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RESEARCH ARTICLE

A range-wide postglacial history of Swiss stone pine based on molecular markers and palaeoecological evidence

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Abstract

Aim: Knowing a species' response to historical climate shifts helps understanding its perspectives under global warming. We infer the hitherto unresolved postglacial history of *Pinus cembra*. Using independent evidence from genetic structure and demographic inference of extant populations, and from palaeoecological findings, we derive putative refugia and re-colonisation routes.

Location: European Alps and Carpathians.

Taxa: *Pinus cembra*.

Methods: We genotyped nuclear and chloroplast microsatellite markers in nearly 3000 individuals from 147 locations across the entire natural range of *P. cembra*. Spatial genetic structure (Bayesian modelling) and demographic history (approximate Bayesian computation) were combined with palaeobotanical records (pollen, macrofossils) to infer putative refugial areas during the Last Glacial Maximum (LGM) and re-colonisation of the current range.

Results: We found distinct spatial genetic structure, despite low genetic differentiation even between the two disjunct mountain ranges. Nuclear markers revealed five genetic clusters aligned East–West across the range, while chloroplast haplotype distribution suggested nine clusters. Spatially congruent separation at both marker types highlighted two main genetic lineages in the East and West of the range. Demographic inference supported early separation of these lineages dating back to a previous interstadial or interglacial *c*. 210,000 years ago. Differentiation into five biologically meaningful genetic clusters likely established during postglacial re-colonisation.

Main Conclusions: Combining genetic and palaeoecological evidence suggests that *P. cembra* primarily survived the LGM in 'cold period' refugia south of the Central European Alps and near the Carpathians, from where it expanded during the Late Glacial into its current Holocene 'warm period' refugia. This colonisation history has led to the distinct East–West structure of five genetic clusters. The two main genetic

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lineages likely derived from ancient divergence during an interglacial or interstadial. The respective contact zone (Brenner line) matches a main biogeographical break in the European Alps also found in herbaceous alpine plant species.

KEYWORDS
gene flow, genetic structure, macrofossils, microsatellite markers, pollen, postglacial colonisation, refugial areas

1 | INTRODUCTION

Massive climate oscillations during Pleistocene glacial–interglacial cycles (Williams et al., 1998) have forced species to shift their ranges according to the prevailing climatic conditions (Lumibao et al., 2017). These migrations have often induced distinct spatial genetic patterns, in particular in mountainous areas with their rugged topography and prominent ecological constraints, such as the spatial distribution of suitable substrate (Alvarez et al., 2009; Thiel-Egenter et al., 2011).

Many plant species have been investigated with regard to their inferred (post)glacial history (e.g., Larsson et al., 2022; Lumibao et al., 2017; Lyman & Edwards, 2022; Marske & Boyer, 2022; Nieto Feliner, 2014), but most such studies relied either on population genetic inference only or on palaeoecological evidence. Tree species are well suited for combining both approaches. Their large quantities of pollen accumulate in sediments and provide a spatiotemporal record of past species' presences and abundances. Moreover, larger tissue parts (fruits, leaves, wood) can be incorporated into sediments (Schwörer et al., 2022). Such macro-remains (macrofossils) enable inferring the local presence of a species within a few tens of metres around distributional sites, because they are less widely transported than wind-borne pollen. Analysing macro-remains may also yield taxonomic resolution unreached by pollen identification. Interpolation among sites where a species was recorded with micro- or macrofossils allows investigating species' range shifts over time in response to external drivers such as climate, disturbance or human interference (Birks, 2019). While combined pollen and macrofossil approaches give insights into the spatiotemporal dynamics of species, intraspecific variation remains obscured, concealing the evolutionary component of these processes.

Complementary to palaeoecological inference, the spatial distribution of genetic diversity in extant populations informs about the genetic relationships among them (phylogeography; Avise, 2000). Subsequently, one can deduce demographic processes which have led to today's spatial genetic structure. However, one can only speculate about the location of refugial areas and respective migration routes based on genetic patterns, and it is even more difficult to elucidate the temporal aspects of these dynamics because absolute dating is not feasible without independent evidence, for example, from palaeoecology. Only the combination of phylogeographical and palaeoecological inference allows understanding the dynamics of species' demographic histories (Gavin et al., 2014). To date, only a few, largely common and widespread, forest tree species have been studied based on their fossil records in combination with population genetic data from extant stands, for example, Norway spruce (*Picea abies*; Tollefsrud et al., 2008), European white oaks (*Quercus* spp.; Petit et al., 2002), Scots pine (*Pinus sylvestris*; Cheddadi et al., 2006), silver fir (*Abies alba*; Liepelt et al., 2009), European beech (*Fagus sylvatica*; Magri et al., 2006), European larch (*Larix decidua*; Wagner et al., 2015), as well as conifer species from North America and Asia like Douglas fir and Qinghai spruce (*Pseudotsuga menziesii*, *Picea crassifolia*; reviewed in Gavin et al., 2014), mountain hemlock and western redcedar (*Tsuga mertensiana*, *Thuja plicata*; Fernandez et al., 2021), or Manchurian red pine (*Pinus tabulaeformis*; Hao et al., 2018). Among these species, *L. decidua* stands out because it is largely confined to the uppermost forests of the European Alps and the Carpathians. These areas were most directly affected by Pleistocene glaciations. As a cold-adapted treeline species, the population size and range of larch reached its maximum during warmer intervals of the overall colder glacial period (interstadials; Wagner et al., 2015). During the coldest and driest phases of the last glacial and during the previous and current interglacial periods, the range was shifted to favourable locations such as the foothills of the Southern Alps and Carpathians during the Last Glacial Maximum (LGM; 'cold period' refugia) and the current range at high elevations in these mountain ranges ('warm period' refugia).

Along with *L. decidua* in large parts of its range, Swiss stone pine (*Pinus cembra*) is a keystone species of the high-elevation timberline ecotone. *Pinus cembra* often follows *L. decidua* as a late-successional species. Scattered individuals of *P. cembra* also colonise areas above the current timberline (Neuschulz et al., 2018); hence, the species has the potential to expand uphill into yet unforested area under benign microsite conditions (but see Dauphin et al., 2021).

Population genetic studies of *P. cembra* from parts of the range indicated weak genetic differentiation between the Alps and Carpathians (Dzialuk et al., 2014; Höhn et al., 2009), suggesting recent common ancestry of extant populations. Moreover, haplotype diversity in Swiss populations gradually declines from East to West (Gugerli et al., 2009), interpreted as a result of westward migration from a presumed refugial area at the (south)eastern fringes of the Alps. Within the Carpathians, low levels of genetic differentiation among disjunct populations were explained by the disruption of a previously contiguous occurrence (Lendvay et al., 2014). However, except for a regional investigation in southwestern France (Toth et al., 2019), we lack an account of the species' history by combining phylogeographical and palaeoecological inference.

