Altitudinal segregation partially predicts survival of three alpine plant species across nunatak and peripheral glacial refugia

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Abstract
Survival of Quaternary cold stages in peripheral refugia and/or ice-free peaks within ice-sheets (nunataks) has likely (co)shaped the genetic structure of temperate mountain biota. We used three altitudinally segregated species endemic to the Dolomites and the adjacent Carnic Prealps in the southeastern European Alps to perform genetic structure analyses and demographic modeling based on RADseq data and retrospective species distribution models to test the following hypotheses. (i) The deep Piave valley forms the deepest genetic split in the species distributed across it. (ii) The montane to alpine species Campanula morettiana and Primula tyrolensis survived the Quaternary cold stages in peripheral refugia, while high-alpine to subnival Saxifraga facchinii likely survived in several nunatak refugia. (iii) The lower-elevation species suffered a strong population decline during the Quaternary glaciation. By contrast, the higher-elevation species shows long-term stability of population sizes due to survival on permanently ice-free peaks. We found peripheral refugia on both sides of the Piave Valley, which acted as a major genetic barrier. Demographic modeling confirmed nunatak survival not only for S. facchinii, but also for C. morettiana; results were inconclusive for P. tyrolensis. Altitudinal segregation influenced the species’ demographic fluctuations, with the lower-elevation species showing a significant population increase at the end of the Quaternary cold stages, and the higher-elevation species either showing decrease towards the present or stable population sizes with a short bottleneck. Our results highlight the role of both nunatak survival and of species ecology in the demographic history of mountain species.

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Running title: Phylogeography of Southeastern Alps endemics

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Abstract

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Keywords 4-6

endemic alpine plants, Dolomites, glacial refugia, phylogeography, RADseq, species distribution models

Introduction

The alternation of cold (glacial) and warm (interglacial) periods during the Quaternary caused large-scale expansions and contractions of glaciers and ice sheets in high latitudes and mountainous areas (Ehlers et al., 2011), such as the European Alps (Seguinot et al., 2018). During the Last Glacial Maximum (LGM, 20 ka; Clark et al., 2009), most of the Alps were covered by ice (Ivy-Ochs et al., 2008), which forced the distribution ranges of mountain plants to contract to three main types of refugia (Holderegger & Thiel-Egenter, 2009). (i) Nunatak refugia were located on summits and ridges protruding from the ice sheet, potentially allowing the survival of extremely cold-hardy species (Schonswetter et al., 2005) in interior parts of the Alps. (ii) Peripheral glacial refugia were mountainous areas, which remained largely unglaciated and provided less challenging conditions; in the Alps they were mainly situated at their southwestern, southern and eastern margin (van Husen, 1987; Schonswetter et al., 2005). Finally, (iii) lowland glacial refugia were located in the plains surrounding the Alps beyond the limits of the ice sheet (Holderegger & Thiel-Egenter, 2009). The latter refugia are expected to play a negligible role for high-elevation species of the Alps as they were covered by steppe, wetlands, and coniferous forest even at the LGM (Ravazzi et al., 2014; Wesche et al. 2016).
As long as no massive postglacial expansion replaced local lineages through genetic swamping (Todesco et al., 2016), survival in nunatak and peripheral refugia can in principle be distinguished by differences in the spatial genetic structure and diversity of extant populations (Bettin et al. 2007; Lohse et al., 2011; Schonswetter & Schneeweiss, 2019; Stehlik et al., 2002; Stehlik et al., 2001; Westergaard et al., 2011). Specifically, genetic groups exclusive to strongly glaciated areas, which do not overlap with potential peripheral refugia, are interpreted as descendants of nunatak survivors. Such groups are additionally expected to exhibit reduced within-population diversity as they likely underwent massive bottlenecks. In contrast, a genetic group overlapping with an area with weak LGM glaciation exhibiting high to moderate levels of genetic diversity is likely derived from recent range expansion from a peripheral refugium (Excoffier et al., 2009; Mraz et al., 2007; Tonin et al. 2023).

