

Geography determines genetic relationships between species of mountain pine (*Pinus mugo* complex) in western Europe

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ABSTRACT

Aim Our aims were to test whether morphological species of mountain pines were genetically supported in the western part of the distribution range of the *Pinus mugo* species complex (*Pinus mugo* Turra *sensu lato*), to resolve genetically homogeneous clusters of populations, to determine historical demographic processes, and to assess the potential hybridization of mountain pines with Scots pine, *Pinus sylvestris* L.

Location Populations were sampled in the Iberian System, the Pyrenees, the French Mont Ventoux, Vosges and Jura mountains, the German Black Forest and throughout the Alps. This corresponded to a range-wide sampling for mountain pine *sensu stricto* (*Pinus uncinata* Ram.) and to a sampling of the western parts of the ranges of dwarf mountain pine (*Pinus mugo* Turra *sensu stricto*) and bog pine/peatbog pine [*Pinus rotundata* Link/*Pinus* × *pseudopumilio* (Willk.) Beck].

Methods In total, 786 individuals of *P. mugo sensu lato* from 29 natural populations, and 85 individuals of *P. sylvestris* from four natural populations were genotyped at three chloroplast microsatellites (cpSSRs). Populations were characterized for standard genetic diversity statistics and signs of demographic expansion. Genetic structure was explored using analysis of molecular variance, differentiation statistics and Bayesian analysis of population structure (BAPS).

Results One hundred haplotypes were identified in *P. mugo sensu lato*. There was a stronger differentiation between geographical regions than between morphologically identified taxa (*P. mugo sensu stricto*, *P. uncinata* and *P. rotundata/P.* × *pseudopumilio*). Overall genetic differentiation was weak ($G_{ST} = 0.070$) and displayed a clear phylogeographic structure [$N_{ST} = 0.263$, $N_{ST} > N_{ST}$ (permuted), $P < 0.001$]. BAPS identified a Pyrenean and an Alpine gene pool, along with several smaller genetic clusters corresponding to peripheral populations.

Main conclusions The core regions of the Pyrenees and Alps were probably recolonized, respectively by *P. uncinata* and *P. uncinata/P. mugo sensu stricto*, from multiple glacial refugia that were well connected by pollen flow within the mountain chains. *Pinus rotundata/P.* × *pseudopumilio* populations from the Black Forest, Vosges and Jura mountains were probably recolonized from various glacial populations that kept their genetic distinctiveness despite late glacial and early Holocene expansion. Marginal *P. uncinata* populations from the Iberian System are compatible with elevational shifts and long-term isolation. The causes of haplotype sharing between *P. mugo sensu lato* and *P. sylvestris* require further research.

Keywords

Chloroplast microsatellites, conservation, genetic clusters, haplotype sharing, historical demography, hybridization, *Pinus mugo* complex, *Pinus sylvestris*, post-glacial recolonization, western Europe.

INTRODUCTION

The Pleistocene climate oscillations affected the distribution of genetic variation within plant species in complex ways (Petit *et al.*, 2003; Tribsch & Stuessy, 2003). During the cold stages, some cold-adapted tree species of the Northern Hemisphere were not restricted to southern glacial refugia but maintained fairly extensive populations close to the ice sheets (Tarasov *et al.*, 2000). These populations expanded during warm stages and experienced extensive gene flow (Maliouchenko *et al.*, 2007). As a consequence, woody species with boreal-temperate contemporary distributions show significantly lower genetic differentiation at maternally inherited markers than species that were restricted to southern glacial refugia (Petit *et al.*, 2003; Aguinalde *et al.*, 2005). At lower latitudes, in contrast, cold-adapted tree species commonly thrived in the mountain foothills during cold stages and migrated to higher elevations during warmer interglacials (Davis & Shaw, 2001; Robledo-Arnuncio *et al.*, 2005). Decreased gene flow and increased genetic drift may produce substantial differentiation among such interglacial high-mountain populations (Robledo-Arnuncio *et al.*, 2005), leading even to speciation events (Aguirre-Planter *et al.*, 2000; Jaramillo-Correa *et al.*, 2008).

Pinus mugo Turra *sensu lato* (s.l.), the mountain pine complex, is a very polymorphic species complex with a montane distribution in southern and central Europe, in which 16 species, 91 varieties and 19 forms had been described prior to the taxonomic revision of Christensen (1987a). The delimitation of taxa is difficult because of a high morphological variability in growth habit (single versus multi-stemmed, erect versus prostrate), cone and needle characters (Christensen, 1987a,b; Boratyńska & Boratyński, 2007, and references therein; Marcysiak & Boratyński, 2007), and possible hybridization with Scots pine, *Pinus sylvestris* L. (Christensen, 1987a,b; Wachowiak & Prus-Głowacki, 2008). Taxonomic resolution has also been impaired because many studies examined only small parts of the complex's distribution range or made unsuitable interpretations of phenotypic traits or taxon names (see Businský, 1999; Hamerník & Musil, 2007). At present, the two most complete revisions of the group differ in their conclusions: Christensen (1987a,b) recognized two subspecies, *Pinus mugo* Turra ssp. *mugo* and *P. mugo* Turra ssp. *uncinata* (Ram.) Domin, and one 'hybrid taxon', *P. mugo* Turra nothosp. *rotundata* (Link) Janchen & Neumayer, which he believed to have arisen from hybridization between the other two species. Businský (1999) listed *Pinus mugo* Turra, *Pinus uncinata* Ramond and *Pinus rotundata* Link as separate species. Hamerník & Musil (2007) provided comparative tables for the taxon names used in the literature to refer to specific morphologies and distribution ranges, and here we follow their nomenclature (see figures 2 and 3 in Hamerník & Musil, 2007, for growth habit and cone morphology, and figures 4 and 5 in Hamerník & Musil, 2007, for distribution ranges). *Pinus uncinata* Ramond, mountain pine *sensu stricto* (s.s.), is usually a monocormic (single-stemmed) tree with asymmetrical cones and grows in the subalpine vegetation belt up to the upper

forest limit, and even up to the upper tree limit (1000–2300 m elevation) in the western part of the range of the complex. It is found in the Iberian System (Sierra de Gúdar and Sierra de Cebollera) in Spain, the Pyrenees, the Massif Central, the Mont Ventoux and the western Alps (Cantegrel, 1983; Christensen, 1987a; Hamerník & Musil, 2007). It overlaps with *Pinus mugo* Turra s.s., dwarf mountain pine, in eastern Switzerland and western Austria, where transitive forms occur. The latter has a shrubby (polycormic) growth habit and symmetrical cones, and forms large mats in subalpine habitats of the eastern Alps, the Erzgebirge, the Dinaric Alps, the Carpathians and the Rila and Pirin mountains of Bulgaria, with an isolated population in the Italian Apennines and other outlier populations in the western Alps (Christensen, 1987a; Hamerník & Musil, 2007). *Pinus rotundata* Link, bog pine, is ecologically highly specialized to peat bog habitats and occurs in the northern part of the distribution range, from the German Black Forest in the west to the Polish Table Mountains in the east. It is generally monocormic and has weakly to strongly asymmetrical cones. A common synonym used mostly for eastern European bog pine is *Pinus uliginosa* Neumann (Businský, 1999; Hamerník & Musil, 2007), although recent work suggests that *P. rotundata* and *P. uliginosa* might be distinct species (Businský & Kirschner, 2006). Polycormic specimens of different heights (sometimes even prostrate) found mainly on raised bogs are thought to be of hybrid origin between *P. rotundata* and *P. mugo* s.s. and are classified as *Pinus* × *pseudopumilio* (Willk.) Beck, peatbog pine (Businský, 1999; Hamerník & Musil, 2007). *Pinus sylvestris* is closest to *P. uncinata* with respect to needle sclerenchyma characters (Boratyńska & Boratyński, 2007), whereas its cone morphology more closely resembles that of *P. mugo* s.s. (Christensen, 1987b; Marcysiak & Boratyński, 2007). Divergence within the *P. mugo* species complex is thought to have initiated in the Pliocene, when increased ice and snow cover in the Alps broke up a large distribution range, leading to separation into individual Pleistocene refugia (Sandoz, 1983; Christensen, 1987a).