Here, we present the first range-wide study of the postglacial history of Swiss stone pine based on dense sampling for both phylogeographical and palaeoecological evidence. Our main questions are as follows: (i) What is the spatial genetic structure of extant populations of *P. cembra* across its natural range, and is there congruence between the patterns derived from nuclear and chloroplast markers? (ii) Can we infer the location of the refugial areas of *P. cembra* during the LGM and re-colonisation routes drawing on palaeoecological evidence? (iii) Does the inferred demographic history of *P. cembra* coincide with putative refugia, from where migration routes led the species to re-colonise the Alpine and Carpathian ranges? We further evaluate whether the spatial genetic structure found in *P. cembra* matches known genetic discontinuities in other high-altitude plant species. We hypothesise that (i) the phylogeographical pattern is similar between marker types, and that this pattern relates to the separation of the two mountain ranges, (ii) refugial areas are mainly located along the southeastern fringe of the Alps and around the Carpathians and (iii) that the distribution of extant genetic variation coincides with refugial areas from which the populations originate.

2 | MATERIALS AND METHODS

2.1 | Study species

Pinus cembra L. predominantly occurs in areas with continental climate in the Central Alps and the Carpathians, but also shows scattered stands in more oceanic, mainly peripheral areas in the Alps. Even within the core range, stands are often disjunct owing to the rugged topography, but also over-exploitation by humans during past centuries.

This long-lived, wind-pollinated species is primarily outcrossed (Salzer & Gugerli, 2012). As a closed-cone pine, *P. cembra* has unwinged seeds dispersed by the European nutcracker (*Nucifraga caryocatactes*). This bird collects the nutritious seeds and caches them as winter food reserves; seeds from unexploited caches may germinate and eventually establish (Neuschulz et al., 2015). Seed dispersal distances reach several hundred metres, but exceptional long-distance dispersal may be assumed because nutcrackers cover distances of several kilometres (Sorensen et al., 2022), even with seed-filled pouches (Mattes, 1982).

2.2 | Sampling

Sampled populations covered the entire natural range of Swiss stone pine. We collected needles from 5 to 33 primarily adult trees (mean: 20) in 147 stands (Alps 133, Carpathians 14; Appendix S1; Figure S1.1; Table S1.1) between 1997 and 2014, respecting 30 m distance between sampled individuals where possible to minimise potential effects due to spatial autocorrelation. A subset of samples was previously analysed (Gugerli et al., 2009; Höhn et al., 2009; Lendvay et al., 2014). In total, 2989 and 2988 individuals were genotyped for nuclear and chloroplast markers, respectively (see below).

2.3 | Genotyping

DNA extracts (methods detailed in Gugerli, Senn, et al., 2001; Gugerli et al., 2009; Höhn et al., 2009; Mosca et al., 2012) were amplified at 11 nuclear microsatellites (simple sequence repeats, nSSRs; Salzer et al., 2009; Lendvay et al., 2014) and four chloroplast microsatellites (cpSSRs; Vendramin et al., 1996). Primer information, PCR conditions and thermocycler profiles are described in Lendvay et al. (2014) and listed in Table S1.2. We initially tested for linkage disequilibrium ($p < 0.001$, 10,000 permutations) among pairs of loci within populations in Arlequin v. 3.5 (Excoffier & Lischer, 2010).

2.4 | Genetic diversity

Based on nSSR genotypes, we computed mean allelic richness (A_r) per population using the PopGenReport R package v. 3.0.4 (Adamack & Gruber, 2014) and observed/expected heterozygosity (H_O, H_E) per population in Arlequin. The latter were used to test for population-wise deviations from Hardy–Weinberg equilibrium ($F_{IS} \neq 0$) across loci using 1,000,000 steps of Markov chains. For cpSSR haplotypes, we calculated haplotypic diversity, haplotype richness (relative to sample number) and numbers of population-specific haplotypes. Estimates of genetic diversity were tested for longitudinal trends using linear models with the *lm* function in R (R Development Core Team, 2021).

For both marker types, we performed a hierarchical analysis of molecular variance (AMOVA), implemented in Arlequin, to assess the partitioning of genetic variation among groups of populations (F_{CT}), among populations within groups (F_{SC}), and within populations. We grouped populations according to (i) the two mountain ranges and (ii) the two main genetic lineages identified using STRUCTURE ($K = 2$; see below). For the latter, we grouped populations based on the majority of assignment probability ($q > 0.5$), except for population CH54, considered as being non-autochthonous (retained in western lineage). Levels of significance were tested using default settings.

2.5 | Genetic structure

We assessed the spatial genetic structure using Bayesian models implemented in STRUCTURE v. 2.3.4 (Pritchard et al., 2000) for nSSRs and BAPS v. 6.0 (Corander et al., 2003) for cpSSRs. We further performed discriminant analysis of principal components (DAPC) as a multivariate analysis without a priori assumptions of a population genetic model (detailed in Appendix S2).

We tested for isolation by distance (IBD) for the overall data set as well as for the Alpine and Carpathian populations separately, using pairwise geographical and linearised genetic distances $F_{ST}/(1 - F_{ST})$ (Rousset, 2000; details given in Appendix S2). We further carried out a hierarchical clustering analysis from the populations assigned to one of the five genetic groups (nSSR-based, $q > 0.5$; see Results) to infer phylogenetic relationships and divergence events among geographical areas (see below). We used the Euclidean method to compute the distance matrix from pairwise F_{ST} values and the Ward D as agglomeration method with the *dist* and *hclust* functions of the STATS R package v. 3.6.1, respectively.

2.6 | Demographic analysis

To infer the demographic history, we applied approximate Bayesian computation (ABC; Beaumont et al., 2002) using DIYABC 2.0 (Cornuet et al., 2014), detailed in Suppl. Information (Appendix S2). We based this analysis on the identified five genetic clusters (see Results): Carpathians (C), Eastern Alps (EA), mid-Eastern Alps (MEA), mid-Western Alps (MWA) and Western Alps (WA).

According to preliminary analyses, we chose three scenarios that differed only in the splitting times of WA and MEA from MWA (Appendix S2). To date the demographic history, we assumed a generation time of 40 years (Ulber et al., 2004).

2.7 | Palaeoecological records

To identify refugia during the LGM and track postglacial spread in response to climatic changes, we analysed existing pollen and macrofossil records from Central Europe (Appendix S3). We determined the empirical limit at 71 pollen records (Table S3.1) to infer the timing of local population establishment at individual sites. Pollen records were individually selected from the European Pollen Database (EPD; Giesecke et al., 2014) and the Alpine Pollen Database (ALPADABA), accessed via Neotoma (Williams et al., 2018). All selected pollen records feature reliable chronologies, most based on radiocarbon dating of terrestrial plant macrofossils.

We additionally compiled a data set with the first presence of *P. cembra* macrofossils at 36 records in Central Europe (Table S3.2). Macrofossils unambiguously document the local presence of *P. cembra* at these locations. However, due to lower numbers that are preserved in the sediment compared to pollen, they might underestimate the timing of local species establishment, that is the minimum age of occurrence.

