085	40	6.60
	M TM 2	33	25	75.8	24.11	12	17.29	0.972	0.078	37	6.16
Sudetes	M GM 4	31	21	67.7	20.64	6	14.79	0.963	0.064	32	5.52
	M GM 5	33	20	60.6	19.44	5	13.79	0.956	0.078	36	8.35
	M GM 6	32	21	65.6	20.54	5	15.06	0.964	0.068	30	5.92
Balkan Mts.	M RM 1	30	28	93.3	27.59	17	26.47	0.995	0.085	39	8.08
	M RM 2	30	18	60.0	17.76	8	7.03	0.887	0.115	42	5.17
	M RM 3	30	26	86.7	25.65	18	23.68	0.991	0.091	33	9.16
	M VIT	31	30	96.8	29.31	20	29.12	0.998	0.097	35	7.06
Apennines	M App	30	21	70.0	20.75	16	15.52	0.968	0.161	42	4.42
P. uncinata		**300**	**159**	**53.0**	**19.74**		**57.92**	**0.986**			**8.24**
Alps	U A	30	20	66.7	19.76	8	12.50	0.952	0.108	34	4.65
Cordillera Iber.	U CEB	30	25	83.3	24.65	24	20.46	0.984	0.150	42	12.67
	U GUD	30	13	43.3	12.84	7	5.11	0.832	0.240	43	7.57
Massiff Central	U MAS 1	30	26	86.7	25.62	11	20.46	0.984	0.107	35	6.29
	U MAS 2	30	30	100.0	29.53	26	30.00	1.000	0.129	42	12.46
Pyrenees	U PYR 1	30	22	73.3	21.75	7	18.00	0.977	0.116	37	5.71
	U PYR 2	30	23	76.7	22.70	4	16.07	0.970	0.101	35	5.37
	U PYR 3	30	25	83.3	24.67	11	21.43	0.986	0.101	36	5.24
	U PYR 5	30	22	73.3	21.75	6	17.31	0.975	0.140	38	3.21
	U PYR 7	30	22	73.3	21.73	9	15.52	0.968	0.142	38	3.73
P. uliginosa		**91**	**69**	**75.8**	**19.11**		**50.80**	**0.991**			**10.29**
Sudetes	UL BAT	30	17	56.7	16.84	6	10.98	0.940	0.108	41	5.79
	UL TOP	30	23	76.7	22.73	18	19.57	0.982	0.108	42	11.03
Silesian Lowl.	UL WEG	31	29	93.5	28.36	29	27.46	0.996	0.194	42	7.92
***P. mugo* s.l.**		**1331**	**757**	**56.9**	**24.28**	**14**	**19.11**	**0.997**			**8.98**
χ^2			0.49	0.55	0.53	2.75	0.42	0.38	4.24	2.57	2.34
P			0.78	0.75	0.76	0.25	0.81	0.83	0.12	0.26	0.31

n – a sample size; A_h – observed number of haplotypes; PD – proportion distinguishable; H_r – rarefaction standardized haplotype richness; P_h – number of private haplotypes, N_e – effective number of haplotypes; H_e – unbiased haplotype diversity; F_{ST} – mean pairwise F_{ST}; $\#F_{ST}$ – number of pairwise F_{ST} with P<0.05; D^2_{sh} – within population genetic distance between haplotypes; χ^2 and P –a Kruskal-Wallis test for the difference in genetic diversity parameters between species.

Table 4 Results of the analyses of molecular variance (AMOVA) assuming no population structuring, taxonomic and geographic grouping