The location of glacial populations of the *P. mugo* complex is poorly known to date because *P. mugo* s.l. pollen is very similar to that of the more common *P. sylvestris* and *Pinus nigra*, and therefore palynological records seldom distinguish these species (Willis *et al.*, 1998). Even macrofossil remains are often not sufficiently well preserved to enable unambiguous identification of *P. mugo* and *P. sylvestris* (García-Amorena *et al.*, 2007). In the Iberian Peninsula, fossil pollen indicates glacial populations of *P. sylvestris*/*P. mugo* in the Cantabrian Atlantic Mountains, in the Iberian System – including the Sierra de Cebollera, where *P. uncinata* grows today (Franco Múgica *et al.*, 1998; Ramil-Rego *et al.*, 1998; Gil García *et al.*, 2002), in eastern Spain (Carrión, 2002), and in the Betic System (Pons & Reille, 1988). Fossil *P. sylvestris*/*P. mugo* trunks dated from c. 20,000 to c. 34,000 yr BP indicate refugia on the Portuguese coast (García-Amorena *et al.*, 2007). Glacial refugia existed very probably in the Alps, where travertine formations dated to 11000–10000 yr BP unambiguously reveal cone imprints of *P. uncinata* (in the southern French Alps at

2200 m elevation, Ali *et al.*, 2003) and of *P. uncinata* and *P. mugo* s.s. (in the Susa Valley, Italy at c. 1300 and 1900 m elevation, Ali *et al.*, 2006). Fossil pollen records additionally suggest *P. sylvestris*/*P. mugo* presence up to c. 1400 m elevation in the Swiss central and southern Alps during the Oldest Dryas (15,000–13,000 yr BP, Burga, 1988). In eastern Europe, Feurdean *et al.* (2007) reported that *P. mugo* formed part of the late glacial (14,700 yr BP) vegetation at mid-altitude in the Carpathians, based on fossil pollen. Furthermore, *P. mugo* charcoal remains prove the glacial-period presence on several sites at latitudes as far north as the Czech Republic (48.87° N, Willis & van Andel, 2004). The fossil pollen studies clearly show a wider natural range and higher levels of *P. sylvestris*/*P. mugo* pollen representation than at present, suggesting an early range-wide post-glacial expansion starting about 13,000 yr BP (Burga, 1988; Ramil-Rego *et al.*, 1998; Rösch, 2000; Gil García *et al.*, 2002; Cheddadi *et al.*, 2006; Feurdean *et al.*, 2007). Milder temperatures than those of the Last Glacial Maximum (LGM, 18,000 yr BP) were common during the Weichselian glacial stage (110,000–10,000 yr BP, van Andel, 2002; Van Meerbeek *et al.*, 2009), so that numerous *P. sylvestris*/*P. mugo* populations could have been supported (see also West, 1980; Field *et al.*, 2000). Given that today *P. uncinata* and *P. mugo* s.s. are species of the subalpine vegetation, the interglacial retreat to high elevations may also have played an important role in shaping their genetic structure.

In the last few decades, *P. mugo* s.l. populations have started expanding into higher elevations in response to decreased grazing pressures and climate warming (Ozenda, 1988; Camarero *et al.*, 2005; Dirnböck *et al.*, 2008). Upslope migration is also observed in species that occur in the subalpine vegetation

belt below, and occasionally in sympatry with *P. mugo* s.l., such as *P. sylvestris* and the more shade-tolerant *Picea abies* (Ozenda, 1988; Camarero *et al.*, 2005). The upward pressure by *P. sylvestris* is especially important in marginal populations of *P. uncinata* that have already reached the mountain tops of low mountains in Spain (Camarero *et al.*, 2005). *Pinus sylvestris* and *P. mugo* s.l. are partially interfertile (Schmid & Bogenrieder, 1998; Wachowiak *et al.*, 2005), although the natural extent of introgression between them appears to be low (Christensen, 1987b). The sub-optimal growth conditions of the marginal *P. uncinata* populations and the competition and potential hybridization with *P. sylvestris* may represent a significant threat to their conservation. Furthermore, if marginal *P. uncinata* populations are genetically distinct from core populations, their loss may represent a significant diminution of diversity and adaptive potential for the species (Hampe & Petit, 2005; Eckert *et al.*, 2008).

In this paper we investigate the distribution of genetic diversity in *P. uncinata* and its genetic relationships with *P. rotundata*/*P. × pseudopumilio* and *P. mugo* s.s. The geographical pattern of genetic diversity has recently been described for *P. uncinata* (Dzialuk *et al.*, 2009), and a lack of genetic differentiation between *P. uncinata* and *P. mugo* s.s. has been observed in the Alps (Monteleone *et al.*, 2006). However, the phylogeographic history of the species complex has so far not been investigated at a larger geographical scale. We genotyped 29 populations of mountain pine morphologically classified as *P. mugo* s.s., *P. uncinata* or *P. rotundata*/*P. × pseudopumilio*, using three (out of 18 tested) hypervariable chloroplast DNA microsatellites (cpSSRs) that resolved 100 haplotypes. In particular, we (1) tested whether morpho-

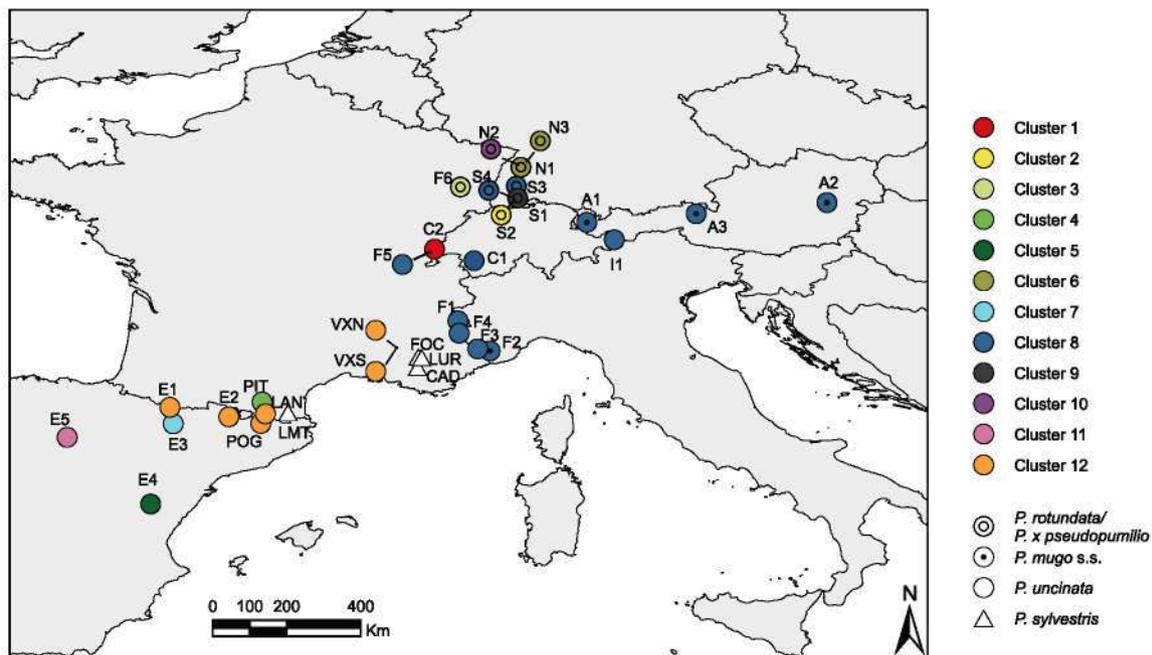


Figure 1 Results of the spatial Bayesian cluster analysis, BAPS, showing genetically homogenous groups of populations of the *Pinus mugo* species complex in western Europe.

logically identified species were genetically differentiated, (2) checked for the presence of genetically homogeneous clusters of populations, and (3) investigated historical demographic processes operating in populations. We also genotyped four populations of *P. sylvestris* sampled in the vicinity of *P. uncinata*, aiming to evaluate *in situ* hybridization.

MATERIALS AND METHODS

Plant material and cpSSR genotyping

A total of 786 individual trees were sampled from 29 natural populations of *P. mugo* s.l. located in the Iberian System, the Pyrenees, the French Mont Ventoux, Vosges and Jura mountains, the German Black Forest and throughout the Alps (Fig. 1, Tables 1 & 2). Populations were morphologically classified as *P. uncinata*, *P. mugo* s.s. or *P. rotundata*/*P. × pseudopumilio*. Bog-affected populations of the northern Black Forest are generally polycormic (*P. × pseudopumilio*),

whereas those of the southern Black Forest are generally monocormic (*P. rotundata*), but they were treated here as the same species category as they have similar ecology, occur in geographical proximity and their taxonomy is very uncertain. Our sample represents a range-wide sample for *P. uncinata*, but covers only the western part of the distribution of *P. mugo* s.s. and of *P. rotundata*/*P. × pseudopumilio*. Populations from the Alps and Pyrenees are relatively extensive and can be considered core populations, whereas the peripheral populations grow in more fragmented parts of the range.