To further illustrate the spatial expansion of *P. cembra* through time, we calculated isochrones for both data sets in 2000-year steps using nearest neighbour spatial interpolation in ArcGIS 10.8 (ESRI, Redlands, California, USA), which is based on the weighted values of neighbouring datapoints. The isochrones connect areas with similar timing of local establishment, based on the empirical limit of pollen or the first presence of macrofossils, respectively.

3 | RESULTS

3.1 | Genetic diversity

We detected between two (Pc35) and 44 alleles (Pc23) per nSSR locus (Figure S2.1a). Linkage disequilibrium between pairs of loci per population occurred in only 43 out of 8085 cases (0.53% at $p < 0.001$, data not shown), with no locus pair standing out. None of the loci showed substantial deviation from HWE within populations, with a slight heterozygote excess at all loci over the entire data set (Figure S2.1b). Expected heterozygosity H_E revealed a significant, but only slight trend of decreasing towards the West across both the entire range and the Alps only ($r_{AC} = 0.0034$, $r_A = 0.0044$, $p < 0.001$; Figure 1a). For allelic richness A_R, this trend was significant only across the Alpine, but not the entire range ($r_A = 0.0115$, $p < 0.001$, $r_{AC} = 0.0005$, $p = 0.751$; Figure 1b). Notably, A_R was also elevated in southwestern Alpine populations (Figure 1b).

At the four cpSSR loci, between five and 11 alleles combined into 79 distinct haplotypes (68 in the Alps, 37 in the Carpathians). Of these, the most abundant haplotype A occurred in all but five populations, reaching 42.9% in total (Alps 45.0%, Carpathians 16.3%; Figure S2.2). Most other haplotypes were rare. We found 42 haplotypes specific for the Alps and 11 for the Carpathians; 29 haplotypes were population-specific overall, 21 of which occurred within Alpine and eight within Carpathian populations (Figure 1c). Haplotypic diversity *H* was mildly, but significantly higher in the eastern Alps and the Carpathians, and relatively low in the western part of the range ($r_{AC} = 0.0161$, $r_A = 0.0381$, $p < 0.001$), except for a few Italian populations in the Western Alps (Figure 1c).

AMOVA showed that genetic differentiation between the two mountain ranges was generally low, but highly significant ($p < 0.001$; Table S2.1): for nSSRs, overall F_{ST} was 0.082 and F_{CT} reached only 0.029, whereas the majority of genetic variation was attributed to the within-population level (91.75%). As expected for haploid, uniparentally inherited markers, genetic differentiation based on cpSSRs was slightly higher, with overall $F_{ST} = 0.120$. Differentiation between mountain ranges was $F_{CT} = 0.061$, and as for nSSRs, most of the total genetic variation resided within populations (87.98%; Table S2.1).

The outcome of AMOVA when grouping populations according to their assignment probability in STRUCTURE at $K = 2$ (see below) yielded similar, but slightly lower values for the partitioning of total genetic variation among groups (F_{CT}) and among populations (F_{SC}, F_{ST}) in both marker types (Table S2.1).

3.2 | Genetic structure

To determine the number of clusters that best explain the genetic structure in *P. cembra* at nSSRs, we evaluated the plot of LnP(D) vs. *K*, considering maximum likelihood and variance among runs (Figure S2.3), as suggested in the manual of STRUCTURE. Moreover, we considered biological relevance and the degree of admixture as criteria for choosing the most informative level of clustering,

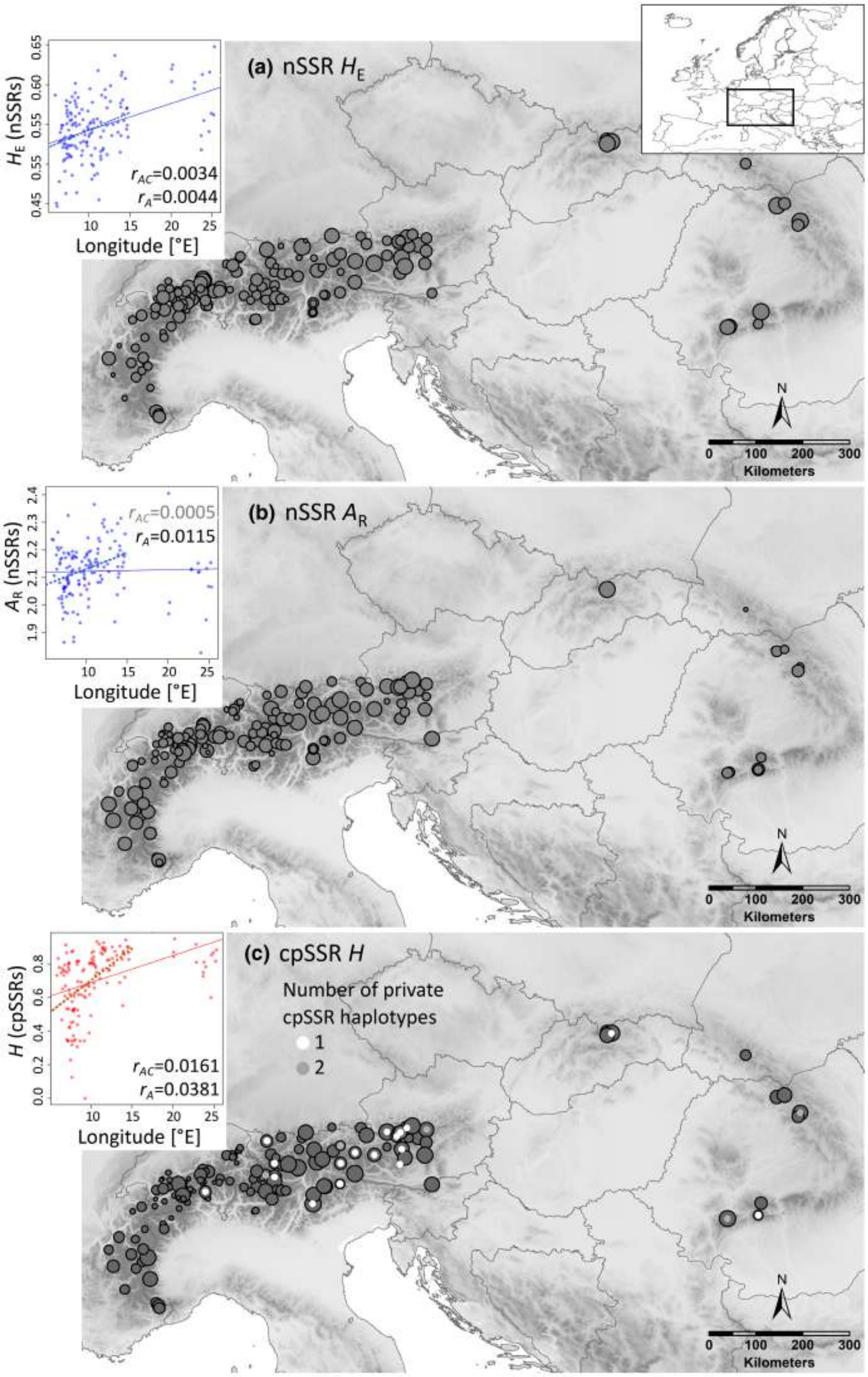

FIGURE 1 Geographical distribution of genetic diversity measures estimated based on nuclear and chloroplast microsatellites (simple-sequence repeats; nSSRs and cpSSRs). (a) Expected heterozygosity (H_E) and (b) allelic richness (A_R) at nSSRs, (c) haplotype diversity (H) at cpSSRs. Circle sizes indicate levels of diversity, with the number of private cpSSR haplotypes indicated by overlaid symbols in (c). Inset plots on the left display genetic diversity measures (nSSRs: blue; cpSSRs: red) vs. longitude, with linear regressions for the entire range-wide (solid lines, r_{AC}) and for Alpine populations (dashed lines, r_A); all models are significant at $p < 0.001$, except for range-wide A_R (in grey, $p = 0.751$). The location of the study range is indicated in the inset map of Europe (top right).