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*F*-statistics
		No population structuring			
Among Pops	43	545.509	0.3380	12.06	F_{ST} = 0.121*
Within Pops	1287	3170.446	2.4634	87.94	
Among Pops	43	3026.137	2.0634	20.59	R_{ST} = 0.206*
Within Pops	1287	10244.478	7.9510	79.41	
		Taxonomic grouping			
Among Taxa	2	108.215	0.1465	5.08	F_{CT} = 0.051*
Among Pops	41	437.294	0.2712	9.41	F_{SC} = 0.099*
Within Pops	1287	3170.446	2.4634	85.5	F_{ST} = 0.144*
Among Taxa	2	434.261	0.5190	5.04	R_{CT} = 0.050*
Among Pops	41	2591.875	1.8267	17.72	R_{SC} = 0.187*
Within Pops	1287	10244.478	7.9510	77.24	R_{ST} = 0.228*
		Geographic grouping			
Among Regions	10	263.814	0.1575	5.58	F_{CT} = 0.056*
Among Pops	33	281.695	0.2009	7.12	F_{SC} = 0.075*
Within Pops	1287	3170.446	2.4634	87.30	F_{ST} = 0.127*
Among Regions	10	1439.538	0.8458	8.35	R_{CT} = 0.083*
Among Pops	33	1586.599	1.3269	13.10	R_{SC} = 0.143*
Within Pops	1287	10244.478	7.9510	78.56	R_{ST} = 0.214*

* P<0.001

Table 5 Results of the analyses of molecular variance (AMOVA) among pairs of taxa.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*F*-statistics
		P. mugo s.s. *vs P. uncinata*			
Among Taxa	1	94.866	0.1862	6.42	$F_{CT} = 0.064^{**}$
Among Pops	39	398.444	0.2566	8.85	$F_{SC} = 0.095^{**}$
Within Pops	1199	2943.966	2.4554	84.72	$F_{ST} = 0.153^{**}$
Among Taxa	1	359.792	0.6534	6.32	$R_{CT} = 0.063^{**}$
Among Pops	39	2452.185	1.8188	17.59	$R_{SC} = 0.188^{**}$
Within Pops	1199	9432.454	7.8670	76.09	$R_{ST} = 0.239^{**}$
		P. mugo s.s. *vs P. uliginosa*			
Among Taxa	1	16.745	0.0326	1.14	$F_{CT} = 0.011^{ns}$
Among Pops	32	362.714	0.2902	10.15	$F_{SC} = 0.103^{**}$
Within Pops	997	2528.246	2.5359	88.71	$F_{ST} = 0.113^{**}$
Among Taxa	1	66.742	0.0579	0.62	$R_{CT} = 0.063^{ns}$
Among Pops	32	1826.742	1.6329	17.62	$R_{SC} = 0.177^{**}$
Within Pops	997	7554.744	7.5775	81.76	$R_{ST} = 0.182^{**}$
		P. uncinata vs P. uliginosa			
Among Taxa	1	23.366	0.0931	3.50	$F_{CT} = 0.035^{*}$
Among Pops	11	113.430	0.2670	10.03	$F_{SC} = 0.104^{**}$
Within Pops	378	868.680	2.2981	86.47	$F_{ST} = 0.135^{**}$
Among Taxa	1	161.250	0.5622	4.59	$R_{CT} = 0.046^{*}$
Among Pops	11	904.824	2.4282	19.82	$R_{SC} = 0.208^{**}$
Within Pops	378	3501.757	9.2639	75.60	$R_{ST} = 0.244^{**}$

* $P < 0.05$
** $P < 0.001$
^{ns} values not significantly different from zero ($P \geq 0.05$)

Table 6. Matrix of pairwise F_{ST} between taxa of *P. mugo* complex.

Taxon	*P. mugo* s.s.	*P. uncinata*
P. uncinata	0.699	
P. uliginosa	0.029	0.558

Table 7 Significance test for the presence of the phylogeographical structure in *P. mugo* complex performed using SPAGeDi 1.3

Taxon	R_{ST}	pR_{ST}	*P*
P. mugo s.s.	0.178	0.090	0.0005
Alps	0.251	0.118	0.0020
Sudetes	-0.002	-0.004	0.7653
SE Carpathians	0.085	0.026	0.0003
W Carpathians	0.050	0.024	0.1597
Balkan Mts.	0.090	0.069	0.3966
P. uncinata	0.214	0.089	0.0086
P. uliginosa	0.178	0.132	0.2433
P. mugo complex	0.206	0.126	0.0012