Samples consisted of silica-dried needles or seeds; the latter were germinated before DNA extraction from the embryo. Because we suspected introgression of chloroplast DNA (cpDNA) haplotypes through hybridization with *P. sylvestris*, 85 individuals of *P. sylvestris* from four populations were also sampled at a distance of < 60 km from two *P. mugo* s.l. populations (Fig. 1, Tables 1 & 2). DNA was extracted using the DNeasy™ Plant Mini Kit (Qiagen, Hilden, Germany) and amplified separately at three paternally inherited cpSSRs

Table 1 Characteristics of sampled populations of the *Pinus mugo* species complex in western Europe. Latitude and longitude are given in decimal degrees.

Species	ID	Locality	Latitude	Longitude	Region	Site	Elevation (m)
<i>P. mugo</i> s.s.	F2	Mont Ourne	N 44.10	E 7.53	Alpes Maritimes (France)	Mineral soil	2100–2200
<i>P. mugo</i> s.s.	A1	Kellaspitze	N 47.22	E 9.88	Lechtaler Alpen (Austria)	Mineral soil	1900–2000
<i>P. mugo</i> s.s.	A3	Wildseeloder	N 47.43	E 12.54	Kitzbühler Alpen (Austria)	Mineral soil	1700–1800
<i>P. mugo</i> s.s.	A2	Raxalpe	N 47.70	E 15.72	Steiermark (Austria)	Mineral soil	1700–1900
<i>P. rotundata</i>	F6	Bas Beillard	N 48.08	E 6.81	Vosges (France)	Bog	600
<i>P. rotundata</i>	S2	Ibacher Moor	N 47.72	E 8.07	Southern Black Forest (Germany)	Bog	910
<i>P. rotundata</i>	S4	Rotmeer	N 47.87	E 8.10	Southern Black Forest (Germany)	Bog	965
<i>P. rotundata</i>	S3	Briglirain	N 48.10	E 8.17	Southern Black Forest (Germany)	Bog	990
<i>P. rotundata</i>	S1	Steerenmoos	N 47.81	E 8.20	Southern Black Forest (Germany)	Bog	1000
<i>P. × pseudopumilio</i>	N1	Grosses Muhr	N 48.62	E 8.21	Northern Black Forest (Germany)	Bog	1060
<i>P. × pseudopumilio</i>	N2	Saumisse	N 48.56	E 8.28	Northern Black Forest (Germany)	Bog	895
<i>P. × pseudopumilio</i>	N3	Hohlhohsee	N 48.70	E 8.42	Northern Black Forest (Germany)	Bog	981
<i>P. uncinata</i>	E5	Vinuesa	N 42.00	W 2.75	Sierra de Cebollera, Soria (Spain)	Mineral soil	1900–2100
<i>P. uncinata</i>	E4	Alcalá de la Selva	N 40.38	W 0.72	Sierra de Gúdar, Teruel (Spain)	Mineral soil	1800–2000
<i>P. uncinata</i>	E1	Panticosa	N 42.73	W 0.25	Pirineos Centrales (Spain)	Mineral soil	1600–2200
<i>P. uncinata</i>	E3	Sierra de Guara	N 42.33	W 0.17	Prepirineos (Spain)	Mineral soil	1800–2000
<i>P. uncinata</i>	E2	Llavorsi	N 42.50	E 1.18	Pirineos Orientales (Spain)	Mineral soil	1600–2200
<i>P. uncinata</i>	POG	La Molina	N 42.34	E 1.96	Pirineos Orientales (Spain)	Mineral soil	1600–2200
<i>P. uncinata</i>	PIT	Pinet	N 42.86	E 1.99	Pyrénées orientales (France)	Bog	800
<i>P. uncinata</i>	LAN	Les Angles	N 42.57	E 2.07	Pyrénées orientales (France)	Mineral soil	1800
<i>P. uncinata</i>	VXN	Ventoux sud	N 44.17	E 5.25	Provence (France)	Mineral soil	1700
<i>P. uncinata</i>	VXS	Ventoux nord	N 44.18	E 5.27	Provence (France)	Mineral soil	1500
<i>P. uncinata</i>	F5	Les Rousses	N 46.50	E 6.08	Jura Mountains (France)	Bog	1060
<i>P. uncinata</i>	C2	Vallée de Joux	N 46.57	E 6.18	Jura Mountains (Switzerland)	Bog	1040
<i>P. uncinata</i>	F1	Le Blétonnet	N 44.83	E 6.75	Hautes Alpes (France)	Mineral soil	2100–2200
<i>P. uncinata</i>	F4	Pinatelle	N 44.53	E 6.78	Alpes de Haute-Provence (France)	Mineral soil	1700–2000
<i>P. uncinata</i>	C1	Solalex	N 46.29	E 7.14	Berner Alpen (Switzerland)	Mineral soil	1670–1810
<i>P. uncinata</i>	F3	Col de Salèse	N 44.15	E 7.23	Alpes Maritimes (France)	Mineral soil	1800–2000
<i>P. uncinata</i>	I1	Endkopf	N 46.80	E 10.55	Ötztaler Alpen (Italy)	Mineral soil	1900–2000
<i>P. sylvestris</i>	LMT	La Matte	N 42.60	E 2.62	Pyrénées orientales (France)	Mineral soil	1550
<i>P. sylvestris</i>	CAD	Cadarache	N 43.70	E 5.77	Provence (France)	Mineral soil	300
<i>P. sylvestris</i>	FOC	Forcalquier	N 43.95	E 5.78	Provence (France)	Mineral soil	500
<i>P. sylvestris</i>	LUR	Lure	N 43.93	E 5.87	Provence (France)	Mineral soil	450

Table 2 Diversity and differentiation statistics of the *Pinus mugo* species complex in western Europe. BAPS group: cluster assigned in the Bayesian analysis of population structure (see text); n : sample size; A_R (13): allelic richness based on a sample size of $n = 13$; H_E : haplotypic diversity; mean pairwise F_{ST} : average differentiation between the focal population and all other populations measured with F_{ST} ; no. pairwise F_{ST} with $P < 0.05$: number of pairwise differentiation tests significant at a level of 0.05 after Bonferroni correction; $\bar{D}_{sh}^2(i, j)$: genetic distance between haplotypes within populations (see text for definition).