as argued by Janes et al. (2017). The first separation (K = 2) in the hierarchical suite of cluster separation occurred in the central Alpine range (Figure S2.4a), and a further genetic cluster emerged at K = 3 in the western Alps (Figure S2.4b). A certain distinction between populations from the Alps and the Carpathians became visible only at K = 4 (Figure S2.4c), while the southeasternmost Alpine populations still showed medium assignment probabilities to the cluster dominant in the Carpathian populations. The most informative genetic structure emerged at K = 5 (Figure 2a), highlighting the East–West alignment of these five clusters across the species' range.

For cpSSRs, BAPS identified $K = 9$ as the most probable number of genetic clusters (Figure 2b). Three of these clusters predominantly occurred in the eastern range (4, 6, 7; Figure S2.5b), with cluster 7 limited to Carpathian populations (Figure S2.6g). In turn, four clusters were largely restricted to the populations in the Western Alps (1–3, 9; Figure S2.5c). Of the remaining two clusters, cluster 5 was widespread and cluster 8 occurred in only three populations in the Alps (Figure S2.6e,h).

The spatial clustering of the two marker types revealed a common signal at K = 2 for nSSRs that coincided with the eastern and western groups of cpSSR-derived clusters (Figure S2.5b,c).

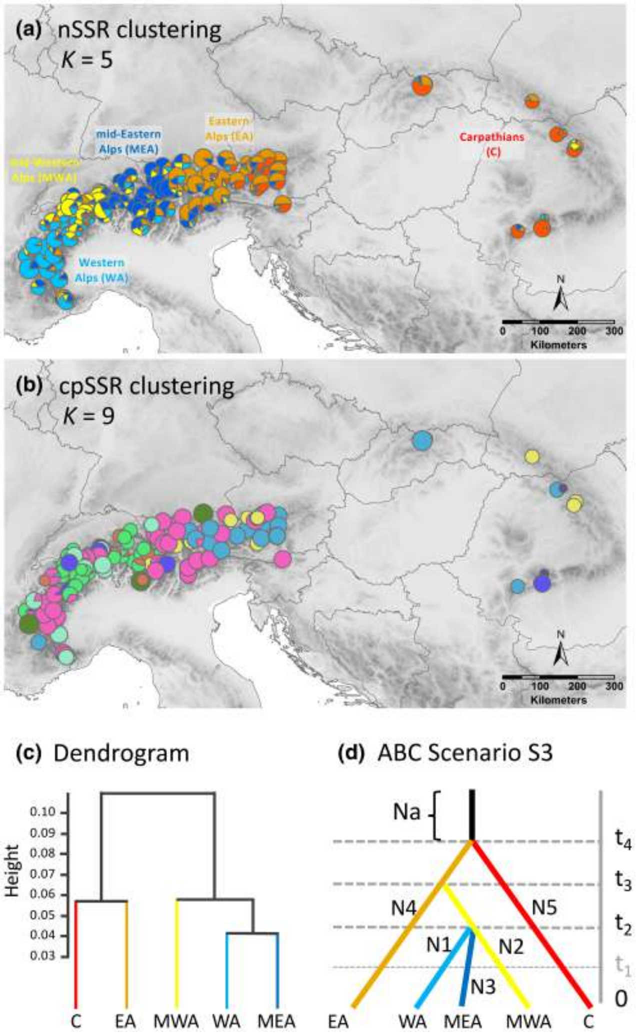

FIGURE 2 Spatial range-wide genetic structure in *Pinus cembra*, assessed based on nuclear and chloroplast microsatellites (simple-sequence repeats; nSSRs and cpSSRs). Results of Bayesian hierarchical clustering analysis using (a) 11 nSSR markers for five genetic clusters ($K = 5$), and (b) four cpSSR markers for nine genetic clusters ($K = 9$). Pie charts display population-level assignment probabilities, sizes are proportional to the sample numbers per population. (c) Hierarchical clustering of the five genetic clusters based on Bayesian clustering analysis at $K = 5$ as in (a). (d) Demographic scenario S3 best supported by approximate Bayesian computation framework; the tested scenarios (see Figure S1.3a) are based on the same five genetic clusters as above; t_n denotes time points of cluster separation (t_1 not relevant in this scenario), Nx refers to respective estimates of effective population sizes, with Na being that of the ancestral population. Colours and abbreviations in (c) and (d) follow those of the five clusters in (a): Carpathians (C, red), Eastern Alps (EA, orange), mid-Eastern Alps (MEA, blue), mid-Western Alps (MWA; yellow) and Western Alps (WA, light blue); colours in (b) do not relate to those of the nSSR-derived clusters.

Hereafter, we consider these two groups of populations as eastern and western lineages of *P. cembra*. While $K = 5$ best explains the genetic structure of nSSR data, cpSSRs suggest $K = 9$ as the most likely number of genetic groups. Besides the mentioned split into two lineages, we based further analyses on five clusters for several reasons. (i) Other than for nSSRs, cpSSR clusters were not spatially aggregated in all cases, (ii) two cpSSR clusters were either ubiquitous or rare, that is spatially not informative and (iii) the paternally inherited cpSSRs, which are dispersed in two sequential steps (via pollen, then via seed in the fertilised embryo), commonly show less clear phylogeographical structuring (Figure S2.6).

Consistent with low estimates of genetic differentiation (F_{ST}) and high variation within populations (AMOVA), we found large genotypic variation along the first axes of the PCA without structuring among populations. For the DAPC, we retained 60 principal components (PCs) based on the α-score statistics and kept 60 discriminant functions (DFs) for clustering analysis. DF1 and DF2 summarised 16.4% and 11.9% of the variance, respectively (Figure S2.1c). These DFs segregated populations RO126 and, to a lesser extent, RO147 from the Southern Carpathians (Figure S2.1c). Interestingly, though, these populations were assigned to and well-integrated within the common eastern Alpine cpSSR clusters 4 and 2, respectively (Figure S2.6b,d).