However, different processes may result in convergent patterns of genetic structure and diversity, challenging a proper interpretation (Lawson et al., 2018). For instance, due to a founder event in the course of range expansion from a peripheral refugium, a postglacially founded population might appear strongly differentiated, leading to the incorrect inference of nunatak survival. The advent of genetic demographic modeling approaches in recent years provided a framework to discern both scenarios as they allow for testing different models of divergence between populations and establishing estimates of divergence time (Massatti & Knowles, 2016; Pan et al., 2020). A better understanding of the temporal processes ultimately enables the discrimination between nunatak survival and postglacial colonization. Furthermore, modeling the fluctuation of effective population size can provide detailed insights into the response of populations to glaciation (e.g., Carnicer et al., 2022; Zaveska et al., 2021).

Altitudinally segregated mountain species have likely differentially responded to the Quaternary glaciation (e.g., Guerrina et al., 2022; Kropf et al., 2003; Surina et al., 2011; Theodoridis et al., 2017). While some have expanded their distribution ranges since the LGM, others may have receded; the exact responses being determined by the species’ climatic niches and dispersal limitations (Carnicer et al., 2022; Dullinger et al., 2012a). The most cold-hardy species may have survived the glaciations on nunataks as fragmented relics of formerly widespread, cold-adapted, alpine vegetation (Lohse et al., 2011; Pan et al., 2020; Schonswetter & Schneeweiss, 2019; Stehlik et al., 2002; Westergaard et al., 2011). On the other hand, less cold-adapted species have likely gone extinct in interior ranges and survived in peripheral refugia.

Our study area spans the Dolomites and the southwestern Carnic Preatls (Prealpi Carniche) within the Southern Limestone Alps, i.e., the southern, mostly calcareous ranges of the Eastern Alps (Figure 1). It comprises large areas close to the range’s periphery that remained weakly glaciated or unglaciated during the LGM (van Husen, 1987; Seguinot et al., 2018) and were consequently suggested as peripheral refugia (Escobar Garcia et al., 2012; Gyorgy et al., 2018; Ronikier et al., 2008; Schonswetter et al., 2005; Zaveska et al., 2021). In interior parts of the Dolomites, the surface of the LGM ice sheet reached up to 2000 m above sea level in the central valleys (van Husen, 1987). The area’s unique geomorphological and geological structures, best described as “mainland archipelagos” (Bosellini et al., 2003, Figure 1), produced a landscape of scattered nunatak “islands” surrounded by ice. The highest peaks have likely provided snow-free patches, at least during summers (Seguinot et al., 2018), thus allowing nunatak survival, as suggested for the central Dolomites based on distribution patterns of endemic flowering plants (Pignatti & Pignatti, 2016; Prosser et al., 2019).

The Dolomites and the southwestern Carnic Preatls are hotspots of biodiversity and endemism (Aeschimann et al., 2011; Pawlowski, 1970; Pitschmann & Reisigl, 1957; Tribisch, 2004). The border between both ranges is formed by the northeast-to-southwest running valley of the Piave river; it coincides with range limits of a suite of alpine species (Aeschimann et al., 2004) and delimits an area of endemism (Tribisch, 2004). Hypothetical refugia to the west and to the east of the Piave river may have hosted genetically differentiated lineages of mountain species, whose postglacial range expansion has potentially been constrained by the deep river valley. Similarly, at the scale of the entire Alps, major valleys such as the Aosta Valley or the “Brenner Line” were recurrently identified as important genetic break zones in various species (Gugerli et al., 2023; Thiel-Egenter et al., 2011).
Here, we studied the population genetic structure of three calcicolous plant species endemic to our study area based on RADseq data (Edwards et al., 2015). Subsequently, we tested demographic scenarios related to glacial survival using diffusion approximation (Gutenkunst et al., 2009) and combined this with effective population size modeling. Moreover, we modeled the species' climatic niches using species distribution models (Guisan & Zimmermann, 2000) and projected them to LGM scenarios to unravel past climatic habitat suitability. The study species include montane to alpine *Campanula morettiana* Rchb. (Campanulaceae) and *Primula tyrolensis* Schott ex Rchb. (Primulaceae), and high alpine to nival *Saxifraga facchinii* Koch (Saxifragaceae).