	ID	Population	Species	BAPS group*	n	No. haplotypes	No. private haplotypes	A_R (13)	H_E	Mean pairwise F_{ST}	No. pairwise F_{ST} with $P < 0.05$	$\bar{D}_{sh}^2(i, j)$
Core populations												
Alps												
	A2	Steiermark (Austria)	<i>P. mugo s.s.</i>	A	24	13	0	7.57	0.913	0.040	9	0.92
	A1	Lechtaler Alpen (Austria)	<i>P. mugo s.s.</i>	A	24	16	1	9.01	0.946	0.027	7	2.82
	A3	Kitzbühler Alpen (Austria)	<i>P. mugo s.s.</i>	A	24	14	1	8.24	0.931	0.038	6	1.98
	I1	Ötztaler Alpen (Italy)	<i>P. uncinata</i>	A	24	12	0	7.77	0.931	0.047	12	1.04
	C1	Berner Alpen (Switzerland)	<i>P. uncinata</i>	A	24	15	2	9.03	0.957	0.039	8	2.35
	F1	Hautes Alpes (France)	<i>P. uncinata</i>	A	24	14	0	8.08	0.909	0.050	7	1.43
	F2	Alpes Maritimes (France)	<i>P. mugo s.s.</i>	A	24	15	3	8.97	0.953	0.052	11	1.51
	F3	Alpes Maritimes (France)	<i>P. uncinata</i>	A	48	27	4	9.76	0.966	0.036	6	5.39
	F4	Alpes de Haute-Provence (France)	<i>P. uncinata</i>	A	24	12	1	6.85	0.851	0.064	8	0.89
Pyrenees												
	E1	Pirineos Centrales (Spain)	<i>P. uncinata</i>	P	21	12	2	7.73	0.919	0.036	6	4.93
	E2	Pirineos Orientales (Spain)	<i>P. uncinata</i>	P	22	8	0	5.75	0.879	0.059	11	1.91
	POG	Pirineos Orientales (Spain)	<i>P. uncinata</i>	P	24	11	0	7.27	0.924	0.038	10	0.99
	LAN	Pyrénées Orientales (France)	<i>P. uncinata</i>	P	28	13	2	7.31	0.915	0.040	11	1.13
Mont Ventoux												
	VXN	Provence (France)	<i>P. uncinata</i>	A/P	17	10	1	7.49	0.919	0.060	8	4.78
	VXS	Provence (France)	<i>P. uncinata</i>	A/P	13	10	3	9.00	0.962	0.036	7	19.1
Peripheral populations												
Black Forest												
	S1	Southern Black Forest (Germany)	<i>P. rotundata</i>		48	25	7	9.39	0.959	0.061	24	2.21
	S2	Southern Black Forest (Germany)	<i>P. rotundata</i>		24	6	0	4.23	0.808	0.084	14	0.49
	S3	Southern Black Forest (Germany)	<i>P. rotundata</i>	A	24	13	2	8.40	0.946	0.048	9	4.92
	S4	Southern Black Forest (Germany)	<i>P. rotundata</i>	A	24	10	1	6.57	0.895	0.074	17	0.93
	N1	Northern Black Forest (Germany)	<i>P. × pseudopumilio</i>	NBF	24	12	4	7.49	0.913	0.087	27	1.48
	N2	Northern Black Forest (Germany)	<i>P. × pseudopumilio</i>		24	8	0	4.77	0.786	0.108	20	0.85
	N3	Northern Black Forest (Germany)	<i>P. × pseudopumilio</i>	NBF	24	11	0	7.25	0.917	0.054	10	1.57
Jura Mountains												
	F5	Jura Mountains (France)	<i>P. uncinata</i>	A	24	10	0	6.34	0.862	0.102	23	0.79
	C2	Jura Mountains (Switzerland)	<i>P. uncinata</i>		24	17	3	9.74	0.967	0.057	16	2.53
Vosges												
	F6	Vosges (France)	<i>P. rotundata</i>		24	10	1	6.03	0.815	0.085	14	2.07
Pyrenees												
	E3	Prepirineos (Spain)	<i>P. uncinata</i>		23	9	3	5.46	0.810	0.125	25	1.47
	PIT	Pyrénées Orientales (France)	<i>P. uncinata</i>		91	8	2	3.43	0.758	0.123	28	2.06
Iberian System												
	E4	Sierra de Gúdar (Spain)	<i>P. uncinata</i>		21	5	1	3.44	0.748	0.152	24	5.56
	E5	Sierra de Cebollera (Spain)	<i>P. uncinata</i>		22	7	6	5.20	0.866	0.115	28	5.86
All <i>P. mugo</i> s.l.												
			<i>P. mugo</i> s.l.		786	100		10.44	0.956			
All <i>P. sylvestris</i>												
	LMT	Pyrénées orientales (France)	<i>P. sylvestris</i>		33	18	7	8.53	0.939	0.022	1	1.61
	CAD	Provence (France)	<i>P. sylvestris</i>		17	6	0	4.67	0.846	0.016	0	0.60
	FOC	Provence (France)	<i>P. sylvestris</i>		18	7	1	5.30	0.863	0.023	1	1.03
	LUR	Provence (France)	<i>P. sylvestris</i>		17	14	2	10.13	0.971	-0.007	0	1.90

*A, Alpine cluster (cluster 8 in Fig. 1); P, Pyrenean cluster (cluster 12 in Fig. 1); NBF, northern Black Forest cluster (cluster 6 in Fig. 1).

(Mogensen, 1996): Pt15169, Pt41093 and Pt71936 originally isolated from *Pinus thumbergii* (Vendramin *et al.*, 1996). These loci were chosen for their high polymorphism and non-overlapping product sizes out of 18 loci tested (Schmid, 2000; Appendix S1 in Supporting Information). The 25- μ L mix for polymerase chain reaction (PCR) contained 0.2 mM of each dNTP, 2.5 mM MgCl₂, 0.2 mM of each primer (the forward primer being labelled with a fluorescent dye), 10 \times reaction buffer (GE Healthcare, Waukesha, WI, USA), 25 ng of DNA and 1 unit *Taq* polymerase (GE Healthcare). PCR conditions were 5 min at 95 °C, 25 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, with a final step of 8 min at 72 °C. Amplification products of the three loci were mixed, separated on an ALF sequencer (GE Healthcare) and sized by comparison with internal size standards of 50, 100 and 200 bp using the software FRAGMENT MANAGER ver. 1.2 (GE Healthcare).

Genetic diversity analysis

In order to characterize polymorphism in each population, we recorded the number of haplotypes and the number of population-specific (i.e. private or endemic) haplotypes, and we estimated rarefied haplotypic richness, which is the number of haplotypes expected in each population for a standardized sample size (13 individuals in our case), using the program RAREFAC (Petit *et al.*, 1998). The program SPAGEDi (Hardy & Vekemans, 2002) was used to compute haplotypic diversity, H , corrected for small sample size. In order to estimate divergence between haplotypes within populations, we computed the average distance $\bar{D}_{\text{sh}}^2(i, j)$ between all pairs of individuals i and j , defining the distance between the haplotypes carried by i and j according to a microsatellite stepwise mutation model (Echt *et al.*, 1998; Vendramin *et al.*, 1998):

$$D_{\text{sh}}^2(i, j) = K^{-1} \left[\sum_{k=1}^K |a_{ik} - a_{jk}| \right]^2,$$

where a_{ik} and a_{jk} are the allele sizes of the haplotypes carried by i and j at the k th microsatellite region, and $K = 3$ is the number of microsatellite regions analysed. This distance is based on the Goldstein *et al.* (1995) distance, but treats the non-recombinant chloroplast genome as a single locus (Echt *et al.*, 1998; Vendramin *et al.*, 1998).

Genetic structure analyses

To evaluate the strength of differentiation of each population from all others, we report the number of significant exact tests of pairwise population differentiation based on haplotype frequencies (F_{ST}). Exact tests were computed in ARLEQUIN ver. 3.1 (Excoffier *et al.*, 2005), and a sequential Bonferroni correction (Rice, 1989) was applied. A hierarchical analysis of molecular variance (AMOVA) of haplotype frequencies, implemented in ARLEQUIN ver. 3.1, was used to assess (1) differentiation among species and populations within species, and (2) differentiation among geographical regions (Iberian

System, Pyrenees, Alps, and all populations north of the Alps) and among populations within regions.

SPAGEDi was used to compute overall among-population differentiation based on unordered (G_{ST}) or ordered (N_{ST}) alleles. For the estimation of N_{ST} , a distance matrix between all pairs of haplotypes was computed, defining distances between haplotypes as above. The significance of the two differentiation statistics was tested with 10,000 permutations of individuals among populations. Phylogeographic structure, namely whether phylogenetically close haplotypes are found together in the same population more often than randomly chosen ones (Pons & Petit, 1996), was investigated by comparing N_{ST} with the distribution of N_{ST} in 10,000 permutations of haplotype distances among pairs of haplotypes (Hardy *et al.*, 2003).

The overall geographical structure of genetic diversity was analysed using Bayesian analysis of population structure (BAPS, implemented in the program BAPS ver. 5.1, Corander *et al.*, 2003). We applied both a non-spatial and a spatial genetic mixture analysis (Corander *et al.*, 2008) to groups (sampled populations) of haplotypes. These methods use a Markov chain Monte Carlo simulation approach to group population samples into variable user-defined numbers K of clusters. The best partition of populations into K clusters is identified as the one with the highest marginal log-likelihood. We carried out 10 repetitions of the algorithm for each K ranging between 1 and 29. Finally, to test for isolation by distance in the whole dataset and various subsets (see Results), Mantel tests were conducted between the matrices of pairwise genetic differentiation [$F_{\text{ST}}/(1 - F_{\text{ST}})$] and of the logarithm of pairwise geographical distances among populations (Rousset, 1997).

Demographic inferences from genetic data

We applied a series of analyses to identify signs of population expansion and date them in the *P. mugo* s.l. dataset, excluding potentially introgressed haplotypes. In an expanding population, the genealogy of a random sample of gene sequences is star-shaped, characterized by short internodes and long terminal branches (Slatkin & Hudson, 1991). New mutations on these branches result in a relative abundance of rare alleles or haplotypes, compared with populations of stationary size. Fu's F_S -test statistic is sensitive to such an excess of rare haplotypes and takes a large negative value in expanding populations (Fu, 1997). We used ARLEQUIN ver. 3.1 to test for the significance of Fu's F_S . For this, we coded cpSSR data in a binary way, representing for each locus the number of repeats of the largest variant with '1's and replacing the absent repeats of shorter variants with '0's. Owing to a particular behaviour of the F_S -statistic, a test with $P < 0.02$ was considered evidence for population expansion at the significance level of $\alpha = 0.05$ (Fu, 1997).