IBD over all samples was highly significant (Mantel's $r = 0.507$, $p < 0.001$), with a clearly positive trend up to a distance of about 500 km but flattening out for more distant population pairs (Figure S2.7a). While IBD remained significant in the Alpine range, but with reduced $r = 0.173$ ($p < 0.001$), no pattern emerged from individuals of the Carpathian range only ($r = -0.196$, $p = 0.94$; Figure S2.7b,c).

Hierarchical clustering analysis using the genetic distance matrix among the five Structure clusters revealed two clades formed by clusters C and EA and by clusters MEA, MWA and WA, respectively (Figure 2c).

3.3 | Demographic analysis

Among the three scenarios tested, the highest posterior probability was assigned to scenario S3 (Figure 2d), whose respective values (0.6903, 95% confidence interval: 0.6274–0.7532) were much higher than those for scenarios S1 and S2 (0.2102 and 0.0994, respectively; Table S2.2). The median values of the effective population sizes for scenario S3 were N1 = 14,500 (WA), N2 = 6630 (MWA), N3 = 8370 (MEA), N4 = 29,400 (EA), N5 = 49,800 (C) and Na = 472 for the ancestral population (Table S2.3). This result indicates that the effective population size of the ancestral population (Na) was more than 60–100 times smaller than those of the EA and C clusters, suggesting a substantial expansion event at time t_4 (5310 generations ago). At time t_3 (1630 generations ago), a bottleneck event reduced the population more than fourfold, giving rise to MWA, and at time t_2 (631 generation ago) the two genetic clusters WA and MEA separated from MWA and at the same time increased their effective population sizes. Assuming a generation time of 40 years for *P. cembra*, these results suggest early population growth and a separation of the Carpathian/eastern Alpine cluster 212,000 years ago, as far back as the penultimate interglacial or a chronologically related interstadial (Marine Isotope Stage—MIS 7). Around 65,000 years ago, during the last glacial period (i.e., Greenland stadial 18), the western Alpine clusters separated, which later differentiated into three distinct clusters (MEA, MWA, WA) starting around 25,000 years ago, that is just before or during the LGM. The latter relates to the postglacial recolonisation during the late Pleistocene and Early Holocene, when these distinct genetic clusters immigrated into their present-day ranges and formed the currently detectable contact zones. All the observed summary statistics (mean number of alleles (*A*) and mean expected heterozygosity (H_E) for single populations, and H_E, *A*, and the classification index for population pairs) were not significantly different from the simulated values (Table S2.4), based on parameters drawn from the posterior distributions for scenario S3. This result, together with the principal component analysis comparison implemented in DIYABC (Figure S1.3b), suggests a high goodness-of-fit of observed data to scenario S3.

3.4 | Fossil record

Pollen records provide direct evidence for LGM refugia of *P. cembra* in the forelands of the Southern Alps as well as the Eastern and Western Carpathians (Figure 3a). However, since most lakes in mountainous regions are of glacial origin, few pollen records reached back to the LGM, ca. 23,000–19,000 calibrated years before present (cal. BP). Several sites documented the local presence of *P. cembra* before the first significant Late Glacial warming pulse at 16,700 cal. BP (Samartin et al., 2012), indicating additional, geographically distinct putative refugial areas, that is the Southern Carpathians, the foothills south of the Central Alps and near the Eastern Alps, and notably the southern lowlands of the Bohemian massif in the present-day Czech Republic. *Pinus cembra* was able to expand its range to the foothills of the southern Central Alps and of the Carpathians (Figure 3a) once the area became deglaciated after ca. 19,000 cal. BP. Only after ca. 14,600 cal. BP, populations also expanded in the mountain ranges of the Alps and the Carpathians, with further expansion around 11,700 cal. BP. Noteworthy, we observed a delayed spread to the Western Alps, where the empirical limit was reached only after 9000 cal. BP at most sites; only in the continental Aosta Valley of northwestern Italy, the expansion was as early as in the Swiss Western Alps (Figure 3a).

The above range shifts are also largely reflected in the macrofossil records (Figure 3b), which provide a spatially more explicit reconstruction by documenting the first local presence of *P. cembra* at individual sites. For instance, even though pollen records indicate that *P. cembra* had already spread to large parts of the Alps by the

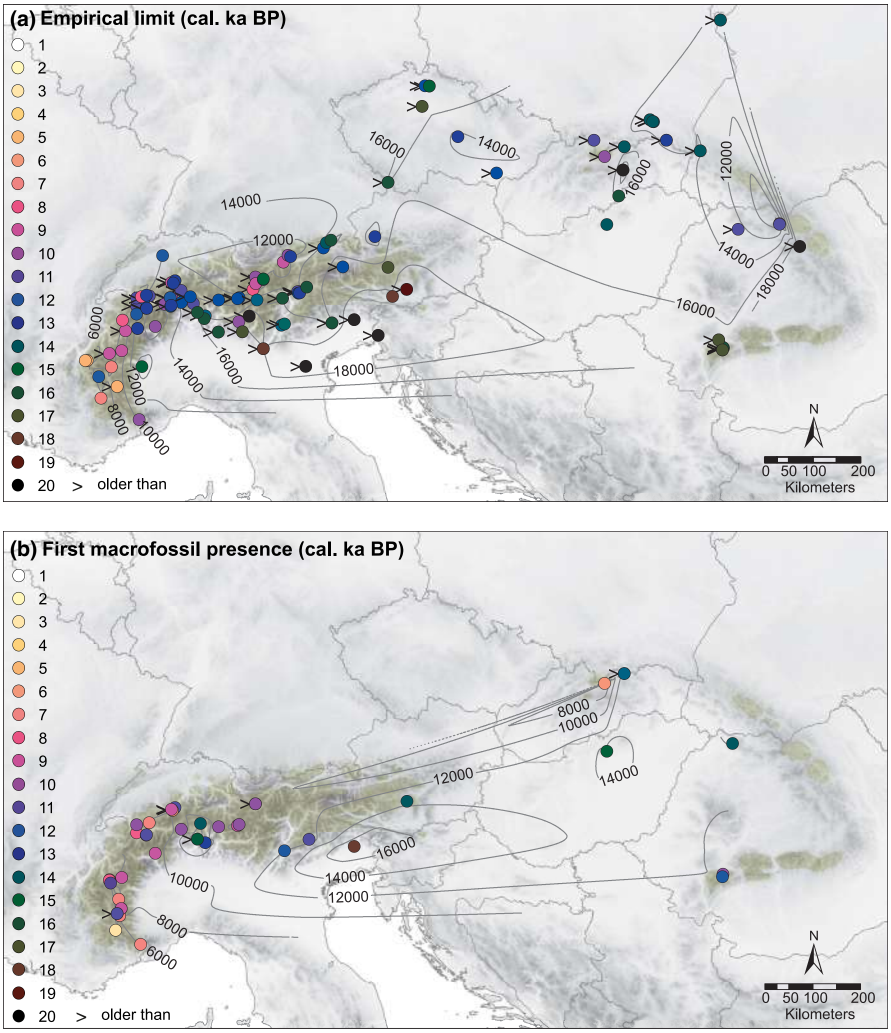

FIGURE 3 Past presence and postglacial expansion of *Pinus cembra* in central Europe inferred from palaeobotanical records (pollen and macrofossils). (a) Age of empirical limits of *P. cembra* pollen records indicating local population establishment, and (b) age of the first local presence unambiguously documented from macrofossils. Ages are rounded to the nearest millennium. Black circles (20 cal. ka BP) suggest refugia of the species during the Last Glacial Maximum, isochrones (grey lines) in 2000-year steps are based on nearest neighbour spatial interpolation. Green shading illustrates the current natural range of P. cembra (Caudullo et al., 2017).

beginning of the Holocene (11,700 cal. BP), it was mostly constrained to low elevations. Only after ca. 10,000 cal. BP, *P. cembra* established at current treeline elevations, reaching highest treeline positions during the Holocene Thermal Maximum (HTM) at 9000–5000 cal. BP.