We focused on the following hypotheses. (1) The deep, southwest-to-northeast running Piave valley does not only form a major break in the distribution ranges of *C. morettiana* and *P. tyrolensis* (Figure 1), but also coincides with the deepest genetic split in these species. As unglaciated or weakly glaciated limestone areas existed on both sides of the valley, we accordingly hypothesize one peripheral refugium in the southern Dolomites to the west of the Piave river and one in the southwestern Carnic Prealps to the east of it. Consequently, we hypothesize a vicariance scenario or a founder event predating the LGM. (2) According to their adaptation to different elevations, the study species are expected to show differential patterns of genetic structure and glacial survival within the Dolomites. Specifically, (2a), we hypothesize that montane to alpine *C. morettiana* and *P. tyrolensis* exhibit a shallow genetic structure as they likely survived in a peripheral refugium and recolonized northerly adjacent areas after the LGM through founder events. In contrast (2b), high-alpine to subnival *S. facchinii* is expected to show a marked genetic structure as it likely survived in several separate nunatak refugia in central and northern massifs of the Dolomites. (3) We hypothesize that *C. morettiana* and *P. tyrolensis* suffered a strong glaciation-induced population decline because much of their present-day distribution ranges were covered by ice. By contrast, cold-adapted *S. facchinii* is expected to show long-term stability of population sizes as it likely survived the Pleistocene climatic oscillations on permanently ice-free peaks.

Material and Methods

Study species

We selected our study species *C. morettiana*, *P. tyrolensis*, and *S. facchinii* (Table 1, Figure 1) among the species with occurrence databases from Rota et al. (2022). The selected species had to match the following criteria. (i) Their distribution should be restricted to the southwestern Carnic Prealps and/or the Dolomites. (ii) Their altitudinal distribution should differ, but in principle span the montane to nival belts between 1400 and 3300 m a.s.l. (iii) They should not be closely related and (iv) grow in similar rocky habitats on carbonate bedrock.

Plant material

Leaf samples were collected in 43 sites in 2019 and 2020 covering the entire distribution range of each species, totalling 18 sites for *C. morettiana*, 12 for *P. tyrolensis* and 13 for *S. facchinii* plus suitable outgroups (Table S1). Young and healthy leaves of three to five individuals per population were sampled and stored in silica gel for the extraction of DNA; in population number 1 of *C. morettiana* ten individuals were collected. In some small populations the number of sampled individuals was reduced (Table S1). Herbarium specimens were not collected given the rarity and conservation status of the species. Collection permits were obtained from the administrative bodies when needed. Flow cytometry was used to establish the ploidy level of each investigated population (Supplementary Text 1).

DNA extraction

Total genomic DNA was extracted from ca. 10–20 mg dried leaf material following a CTAB protocol (Doyle and Doyle 1987) with modifications (Tel-zur et al., 1999). The ground leaf material was washed with a wash buffer containing sorbitol, three times for *C. morettiana* and *P. tyrolensis*, and twice for *S. facchinii*. The quality of the extracts was examined with a Nanodrop spectrophotometer (NanoDrop ND-2000, Thermo Scientific), subsequently they were purified with the Nucleospin gDNA clean-up kit (Macherey-Nagel). DNA
concentration was estimated using a Qubit 4 fluorometer (ThermoFisher Scientific).

RADseq: library preparation, identification of RADseq loci and SNP calling

Single-digest restriction-site associated (RADseq) libraries were prepared using the restriction enzyme PstI (New England Biolabs) and a protocol adapted from Paun et al. (2016). Briefly, we started with 95–140 ng DNA per individual and ligated 100 mM P1 adapters to the restricted samples. Shearing by sonication was performed with a M220 Focused-ultrasonicator (Covaris) with settings targeting a size range of 200–800 bp and a mode at 400 bp (peak in power: 50, duty factor 10%, 200 cycles per burst and treatment time 90 s at 20 degC). Libraries were sequenced on Illumina HiSeq as 100 bp single-end reads or NovaSeq 6000 SP as 150 bp paired-end reads at VBCF NGS Unit (http://www.vbcf.ac.at/ngs/). Sequences were further processed and analyzed using the LEO HPC infrastructure of the University of Innsbruck.