We also examined the shape of the distribution of pairwise size differences between haplotypes within populations. We refer to this as a 'mismatch distribution', as is common practice in the literature (Harpending *et al.*, 1993), although

size differences do not strictly correspond to nucleotide mismatches. Mismatch distributions in expanding populations are typically monomodal (Slatkin & Hudson, 1991), whereas they are ragged and multimodal in populations with a stationary size. From the mismatch distribution, the population growth parameters $\tau = 2\mu t$ (expansion time), $\Theta_0 = 2\mu N_0$ (initial population size scaled by mutation rate) and $\Theta_1 = 2\mu N_1$ (present population size scaled by mutation rate), where μ is the mutation rate, t the number of generations since population expansion and N_0 and N_1 the population sizes before and after expansion (Rogers & Harpending, 1992), can be estimated following the procedure of Schneider & Excoffier (1999). Navascués *et al.* (2006) have shown that the accuracy of parameter estimates decreases if there is homoplasy in the data, as is common for cpSSRs (Provan *et al.*, 2001). We used their new maximum pseudo-likelihood estimation procedure for population growth parameters that takes homoplasy into account, implemented in the program LMSE (Navascués *et al.*, 2009).

RESULTS

Levels and structure of genetic diversity

The numbers of size variants identified at the loci Pt15169, Pt41093 and Pt71936 in *P. mugo* s.l. were 9, 14 and 8, respectively. They combined into 100 haplotypes (see Table S1), resulting in a high total haplotypic diversity of $H_E = 0.96$ (Table 2). In the four *P. sylvestris* populations, the same loci displayed 5, 4 and 7 size variants, which resolved 23 haplotypes (see Table S2). The variant size ranges were roughly the same in both species for locus Pt71936. For locus Pt15169, *P. sylvestris* carried mostly variants [127, 128 and 129 base pairs (bp), range 126–130 bp] that corresponded to the upper part of the variant size range found in *P. mugo* s.l. (range: 119–129 bp). For locus Pt41093, the common variants in *P. sylvestris* (77 and 78 bp, range: 76–79 bp) corresponded to the shorter variants of *P. mugo* s.l. (range: 77–92 bp). Eight *P. mugo* s.l. individuals displayed a total of seven haplotypes characterized by a long variant at Pt15169 and a short variant at Pt41093, indicating that they might be introgressed from *P. sylvestris*. Four of these haplotypes were indeed shared between the two species; they occurred in *P. mugo* s.l. populations VXS (haplotypes 84, 88 and 100), F3 (haplotype 88) and PIT (haplotype 94). The shared haplotypes of the VXS population from southern Mont Ventoux occurred in the *P. sylvestris* populations CAD, FOC and LUR, which are all within 60 km of VXS, but not in the *P. sylvestris* population from the Pyrenees. The three other suspected introgressed haplotypes occurred in population VXS (haplotype 97), and in the two Spanish populations E1 (haplotype 99) and E4 (haplotype 95), where *P. uncinata* and *P. sylvestris* grow sympatrically. The *P. mugo* s.l. populations with (suspected) introgressed haplotypes were generally among those with the highest divergence between haplotypes [$\bar{D}_{\text{sh}}^2(i, j)$ around 5 or higher, Table 2].

The *P. mugo* s.l. populations with the lowest diversity, displaying an allelic richness of 5.5 haplotypes or fewer in a random sample of 13 individuals (Table 2), were peripheral: E3–E5 in Spain, PIT in southern France, and N2 and S2 from the Black Forest. Together with additional populations from the Black Forest, these populations also showed the highest number of significant pairwise differentiation tests (Table 2). Conversely, the most polymorphic populations, displaying an allelic richness of over eight haplotypes, belonged mostly to the Alpine range of *P. mugo* s.l., and these populations were also among the least differentiated.

In a hierarchical AMOVA framework, species belonging to *P. mugo* s.l. were not significantly differentiated from each other in the sampled range ($F_{\text{CT}} = -0.004$, $P > 0.05$), but populations were significantly differentiated within species ($F_{\text{SC}} = 0.078$, $P < 0.001$). Geographical regions were significantly differentiated ($F_{\text{CT}} = 0.013$, $P < 0.05$), as were populations within regions ($F_{\text{SC}} = 0.069$, $P < 0.001$). These results indicate that geography is a stronger determinant of genetic structure at cpSSRs in the sampled range of *P. mugo* s.l. than taxonomy (see also below). In the overall dataset, among-population differentiation was $F_{\text{ST}} = 0.076$ and $G_{\text{ST}} = 0.070$ based on unordered alleles, and $N_{\text{ST}} = 0.263$ based on ordered alleles (all three statistics significant with $P < 0.001$). The test for phylogeographic structure was significant [$N_{\text{ST}} > N_{\text{ST}}$ (permuted), $P < 0.001$].

We applied a Bayesian analysis of population structure to characterize the overall structure of genetic diversity in *P. mugo* s.l. Results were similar using either a non-spatial or a spatial model for genetic mixture analysis; the best partition contained 14 or 12 clusters, respectively. A large cluster of Alpine populations and a smaller one of Pyrenean populations were identified (clusters 8 and 12 for the spatial model, Fig. 1), whereas most other populations were found in single-population clusters. In the spatial model, the Mont Ventoux populations clustered within the Pyrenean cluster. In the non-spatial model, the eastern Pyrenean populations LAN and POG and the southern Mont Ventoux population VXS clustered within the Alpine cluster, indicating some haplotype sharing with the Alpine range. Differentiation analysis was also carried out in the Alpine and Pyrenean clusters defined from the spatial BAPS analysis (Fig. 1). Populations from the Pyrenean cluster were not significantly differentiated based on unordered alleles ($G_{\text{ST}} = 0.005$, $P > 0.05$), although they were using ordered alleles ($N_{\text{ST}} = 0.139$, $P < 0.001$), essentially as a result of the features of the above-mentioned VXS population. Differentiation in the Alpine cluster was weak but significant [$G_{\text{ST}} = 0.036$, $P < 0.001$; $N_{\text{ST}} = 0.130$, $P < 0.001$; $N_{\text{ST}} > N_{\text{ST}}$ (permuted), $P = 0.001$]. Populations of *P. mugo* s.s. were not significantly differentiated from populations of *P. uncinata* within the Alps ($F_{\text{CT}} = -0.003$, $P > 0.05$), but populations within both species were significantly differentiated ($F_{\text{SC}} = 0.020$, $P < 0.001$). It is also worth noting that the seven populations within the Black Forest belonged to five distinct clusters (Fig. 1); hence they were strongly differentiated despite the short spatial distance separating them

[$G_{ST} = 0.085$, $N_{ST} = 0.337$, $N_{ST} > N_{ST}$ (permuted), all $P < 0.001$]. Tests for isolation by distance were marginally significant in the overall dataset ($P = 0.088$) but non-significant in the Pyrenean and in the Alpine BAPS clusters and in the Black Forest.

Demographic inferences from genetic data

Fu's F_S -test for population expansion was significant in most core populations, but in only a few peripheral populations (Table 3). Mismatch distributions were monomodal or slightly wavy for Alpine and Pyrenean populations, but very irregular for most peripheral populations, in particular for those with low genetic diversity (Fig. 2). This suggests that core and peripheral populations of *P. mugo* s.l. have had different demographic histories. In the Alps, roughly two types of populations could be distinguished: those with strictly monomodal mismatch distributions (I1, A2, F4, and to a lesser extent F1 and F2), and those with wavy, bimodal distributions (A1, A3, C1, F3). The latter were able to maintain a lineage with short variants (81–83 bp) at locus Pt41093 and might represent old populations. The Pyrenean populations' mismatch distributions were all wavy, and, again, this pattern was caused by the geographical distribution of a short variant (85 bp) at locus Pt41093 (Fig. 2). Values of the time of expansion τ varied about 3-fold in Alpine and Pyrenean populations but oscillated around the same values, $1.9 < \tau < 5.5$ (Table 3), which suggests similar expansion times in these two ranges. Interpretations in terms of years since expansion are subject to the imprecision with which mutation rates are known for cpSSRs in pines. If a low mutation rate of $\mu = 10^{-5}$ (Provan *et al.*, 1999) and a long generation time of 100 years (as in Navascués *et al.*, 2006) are assumed for the average $\tau = 3.56$, the Alpine/Pyrenean expansion could be as old as 18×10^6 years. A high mutation rate ($\mu = 10^{-3}$), suggested by the high diversity found in cpSSRs of European pine species (e.g. Robledo-Arnuncio *et al.*, 2005), and a short generation time of 25 years (as in Brown *et al.*, 2004) would still place the expansion at 44,500 years ago. Hence, the genetic signs of expansion resulted from demographic events that pre-dated the LGM. Our results also suggest that the current effective population size of *P. mugo* in core populations ($1.3 \times 10^5 < \Theta_1 < 2.1 \times 10^{13}$) was larger than that in expanding peripheral populations ($36.5 < \Theta_1 < 7.6 \times 10^5$).