4 | DISCUSSION

Multiple processes affect the biogeographical and evolutionary history of species, and strong environmental cues, such as the Quaternary climatic oscillations, have left a distinct legacy in the current distribution of species and their genetic structure. These imprints of demographic dynamics are particularly evident in species whose capacity to track habitat shifts through migration is extrinsically affected by strong topographic and environmental cues, such as in mountainous areas. Our analysis of densely sampled, range-wide data of molecular markers and palaeoecological evidence exemplifies how the combined approaches may unravel the (post) glacial history of *P. cembra*. Despite low levels of genetic differentiation between mountain ranges and substantial admixture within populations, likely due to wide-ranging gene flow through wind-borne pollen, we found a distinct spatial genetic structure in extant populations that reflects pre-LGM and postglacial demography. We identified five genetic clusters according to nSSRs, arranged longitudinally across the species range, and a main split into two genetic lineages backed up by both nuclear and chloroplast markers. The contact zone of these two lineages in the central Alps was located in a known bio- and phylogeographical break zone in high-elevation plant species (Thiel-Egenter et al., 2011). In contrast to our hypothesis, this main split did not coincide with the disjunction of the natural range of *P. cembra* across the two mountain ranges.

Combining these findings with the pollen and macrofossil records allows us to assign the two main genetic lineages of *P. cembra* to putative main refugial areas during the LGM. However, the locations of refugial areas during prior glacial–interglacial cycles cannot be determined because palaeoecological evidence of *P. cembra* is largely missing for such old periods. Demographic inference suggests that the strong split of these two lineages dates back to a spatial separation during the penultimate interglacial period (MIS 7), over 200,000 years ago, in combination with a substantial increase in population size thereafter.

4.1 | Congruent main lineages at nuclear and plastid markers

The combination of both types of molecular markers used in this study reveals a distinct separation of *P. cembra* populations into two main genetic lineages (Figure S2.5b,c), even though the best supported grouping suggests five and nine clusters for the nuclear and chloroplast markers, respectively (Figure 2a,b). These two lineages occur in the East (Carpathians and Eastern Alps) and the West (west of the Swiss–Austrian border); hence, they do not relate to the physical separation of the two mountain ranges. Nevertheless, genetic differentiation was slightly lower between these lineages compared to when grouping populations according to their Alpine vs. Carpathian origins (Table S2.1). This finding reflects a similar degree of evolutionary separation of the two lineages within the Alpine range, as we find it between the spatially separated mountain ranges.

While this pattern may come as a surprise at first, it corroborates the low genetic differentiation observed between the Alps and the Carpathians previously reported (Höhn et al., 2009). Even more so, it coincides with the genetic relationships in other species, in particular herbaceous plants of high-elevation habitats (e.g., Mraz et al., 2007; Thiel-Egenter et al., 2009), whose occurrences in the Alps and the Carpathians are genetically linked through the Western Carpathians (Mraz & Ronikier, 2016). Such a coincidence suggests common drivers of migration acting on species during retraction and re-colonisation in response to glacial cycles. The transition between the two main genetic lineages in *P. cembra* relates to the so-called 'Brenner line', identified in 10 silicicolous herbaceous plant species in the Alps (Thiel-Egenter et al., 2011). This well-known biogeographical zone, where changes in species occurrences are also observed (Kerner, 1870), has previously been highlighted as marking an important genetic contact zone (Schönswetter et al., 2005). The location of this zone relates to relatively low elevations, which is less favourable for the occurrence of high-elevation plant species, hence slowing, if not preventing, effective migration and gene flow. The phylogeographical pattern of *P. cembra* is also similar to that found in *L. decidua* based on nSSR data (Wagner et al., 2015), with a contact zone located slightly west of the Brenner line. *Picea abies*, the dominant tree species of the subalpine zone in the Alps, also shows a similarly located genetic separation between Eastern and Western Alps (Gugerli, Sperisen, et al., 2001; Tollefsrud et al., 2008). This genetic distinction is most prominently visible in mitochondrial haplotype diversity of *P. abies*: high variation is found in the Eastern Alps, while western Alpine populations are nearly monomorphic, suggesting a strong bottleneck during westward re-colonisation (Gugerli, Sperisen, et al., 2001; Tollefsrud et al., 2008). This coincidence is noteworthy because it implies that currently co-occurring species may share demographic histories, which has hitherto received little attention in phylogeographical research (Carstens et al., 2007; Lyman & Edwards, 2022).

Demographic analysis and the hierarchical structure of the five genetic clusters based on nSSRs suggest that the eastern and western parts of the range of *P. cembra* make up distinct evolutionary lineages (Figure 2; Figures S2.4, S2.5) that relate to respective refugial origins during the LGM (Figures 3 and 4; see below). This structure is substantiated by the predominant grouping of cpSSR-based clusters into geographically matching eastern and western lineages (Figure 2b).

4.2 | Inconclusive patterns of genetic diversity

Patterns of genetic diversity estimates were not fully congruent, which reflects the weak population genetic structure encountered.