Raw reads were quality-filtered and demultiplexed based on individual-specific barcodes using Picard BamIndexDecoder included in the Picard Illumina2bam package (https://github.com/wtsinpg/illumina2bam) and the program process_radtags.pl in STACKS v. 2.3 (Catchen et al., 2011; 2013). RADseq loci were further assembled, and SNPs were called using the denovo_map.pl pipeline as implemented in STACKS. To select the optimal parameters, a preliminary optimization step following the 80% rule (Paris et al., 2017) was conducted. First, we selected 12–15 samples of each species and ran denovomap.pl for values of the number of mismatches allowed between stacks to merge them into a putative locus \((-M)\) from 0–8 and a percentage of individuals that must possess a particular locus for it to be included in calculation of population-level statistics \((-r)\) of 80%. The minimum number of raw reads required to form a stack \((-m)\) and the maximum number of differences among loci to be considered as orthologous across multiple samples \((-n)\) were given values equal to \(M\). After optimization, \(M = 5\) was selected as the value optimizing the number of de novo assembled loci for \(C. morettiana\) and \(P. tyrolensis\); \(M = 4\) was selected for \(S. facchinii\). These settings were used for subsequent runs of the pipeline with all individuals.

The program populations implemented in STACKS was used to export the selected loci and generate population genetics statistics, applying different filters for the subsequent analyses. Preliminary exploratory analyses of files generated under different filtering parameters in the R package ADEGENET 2.1.3 (Jombart, 2008) allowed us to select a filtering scheme with a good balance of missing data and amount of informative characters. For phylogenetic tree construction and phylogenetic networks, a vcf file was exported including the outgroup and using a minor allele frequency filter \((-\text{min\_maf})\) of three individuals and a maximum heterozygosity of 65% \((-\text{max\_obs\_het})\), following Rochette and Catchen (2017). The vcf files were then explored in ADEGENET; samples with a content of missing data higher than a certain threshold (50%) were excluded and populations was run again for the new dataset. The vcf files were further filtered with VCFtools 0.1.16 to keep loci with a minimum coverage of 10x and loci present in a minimum of 50% of the samples. For STRUCTURE, a minimum of 80% of individuals in the dataset \((-R)\) had to contain the locus for it to be processed; only the first SNP per locus was kept \((-\text{write\_single\_snp})\) to meet the assumption of unlinked SNPs, and the same minor allele frequency and maximum heterozygosity filters as for the previous analyses were applied.

Analyses of SNP data

The optimal grouping of individuals was determined using Bayesian clustering in STRUCTURE 2.3.4, using the admixture model with uncorrelated allele frequencies (Pritchard et al., 2000). Ten replicate runs for \(K\) (number of groups) ranging from 1 to 10 were carried out using a burn-in of 50,000 iterations followed by 500,000 additional MCMC iterations. CLUMPK (Kopelman et al., 2015) was used to summarize the results across different \(Ks\) and to produce plots. The optimal\(K\) was where the increase in likelihood started to flatten out, the results of replicate runs were similar, and the clusters were non-empty. Additionally, the delta\(K\) criterion was employed, reflecting an abrupt change in likelihood of runs across different \(Ks\)(Evanno et al., 2005). FineRADstructure (Malinsky et al., 2018) was used to infer the coancestry matrix of the same dataset but keeping all SNPs per RAD-locus. We used the program populations in STACKS to estimate summary statistics including number of private alleles and nucleotide diversity \((\pi)\) per population.
Adegenet was used to calculate Nei’s distance matrices (Nei, 1972), basis to construct Neighbor-Nets (Bryant & Moulton, 2004) in SplitsTree 4.14.2 (Huson & Bryant, 2006). To infer phylogenetic relationships among individuals, we computed maximum likelihood (ML) phylogenetic analyses using RAXML v. 8.2.12 (Stamatakis, 2014). Invariant sites were removed from the original phylip format using the script “deleteAlignColumn.pl” (https://www.biostars.org/p/55555/) and Felsenstein’s ascertainment bias correction was used to account for missing invariant sites as recommended (Leaché et al. 2015). Tree searches were done under a General Time Reversible model with disabled rate heterogeneity among sites as recommended in the RAXML v. 8.2.X manual (ASC_GTRCAT; -V; Stamatakis, 2014). The best-scoring ML tree was bootstrapped using 1000 replicates and the frequency-based stopping criterion (Pattengale et al., 2010). Results were visualized with FIGTREE 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Demographic modeling