DISCUSSION

Absence of species differentiation in *Pinus mugo* s.l.

The high morphological variation and adaptation to different ecological conditions in the *P. mugo* complex gave rise to the delimitation of the species *P. uncinata*, *P. mugo* s.s. and *P. rotundata* (Businský, 1999), which have numerous synonyms (Hamerník & Musil, 2007). Genetic variation at neutral cpSSRs was high in *P. mugo* s.l., $H_E = 0.96$, similar to or higher

than in other conifers in Central Europe or the Mediterranean Basin (Gómez *et al.*, 2005; Robledo-Arnuncio *et al.*, 2005; Terrab *et al.*, 2006). Genetic variation was, however, not structured according to morphology, but according to geography. In particular, Alpine populations of *P. uncinata* and *P. mugo* s.s. belonged to a nearly homogeneous gene pool at cpSSR markers, indicating a common history and homogenizing gene flow. This result is in agreement with the absence of differentiation between 15 Alpine populations of *P. uncinata* and *P. mugo* s.s. at nuclear random amplified polymorphic DNA markers (Monteleone *et al.*, 2006), and with a smaller allozyme differentiation between Alpine *P. uncinata* and *P. mugo* s.s. than among *P. rotundata* populations from the Black Forest (Schmid, 2000). Moreover, Lewandowski *et al.* (2000) identified only weak allozyme differentiation between populations belonging to different taxa from the *P. mugo* complex. These results suggest that different growth forms and ecological adaptations evolved in different parts of the distribution range of the complex and were maintained despite the existence of extensive gene flow (Sambatti & Rice, 2006; Savolainen *et al.*, 2007). The evolution of local adaptations and the ensuing high taxonomic diversity paralleled with low genetic differentiation at neutral markers seem to be a common pattern in pines (e.g. Barbéro *et al.*, 1998; Savolainen *et al.*, 2007). For instance, *Flora Europaea* lists 26 varieties for *P. sylvestris* (Gausson *et al.*, 1993) and differentiation is strong for quantitative traits (García-Gil *et al.*, 2003), but cpSSR and allozyme differentiation are weak over large parts of the range (both $c. 7\%$; Cheddadi *et al.*, 2006; Wang *et al.*, 2008). Similarly, in maritime pine, *Pinus pinaster* Ait., at least three subspecies are recognized (Barbéro *et al.*, 1998), and quantitative traits are much more strongly differentiated than allozymes (González-Martínez *et al.*, 2002) or cpSSRs (Bucci *et al.*, 2007). These observations suggest that adaptations in pines probably rest on a few genes while the majority of the genome shows only weak differentiation and reflects essentially demographic history (Scotti-Saintagne *et al.*, 2004; González-Martínez *et al.*, 2008).

Phylogeographic structure

The pattern of differentiation we identified in the surveyed range of *P. mugo* s.l. was weak ($G_{ST} = 0.07$), but had a clear phylogeographic structure [$N_{ST} = 0.263$, $N_{ST} > N_{ST}$ (permuted), $P < 0.001$], with vicariant gene pools in the Pyrenees and Alps and several smaller genetic clusters corresponding mostly to marginal populations. This differentiation pattern suggests that different historical and possibly adaptation processes affected central versus peripheral populations of *P. mugo* s.l. in the studied range. Our results are completely congruent with a recently published phylogeographic study of *P. uncinata*, which also reports a homogeneous Pyrenean gene pool and strongly differentiated populations in the Iberian System (Dzialuk *et al.*, 2009). Although macrofossil data provided unambiguous support for glacial refugia of *P. mugo* s.l. only in the Alps and in the Czech Republic, differentiated

Table 3 Tests for population expansion and estimation of demographic parameters in populations of the *Pinus mugo* species complex in western Europe. BAPS group: cluster assigned in the Bayesian analysis of population structure (see text); Fu's test: test of population expansion (Fu, 1997); Population growth parameters (with homoplasy): maximum pseudo-likelihood method of Navascués *et al.* (2009) to estimate ancestral (Θ_0) and current (Θ_1) population sizes, scaled by mutation rate, and the number of generations (τ) since the beginning of expansion, scaled by mutation rate; $-\log[CL]$: likelihood of the model; n.a.: method not applied because Fu's test was non-significant.

Region	ID	Population	Species	BAPS group*	n	Fu's test		Population growth parameters (with homoplasy)			
						Fu's F_S	P-value	Θ_0	Θ_1	τ	$-\log[CL]$
Core populations											
Alps											
	A2	Steiermark (Austria)	<i>P. mugo</i> s.s.	A	24	-8.0636	0.000	1.16×10^{-4}	2.10×10^3	4.58	39.3158
	A1	Lechtaler Alpen (Austria)	<i>P. mugo</i> s.s.	A	24	-9.8363	0.000	7.15	5.49×10^{12}	1.92	24.2562
	A3	Kitzbühler Alpen (Austria)	<i>P. mugo</i> s.s.	A	24	-7.7800	0.000	4.69	7.50×10^{12}	1.50	31.1874
	I1	Ötztaler Alpen (Italy)	<i>P. uncinata</i>	A	24	-6.0340	0.000	1.52×10^{-4}	1.19×10^{11}	4.20	38.9933
	C1	Berner Alpen (Switzerland)	<i>P. uncinata</i>	A	24	-8.5510	0.001	4.43	1.33×10^{13}	2.98	22.3526
	F1	Hautes Alpes (France)	<i>P. uncinata</i>	A	24	-8.4183	0.000	8.97×10^{-1}	5.28×10^5	3.96	26.6068
	F2	Alpes Maritimes (France)	<i>P. mugo</i> s.s.	A	24	-9.2685	0.000	6.47×10^{-5}	3.56×10^{12}	5.48	26.7261
	F3	Alpes Maritimes (France)	<i>P. uncinata</i>	A	47	-16.0977	0.000	9.13	4.31×10^5	4.50	56.0157
	F4	Alpes de Haute-Provence (France)	<i>P. uncinata</i>	A	24	-6.9137	0.000	7.22×10^{-1}	6.67×10^{12}	2.58	31.7544
Alps (pooled)					239	-26.1068	0.000	4.85	8.39×10^{11}	2.81	879.1600
Pyrenees											
	E1	Pirineos Centrales (Spain)	<i>P. uncinata</i>	P	20	-5.5979	0.001	2.54	7.79×10^5	2.94	25.4610
	E2	Pirineos Orientales (Spain)	<i>P. uncinata</i>	P	22	-0.8389	0.343	n.a.	n.a.	n.a.	n.a.
	POG	Pirineos Orientales (Spain)	<i>P. uncinata</i>	P	24	-4.9505	0.004	9.90×10^{-5}	1.44×10^{12}	3.89	33.5098
	LAN	Pyrénées orientales (France)	<i>P. uncinata</i>	P	28	-6.5923	0.000	9.63×10^{-3}	1.32×10^5	4.20	34.3784
Pyrenees (pooled)					94	-7.7169	0.004	4.86×10^{-1}	2.42×10^5	4.51	306.2670
Mont Ventoux											
	VX	Provence (France)	<i>P. uncinata</i>	A/P	26	-3.0738	0.079	n.a.	n.a.	n.a.	n.a.
Peripheral populations											
Black Forest											
	S1	Southern Black Forest (Germany)	<i>P. rotundata</i>		48	-17.7173	0.000	1.70	6.86×10^4	6.05	64.5111
	S2	Southern Black Forest (Germany)	<i>P. rotundata</i>		24	-0.9583	0.278	n.a.	n.a.	n.a.	n.a.
	S3	Southern Black Forest (Germany)	<i>P. rotundata</i>	A	24	-3.3652	0.062	n.a.	n.a.	n.a.	n.a.
	S4	Southern Black Forest (Germany)	<i>P. rotundata</i>	A	24	-3.0294	0.043	n.a.	n.a.	n.a.	n.a.
	N1	Northern Black Forest (Germany)	<i>P. × pseudopumilio</i>	NBF	24	-5.0145	0.004	1.03×10^{-4}	3.65×10^1	6.03	28.4685
	N2	Northern Black Forest (Germany)	<i>P. × pseudopumilio</i>		24	-2.3595	0.078	n.a.	n.a.	n.a.	n.a.
	N3	Northern Black Forest (Germany)	<i>P. × pseudopumilio</i>	NBF	24	-3.6368	0.024	n.a.	n.a.	n.a.	n.a.
Jura Mountains											
	F5	Jura Mountains (France)	<i>P. uncinata</i>	A	24	-4.3765	0.005	4.73×10^{-6}	5.90×10^5	3.16	38.3271
	C2	Jura Mountains (Switzerland)	<i>P. uncinata</i>		24	-10.9713	0.000	3.50×10^{-6}	7.61×10^5	12.00	23.6352
Vosges											
	F6	Vosges (France)	<i>P. rotundata</i>		24	-2.5319	0.084	n.a.	n.a.	n.a.	n.a.
Pyrenees											
	E3	Prepirineos (Spain)	<i>P. uncinata</i>		23	-2.3433	0.084	n.a.	n.a.	n.a.	n.a.
	PIT	Pyrénées orientales (France)	<i>P. uncinata</i>		90	1.0945	0.735	n.a.	n.a.	n.a.	n.a.
Iberian System											
	E4	Sierra de Gúdar (Spain)	<i>P. uncinata</i>		20	2.3347	0.886	n.a.	n.a.	n.a.	n.a.
	E5	Sierra de Cebollera (Spain)	<i>P. uncinata</i>		22	1.3627	0.754	n.a.	n.a.	n.a.	n.a.