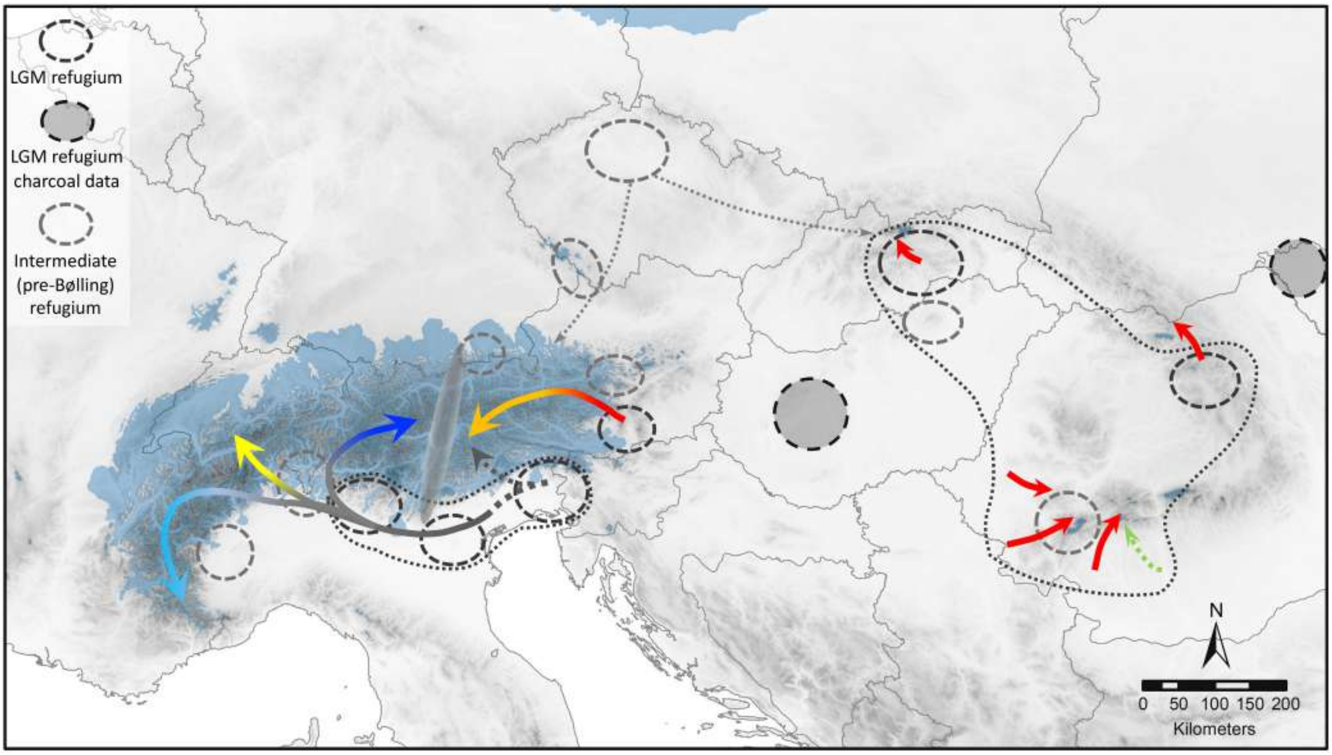

FIGURE 4 Synthesis map of the postglacial history of *Pinus cembra* based on extant population genetic structure, demographic inference and palaeoecological evidence. Inference from charcoal remains of *Larix/Pinus* spp. with partly uncertain taxonomic identification is based on Willis and Van Andel (2004). Coloured arrows refer to putative migration routes of respective genetic clusters as shown in Figure 2; Figure S2.4. The vertical grey bar marks the approximate area of the biogeographical Brenner contact zone, the blue area represents presumed ice cover at the Last Glacial Maximum (Ehlers et al., 2011).

While estimates of allelic richness (A_R) were evenly dispersed at the range-wide scale, we observed a trend towards lower A_R and expected heterozygosity (H_E) in the Western than in the Eastern Alps. Such a gradual trend would conform to the expectations about reduced genetic diversity in newly colonised areas (Widmer & Lexer, 2001) and agree with the previously suggested founder effects during the re-colonisation of the Alpine range from East to West (Gugerli et al., 2009). However, such a trend should theoretically be more pronounced for A_R than for H_E, and we found a few populations in the Southwest of the Alps that were also relatively diverse, though this region was colonised late. Levels of genetic diversity are governed by multiple demographic processes on top of the colonisation history, such as effects of genetic drift related to effective population size, and the degree of population isolation in the context of core–periphery dynamics (Dauphin et al., 2020). Despite these potentially counteracting processes, we detected a highly significant pattern of IBD. The respective plot reveals a classical structure for large-scale population sampling (Figure S2.7): a positive relationship between geographical distance and genetic differentiation up to ca. 500 km, and a wider scatter suggesting effects of genetic drift over longer distances. Such a pattern is in line with ample gene flow, as supported by the high level of admixture seen in the STRUCTURE results (Figure 2a). IBD was not significant among Carpathian populations, as already found by Lendvay et al. (2014).

The nSSR data also displayed elevated estimates of H_E in the westernmost Alpine populations, which could indicate a refugial area in this region. While this has been advocated in a regional study combining molecular and palaeoecological inference (Toth et al., 2019), the reliability of the latter was questioned (Finsinger et al., 2019). We cannot exclude that *P. cembra* also retained a refugial population in or near the southwestern Alps, but its relevance regarding the extant genetic structure is likely minimal.

4.3 | Palaeoecological inference of LGM refugial areas and re-colonisation routes

Based on pollen and macrofossil data (Figure 3), we can identify putative LGM refugial locations of *P. cembra* in the forelands of the southern Central Alps (e.g., Huber et al., 2010) and the Po Plain (e.g., in the North Italian Colli Euganei near Padova; Kaltenrieder et al., 2009), as well as in or near the Western, Eastern and Southern Carpathians (Fărcaş et al., 1999; Feurdean et al., 2011; Kuneš et al., 2008), and possibly the lowlands of the Bohemian massif (Petr & Novák, 2014). Charcoal finds further document the presence of larch–stone pine stands during the LGM in the Hungarian lowlands and in north-western Moldova near the Eastern Carpathians (Willis & Van Andel, 2004). However, there is no reliable evidence of a glacial refugium in or close to the Western Alps (Finsinger et al., 2019).

With the melting of the ice sheets and a first warming pulse after Heinrich event 1 at 16,700 cal. BP (Samartin et al., 2012), *P. cembra* populations rapidly expanded their range into the foothills and valleys of the southern Eastern Alps (from eastern Lombardy to Veneto, Friuli and Western Slovenia), reaching the southern slopes of the Western Alps (from western Lombardy to Ticino and Piedmont; e.g., Finsinger et al., 2011). Further warming during the Bølling–Allerød interstadial (beginning at ca. 14,700 cal. BP) led to an upward shift of *P. cembra* to intermediate elevations (ca. 1000–1600 m a.s.l.) in the southern Central Alps (Vescovi et al., 2007), its establishment in the Northern and Central Alps, and an expansion in the Carpathians and beyond. At the end of the Late Glacial, the range of *P. cembra* reached its largest extent, covering wide areas between the Alps and the Carpathians, and even extending northward into the Central European lowlands as documented by the inferred local presence in Western Ukraine (Figure 3a; Schwörer et al., 2021). With the rapid warming of 2–4°C in the Early Holocene (Heiri et al., 2014), *P. cembra* shifted to higher elevations in the Alps and reached the highest treeline positions, 200–300 m above today's treeline, during the HTM (9000–5000 cal. BP; Tinner & Theurillat, 2003; Fischer et al., 2018). However, the Holocene warming led to an overall range contraction of *P. cembra*, especially in Central and Eastern Europe. These dynamics were mostly driven by competition with temperate tree species, which resulted in the present-day disjunct populations in the Carpathians (Lendvay et al., 2014). In the westernmost Western Alps of France and Italy, *P. cembra* only became widespread during the Mid Holocene, as documented by many pollen and macrofossil records (Figure 3) as well as by charcoal and travertine deposits (Ali et al., 2005). There are a few sites that document its local presence since the Early Holocene (e.g., half-way between Geneva and Nice; Blarquez et al., 2010; Ponel et al., 2011), indicating that the late expansion in the Western Alps was not due to migrational lag, but possibly caused by local to regional environmental conditions. Also many sites in other parts of the Alps show highest abundances of *P. cembra* only after ca. 8000 cal. BP (e.g., Dietre et al., 2014; Schwörer et al., 2021; Tinner & Kaltenrieder, 2005), when the climate became gradually moister due to a large-scale reorganisation of atmospheric circulation patterns (Magny et al., 2003; Tinner & Lotter, 2006). The very dry (summer) conditions during the Early Holocene might have inhibited the widespread expansion in the Western Alps during this period.