To explore alternative glacial survival histories among genetic groups we used the diffusion approximation method δaδi (Gutenkunst et al., 2009) to analyze joint site frequency spectra (JSFS). We fitted two- and three-population demographic models using dadi_pipeline v3.1.4 (Portik et al., 2017; Figure S1). The genetic groups analyzed were chosen according to genetic structure and phylogenetic results. For population pairs with strong differences in sampling size, the JSFSs were computed for a subsample of the individuals of the group with the highest sample size. We fit different models corresponding with three hypotheses. First, related to hypothesis 1, we fitted 23 two-population models comprising vicariance and founder events in alternative directions (Portik et al., 2017; Charles et al., 2018; Záveská et al., 2021; Figure S1) for the divergence between Dolomites and southwestern Carnic Alps in C. morettiana and P. tyrolensis. To test different refugial hypotheses within the Dolomites related to hypothesis 2, nine two-population models comprising vicariance and founder events from the south to the north (west) were fit in the case of C. morettiana (Figure S1). For S. facchinii, five three-population models (Portik et al., 2017; Barratt et al., 2018; Firneno et al., 2020) were fit to explore whether the observed admixture in the central group was caused by gene flow or by hybrid origin (Firneno et al., 2020). Finally, seven two-population models were fit to test whether the strongly differentiated northernmost populations (one in C. morettiana and P. tyrolensis, and three in S. facchinii) originate from nunatak survival or recent founder events (Figure S1).

For all models, we performed consecutive rounds of optimizations to ensure convergence on similar log-likelihood scores (Portik et al., 2017). For each round, we ran multiple replicates using parameter estimates from the best-scoring replicate (highest log-likelihood) to seed searches in the following round. We used the default settings in the dadi_pipeline for each round (replicates = 10, 20, 30, 40; maxiter = 3, 5, 10, 15; fold = 3, 2, 2, 1), and optimized parameters using the Nelder-Mead method (optimize_log_fmin). Across all analyses, we used the optimized parameter sets of each replicate to simulate the JSFS; the multinomial approach was used to estimate the log-likelihood of the JSFS given the model. For each analysis, the AIC (Akaike Information Criterion) and log likelihoods were inspected, and ΔAIC scores were used to calculate relative likelihoods and Akaike weights (ωi). The model with highest Akaike weight was selected as the most likely for each divergence event (Burnham & Anderson, 2002). For the two best models of each analysis, the departure of simulated and empirical JSFSs and the progression of the log-likelihood along optimization rounds were inspected.

To verify that our models were reasonable explanations of the JSFS and to estimate the divergence time between genetic groups, we performed goodness-of-fit tests of the above-mentioned two-population models following Barratt et al. (2018). The three-population models had to be excluded from these tests due to limited computational resources. Shortly, for each population pair modeled with δaδi, we fit the top-ranked model using our optimized parameters, scaled the resulting model JSFS by the inferred theta value and used the model JSFS to generate 100 Poisson-sampled JSFS. We then optimized each simulated JSFS to obtain a distribution of log-likelihood scores and Pearson’s chi-squared test statistic and subsequently determined whether our empirical values were within these distributions. If the estimated statistics were not within the posterior distributions, we concluded that estimation of parameters under the selected model was inaccurate (Wegmann et al., 2010; Barratt et al., 2019). Estimated divergence times for each population pair were
plotted and confidence intervals calculated and transformed to conventional units, using a generation time of 10 years and the mutation rate of *Arabidopsis thaliana* $(7 \times 10^{-9}$, Ossowski et al., 2010) for all study species (Gutenkunst et al., 2009). If the distribution of the divergence times was not gaussian and showed extremely broad confidence intervals, we concluded that parameter estimation failed. Acknowledging the bias of AIC towards more complex models (Cavanaugh & Neath, 2019), the goodness-of-fit protocol was applied to the second-best model if the first failed, but only in the case that it had fewer parameters and the error between the empirical and simulated JSFS was not clearly worse than the best model. The same criteria as above were applied to the second-best model to estimate divergence times.