*A, Alpine cluster (cluster 8 in Fig. 1); P, Pyrenean cluster (cluster 12 in Fig. 1); NBF, northern Black Forest cluster (cluster 6 in Fig. 1).

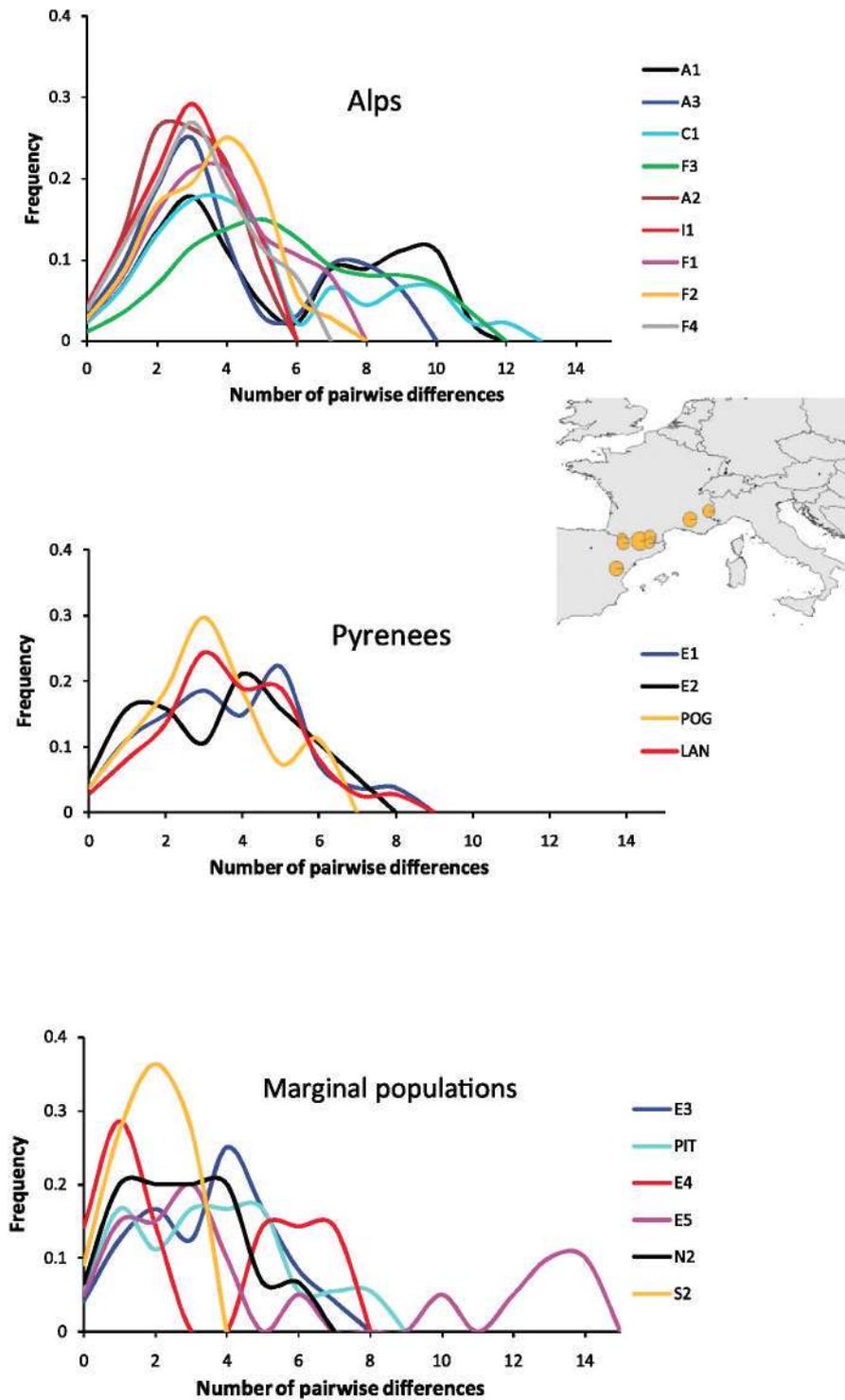


Figure 2 Mismatch distributions of Alpine (top), Pyrenean (middle) and marginal (i.e. peripheral with low diversity, see text) (bottom) populations of the *Pinus mugo* species complex in western Europe. The inset map shows the distribution of the 85-bp variant at Pt40196.

gene pools in the Alps and Pyrenees suggest colonization of both mountain ranges from independent glacial refugia. A third major vicariant gene pool for *P. mugo* s.s. might lie within the Balkan Peninsula (not surveyed in our study), where Slavov & Zhelev (2004) found only very weak

differentiation among populations, $F_{ST} = 0.04$, using allozymes (see also Feurdean *et al.*, 2007). Distinct vicariant gene pools on major mountain chains have also been identified for other conifers of the region, for instance for *Abies alba* Mill. (Vendramin *et al.*, 1999), *P. nigra* (Afzal-Rafii & Dodd, 2007)

and *Pinus cembra* (Höhn *et al.*, 2009). For *P. nigra*, differentiated peripheral populations were also observed (Afzal-Rafii & Dodd, 2007).

Our cpSSR data revealed signatures of population expansion pre-dating the LGM (c. 18,000 yr BP) in Alpine and Pyrenean populations of the *P. mugo* complex. Palaeobotanical evidence indicates that *P. sylvestris*/*P. mugo* s.l. was present in Europe throughout the Pleistocene cold stages and interglacials (West, 1980; Field *et al.*, 2000) and spread early in the post-glacial period from a wide range of locations, including sites in the Iberian Peninsula, the Alps, the Hungarian plains and the Carpathians (Cheddadi *et al.*, 2006; see also the Introduction). Given that average Weichselian temperatures were higher than in the LGM (van Andel, 2002; Van Meerbeek *et al.*, 2009), the earlier spread suggested by our genetic data is conceivable, but supporting quantitative palaeobotanical data are not available, to our knowledge. Similarly to the case for *P. mugo* s.l., bottlenecks followed by population expansion were detected in Eurosiberian, cold-adapted conifers based on nuclear DNA sequence data (dated to 150,000–300,000 yr BP in *Picea abies*, Heuertz *et al.*, 2006; to c. 2 Myr BP in *P. sylvestris*, Pyhäjärvi *et al.*, 2007).