4.4 | Links between extant genetic pattern and palaeoecological evidence

In an attempt to associate the genetic structure of extant populations with the palaeoecological evidence, we suggest that recolonisation from presumed LGM refugial areas into the current range came predominantly from (i) the southern forelands of the Alps (e.g., Piedmont, Lombardy, Veneto) and the Po Plain, and (ii) the fringes of the southern Eastern Alps (e.g., Friuli, Slovenia) as well as the Carpathian forelands and the Hungarian plain into the eastern range, possibly complemented by immigration from Bohemia and a distinct refugium near the Southern Carpathians (Figure 4). The latter hypothesis is supported by a distinct genetic composition of population Muntinul Mare (RO126), already highlighted by Lendvay et al. (2014), which would reflect the still trackable effect of a local refugium. In their study on the Chinese *P. tabulaeformis*, Hao et al. (2018) reached a similar conclusion regarding the impact of northern refugia on the extant genetic structure. In *P. cembra*, additional refugia suggested by palaeo-records, such as the lowlands of the Bohemian massif (Figure 4; Kuneš et al., 2008), may have contributed to current regional populations, but we cannot resolve their impact on the genetic structure with the data at hand. In turn, the available palaeo-data cannot reveal any indication of northern primary refugia, for example, along the unglaciated northern fringe of the Alps, to still show an effect on the extant genetic structure, as has been put forward in other species (Edwards et al., 2014; Stewart & Lister, 2001; Svenning et al., 2008; Zeng et al., 2015).

Estimates of effective population sizes indicate substantial expansion and reduction over the past 200,000 years, reflecting population dynamics from pre-LGM to recent times. Given the species' ecological characteristics and the palaeoecological evidence, range contraction for *P. cembra* and respective decrease in effective population size mainly occurred during interglacials ('warm period' refugia) and glacial periods ('cold period' refugia), whereas the species' range expanded during interstadials under intermediate (cool) climatic conditions. Nevertheless, this range expansion may not have been paralleled by an increase in population size, as the species may have occurred only scattered in locally benign habitats. Given the results of our ABC simulations, we can reasonably reject the alternative explanation of a formerly large population over a wide range that simply contracted and separated into the current range that is restricted to the high-elevation forests within the two European mountain ranges. While such a scenario might apply to the extant populations in the Carpathians (Lendvay et al., 2014), the range-wide demographic history seems better explained by a re-colonisation as described. Likewise, demographic analysis suggests a common origin of the three western genetic clusters that differentiated during westward re-colonisation after the LGM, rather than relating to three separate refugial areas.

4.5 | Refining postglacial migration and future perspectives of *P. cembra*

Given our current knowledge of the history of *P. cembra*, we see various opportunities for further in-depth research along the time axis. From a historical perspective, palaeo-genomics using, for example, macro-remains (Schmid et al., 2017; Schwörer et al., 2022) may enhance our understanding of the spatiotemporal dynamics of migration of specific genetic lineages. For example, lake sediment archives that contain macrofossils (Figure 3b) or currently melting glaciers that occasionally disclose logs and trunks of *P. cembra* at elevations beyond the current tree line (Nicolussi & Schlüchter, 2012), provide evidence of the species' past presence and, thus, stepping-stone populations that could be genetically characterised (Danusevicius et al., 2021; Parducci et al., 2012). Assigning genotypes of such samples to the extant genetic clusters could reveal specific postglacial colonisation routes. Moreover, one could track changes in allele frequencies over time at adaptive loci as imprints of selection due to (rapid) climate change. Complementary hindcasting through species distribution or ecological niche modelling could inform about the occurrence and connectivity of suitable habitat, a pre-requisite for effective migration (Gavin et al., 2014), but sufficiently resolved environmental data are currently lacking.

The distinct spatial genetic structure found in *P. cembra* is a relevant basis for delineating zones of seed sources and designating important areas for conserving forest genetic resources (Lefèvre et al., 2013). Defining a network of conservation-relevant stands should best comprise populations that reflect the species' demographic legacy. Moreover, such a distinct genetic structure allows tracking human long-distance seed transfer, that is when afforestation relied on allochthonous plant material, a valuable information for forest managers. For example, the Structure analysis revealed one population in the Bernese Oberland (CH54), embedded in the yellow WMA cluster (Figure 2a), with high assignment probability to the cluster dominating the eastern Alpine area (orange cluster in Figure 2a). Forestry archives indicated repeated planting in this local stand, also using reproductive material from South Tyrol (U. Ryter, pers. communication). Likewise, known planted stands in the northern Alps of western Switzerland (Fragnière et al., 2022) suggested eastern Swiss and eastern Alpine provenances (V. Sonnenwyl et al., pers. communication).

In view of future climate change, the knowledge of historical dynamics informs models of how *P. cembra* will respond to further increasing temperature (cf. Casalegno et al., 2010; Dauphin et al., 2021). The species will require to colonise areas above the current treeline where areas of suitable habitat will become smaller. In this context, seed dispersal by the nutcracker will play a pivotal role (Neuschulz et al., 2018), but also interactions with ectomycorrhizal and pathogenic fungi deserve attention. Finally, research will shift from neutral to adaptive genetic variation to better understand how selection and adaptation shape the genetic basis of *P. cembra* and may allow the species to persist in an expected warmer and drier climate.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

The full genotype data (nuclear and chloroplast microsatellites) and geographical coordinates of all study populations are available on Dryad repository under doi 10.5061/dryad.866t1g1v6.

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BIOSKETCH

Felix Gugerli and his team have investigated diverse aspects of the ecological genetics of Swiss stone pine, focusing on evolutionary dynamics on various spatial and temporal scales. Together with his colleagues, he studies population genetic patterns and their underlying processes, inferred from neutral and adaptive genetic variation and employing diverse molecular approaches, in forest trees, alpine plants and species of conservation concern.

Author contributions: FG designed the research, compiled and analysed the genetic data and wrote the manuscript, with contributions from BL, BD, FB, CM-M and CS; FG, SB, MH and BL performed the field sampling; FG, SB, BL and MH performed the laboratory work; BL, BD, GGV and FB contributed to population genetic analyses; CS, CM-M, WOvdK and WT collected and synthesised the palaeo-data, and all authors read, critically commented and edited previous versions of the manuscript.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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