To explore population size changes associated with the last glaciation, we modeled the effective population size ($N_e$) using the software Stairway plot (Liu & Fu, 2015). First, we computed the folded SFS of each species and genetic group using easySFS (https://github.com/isaacovercast/easySFS) by downprojecting the datasets to a minimum sample size, maximizing the number of SNPs kept (Gutenkunst et al., 2009). We ran Stairway plot on 200 bootstrap replicates drawn from the calculated SFSs. Median $N_e$ and confidence intervals were obtained based on 200 estimations. In the absence of reliable data for close relatives, we used the mutation rate of *Arabidopsis thaliana* $(7 \times 10^{-9}$, Ossowski et al., 2010). No information on the generation times for the study species is available; we used 10 years as an approximate value.

**SDMs and climatic habitat suitability at LGM**

We projected the species distribution models (SDMs) from Rota et al. (2022) for our study species to LGM conditions. We downloaded 19 bioclimatic predictors from CHELSA database (Karger et al., 2021) for current (1981–2010) and LGM climate (i.e., 21 ka) and used three non-interdependent General Circulation Models (GCMs) (NCAR-CCSM4, MPI-ESM-P, MIROC-ESM) to account for variability in hindcasts at the LGM. To improve the models’ transferability, we used the first two axes of a principal component analysis (PCA) as environmental variables for species distribution modeling, calculated with the bioclimatic variables for current and LGM climates pooled together, with the R package “factoextra” (Kassambra et al., 2017). PCA axes were downscaled through Geographic Weighted Regression as in Rota et al. (2022), from 30 arcsec to 50 m resolution. We generated five projections for current climate (5 algorithms - CTA, GAM, GBM, GLM, RF, Table S3) and 15 projections for the LGM climate scenario (5 algorithms * 3 GCMs) for each species. We calculated the mean value of all projections for each cell, and masked SDM outputs using a carbonate geological layer obtained from the geolithological map of Italy (Geoprtale Nazionale, http://www.pcn.minambiente.it/mattm/). LGM projections were also masked with the ice sheet extent from Ehlers et al. (2011) and Van Husen (1987). SDM analyses were carried out using the ‘biomod2’ v 3.3-7.1 package (Thuiller et al., 2009) in R (version R/3.6.2) on the Vienna Scientific Cluster (VSC).

**Results**

**Genetic structure and phylogenetic relationships**

No intraspecific ploidy variation was encountered (Table S1). The average number of high-quality reads per sample retained after demultiplexing and quality filtering was 1.44 million (SD = 0.85). The data have been deposited in the NCBI sequence read archive (BioProject ######). The resulting number of SNPs per species and filtering scheme is presented in Table S2.