Within the Alpine and Pyrenean *P. mugo* s.l. gene pools, overall genetic structure (measured with G_{ST}) was weak or absent, but phylogeographic structure [$N_{ST} > N_{ST}$ (permuted)] was discovered in each mountain range. These results suggest that each mountain chain was probably colonized by a different series of historically differentiated glacial populations, which became well connected by pollen flow within mountain chains at the latest during post-glacial recolonization, but possibly earlier (see also Liepelt *et al.*, 2002; Heuertz *et al.*, 2004). In the Alps, good candidates for a long-term glacial refugium are the western Alpine populations F2 and F3, harbouring a total of seven endemic haplotypes (see Refugium I of Schönswetter *et al.*, 2005). Populations that were probably old, as indicated by divergent lineages [i.e. populations with high $\bar{D}_{th}^2(i, j)$, bimodal mismatch distributions, and/or numerous high pairwise N_{ST} -values (results not shown)], were widely spread throughout the Alps, from west (C1, F2 and F3) to east (I1, A1 and A3), on southern (F2) or northern flanks (F4, A3), suggesting that colonization of the chain may have happened from west, south, east and north. Fossil pollen data are in agreement with early post-glacial recolonization from the south and the north-west in the Swiss Alps (Burga, 1988). In the Pyrenees, our sampling was fairly limited; however, populations from west and east of the range harboured endemic haplotypes (E1 and LAN), indicating that, similarly to in the Alps, multiple populations may have contributed to the colonization of the current range. More differentiated maternally inherited mitochondrial DNA data (e.g. Liepelt *et al.*, 2002) might provide further information on locations of glacial refugia in areas where cpSSR data are weakly differentiated. However, the mitochondrial region nad3-rpS12, containing microsatellite variation in *Pinus* species (Soranzo *et al.*, 1999), was monomorphic in the surveyed *P. mugo* s.l. range (Schmid, 2000).

The *P. rotundata*/*P. × pseudopumilio* populations native to disjunct bog habitats of the Black Forest had very variable levels of genetic diversity and were strongly differentiated, despite occurring within a small geographical region (the maximum distance between populations was 112 km). The highly divergent gene pools identified by BAPS suggest multiple origins, including a genetic connection between the southern Black Forest and the Alps. High diversity and the presence of endemic haplotypes suggest old age and large population sizes in Steerenmoos (S1), Briglirain (S3) and Grosses Muhr (N1), whereas low diversity indicates strong genetic drift in Saumisse (N2) and Ibacher Moor (S2). The small bog-affected pine populations (typically 2–12 ha, mapped by P. von Sengbusch, University of Freiburg, pers. comm., 2004) are nowadays separated by silver fir and spruce forests (Dierssen & Dierssen, 1984) that inhibit gene flow among them, leading to genetic drift. Selection by the local environment may also have contributed to the high differentiation. As in other regions, *Pinus* showed early post-glacial expansion and subsequent decline in the Black Forest (pollen core of the S1 population, Rösch, 2000). Furthermore, human impact has been intense in the region since about 7600 yr BP, and the present-day bog pine populations seemed to have expanded only after medieval burning (Rösch, 2000, and references therein). Additional distinct gene pools were identified in bog-affected populations: in the isolated population from the French Vosges (F6), in the Jura (with some Alpine influence, Fig. 1, Table 3) and in the French eastern Pyrenees (PIT). Overall, these data suggest multiple glacial *P. mugo* s.l. populations north of the Alps and probably the Pyrenees, which kept their genetic distinctiveness despite late glacial and early Holocene expansion.

The marginal populations of the Iberian Peninsula, namely Prepirineos (E3), Sierra de Gúdar (E4) and Sierra de Cebollera (E5), were the most differentiated populations, all harbouring endemic haplotypes and lacking evidence of population expansion. These results are in agreement with a model of long genetic isolation, probably through several glacial cycles (Dzialuk *et al.*, 2009), with populations experiencing elevational shifts but no considerable growth in size. Robledo-Arnuncio *et al.* (2005) showed that *P. sylvestris* also responded to climate oscillations by elevational shifts in northern Spain, but, in contrast to *P. uncinata*, it maintained large population sizes. Large *P. sylvestris* glacial populations survived in drainage basins, which connected the individual mountain blocks to which the species retreated during warm stages (Robledo-Arnuncio *et al.*, 2005). It is possible that the occupation of the plains by *P. sylvestris* prevented the establishment of *P. uncinata* at lower elevations during cold stages, contributing to the presently observed differentiation in *P. uncinata*.

Haplotype sharing between *P. mugo* s.l. and *P. sylvestris*

We found evidence of haplotype sharing between *P. sylvestris* and *P. mugo* s.l., which could result from hybridization or

sharing of ancestral haplotypes, given that the species are closely related (Filippula *et al.*, 1992). They are also partially interfertile, but the incidence of natural hybridization seems to be very low (Christensen, 1987b). In controlled crosses (Wachowiak *et al.*, 2005) and under natural conditions (Wachowiak & Prus-Głowacki, 2008), viable offspring were obtained between the two species only when *P. mugo* s.l. was the pollen donor. However, our finding of *P. sylvestris* haplotypes in *P. mugo* s.l. would rather suggest *P. sylvestris* to be the pollen donor. Whether our results reflect true introgression from *P. sylvestris* into *P. mugo* is debatable, as the detection power is hampered by our use of few markers and by the suggested high levels of homoplasy of cpSSRs (Provan *et al.*, 2001). In Spain, the much more abundant *P. sylvestris* may literally swamp marginal *P. uncinata* populations with its pollen. Therefore, the issue of introgression is important for the conservation and evolution of *P. mugo* s.l. and requires more attention in future research.

Identification of conservation priorities

It is predicted that in the Iberian Peninsula, montane conifer species such as *P. sylvestris* and *P. uncinata* will suffer intense and rapid reductions of their distribution ranges in the future because global warming will induce their migration to higher elevations, but such areas of sufficient elevation are not available for colonization (Benito Garzón *et al.*, 2008). The *P. uncinata* populations in Sierra de Cebollera and Sierra de Gúdar are in this situation. These populations are strongly differentiated, and, as southern 'rear edge' populations, they may harbour important adaptations relevant to the conservation of the species (Dynesius & Jansson, 2000; Hampe & Petit, 2005). The *P. uncinata* population from Sierra de Cebollera is showing signals of expansion owing to lower grazing pressure and increased mean temperatures; however, recruitment patterns are significantly influenced by the availability of suitable habitats for germination at the edges of *Calluna vulgaris* mats (Camarero *et al.*, 2005) and high minimum September temperatures (Camarero & Gutiérrez, 2007). This suggests that biotic interactions and nonlinear responses of species to temperature need to be considered when predicting the effects of climate change (Davis *et al.*, 1998). In particular, research on competitive interactions and on the pattern of gene flow between *P. uncinata* and *P. sylvestris* is required in order to understand the effects of the climate-driven invasion of *P. uncinata* populations by *P. sylvestris* on low mountains in Spain. The genetic distinctiveness of *P. uncinata* populations from the Iberian System further justifies conservation measures. In these low mountains *in situ* conservation seems challenging, and *ex situ* conservation of seed lots and live collections is urgently needed.

In the Black Forest, many *P. rotundata*/*P. × pseudopumilio* populations show considerable dieback and insufficient natural regeneration. These seem to be delayed consequences of the drainage of bogs for peat collection (von Sengbusch, 2002). After at least 200 years of regular burning of peat bogs in the

Black Forest, fire disturbance has ceased, reducing the availability of open habitats for bog pine regeneration. At the same time, the lowering of water levels has allowed the establishment of a dense undergrowth and invasion of the drier bog margins by the shade-tolerant *Picea abies* (von Sengbusch, 2002). Lower water levels also trigger a plastic response towards a more slender growth habit in straight-stemmed bog pines in the southern Black Forest, which makes the trees more prone to mechanical stress owing to winter snow cover and winds, enhancing the dieback (von Sengbusch, 2002). Because global warming is projected to aggravate the dieback, and these populations are genetically distinct, conservation measures are justified and necessary. A combined strategy of *in situ* conservation of the larger bogs along with the establishment of *ex situ* collections is recommended.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Selection of cpSSR loci.

Table S1 Haplotypes observed in the *Pinus mugo* species complex.

Table S2 Haplotypes observed in *Pinus sylvestris*.

Table S3 *Pinus mugo* s.l. sequencing of cpSSR Pt41093.

Table S4 Pairwise F_{ST} -values between populations of the *Pinus mugo* species complex.

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BIOSKETCH

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