In the STRUCTURE analysis of *C. morettiana* (Figure S2a), the southwestern Carnic Prealps population 1 was split from the populations in the Dolomites at $K = 2$ (Figures 2a, b). At $K = 5$ the same southern, northwestern and northeastern groups detected in the Neighbor-Net for populations in the Dolomites were identified, with the northernmost population 18 constituting a single-population group. A certain degree of admixture was observed across all groups, particularly in the northeastern group. The fineRADstructure analysis showed similar results, with well-defined southwestern Carnic Prealps and Dolomites groups, and a high degree of substructure within the Dolomites (Figure S3a). For *P. tyrolensis* (Figure S2b), at $K = 2$ STRUCTURE resolved the southwestern Carnic Prealps populations 1–3 and populations 4–12 from the Dolomites (Figure 2c, d). Low admixture was detected in populations 4–7. At $K = 4$ the northernmost population 12 was differentiated. FineRADstructure showed similar results (Figure S3b). In *S. facchinii*
(Figure S2c) the best solution at $K = 2$ identified a southern (populations 1, 2) and a northern group (populations 5–13); the two central populations 3 and 4 were strongly admixed. At $K = 4$ the northern populations 10 and 11 constituted two non-admixed single-population groups, while the geographically close populations 12 and 13 formed a separate cluster (Figure 2c, f). The northern populations 5–9 were strongly admixed, with contributions of the three northernmost clusters, while populations 3 and 4 from the center of the distribution area were strongly admixed between the northern and southern genetic groups. The fineRADstructure analysis showed similar results, with well-defined southern and northern groups, which included clearly defined populations (Figure S3c). Similarly to the STRUCTURE analysis at $K = 4$, the two central populations 3 and 4 were admixed. Population genetics summary statistics for all three species are presented in Table S1 and Figure 2.

The Neighbor-Net and the outgroup-rooted RAXML phylogenetic tree of *C. morettiana* showed two main groups, one in the southwestern Carnic Prealps (population 1, BS 100%) and one in the Dolomites (2–18, Figure 2b, S4, BS 100%). Within the Dolomites, a southern (2–9), a northwestern (10–12) and a northeastern group (13–18) could be differentiated in the Neighbor-Net (Figure 2b). Similar to *C. morettiana*, the Neighbor-Net of *P. tyrolensis* showed two main groups, one in the southwestern Carnic Prealps (1–3) and one in the Dolomites (Figure 2d). In the RAXML tree these two groups had low support (BS 51–52%; Figures 2d, S5). The Neighbor-Net of *S. facchinii* showed a strong differentiation of the southern populations (1, 2), a northern group (5–13) with star-like pattern, and the central populations (3, 4) placed along the splits linking the northern and southern groups (Figure 2f). In the RAXML tree, the southern populations 1 and 2 were sister to the central and northern populations (3–13), which constituted a monophyletic group (BS 100%). The central populations were not monophyletic and formed the sister group of the northern clade (5–13, BS 100%). Populations 2, 3, 6, 10, 11, 12 and 13 constituted monophyletic groups with BS > 95% (Figures 2f, S6).

**Demographic modeling**

The number of SNPs used for each two-population scenario is reported in Figure S7; for the three-population model in *S. facchinii*, 30,450 SNPs were used. The log-likelihood increased along optimization rounds as expected for all two- and three-population models simulated with δa. The departure of simulated and empirical JSFSs was in some cases strong, indicating potential model fit problems (Figures S7, S8). The selected models are indicated in Figure S1. The goodness-of-fit tests indicated in some cases that the best model according to the AIC was not able to accurately estimate the parameters, indicated by broad distributions of the time estimation and/or deviations of the empirical log-likelihood or Chi-squared from the estimated distribution. In some of these cases, the second-best model provided a more reliable time estimate.

The divergence between populations from the Dolomites and the southwestern Carnic Prealps was best described by founder event models. However, in *C. morettiana* the estimated proportion of founder individuals from the original population was 0.5, and we therefore considered it vicariance. In *P. tyrolensis*, the direction of dispersal was from the southwestern Carnic Prealps to the Dolomites. In both cases, the divergence largely predated the LGM (Figures 3, S7). Within the Dolomites, modeling the split between the main groups of *C. morettiana* resulted in poor estimates using the best model according to the AIC, indicating lack of ability of the model to estimate the parameters, and therefore the second-best model was used. The divergence times between the northern and southern, as well as between the northern and southern populations predated the LGM. Since no clear grouping of *P. tyrolensis* populations within the Dolomites arose from the genetic structure analyses, no demographic modeling was conducted at this level. In the case of *S. facchinii*, the second-best three-population model showed a much better fit between the modeled and the empirical JSFS (Figure S8). Both models indicated secondary contact as the cause for the admixed pattern observed in the two central populations 5 and 6.

The divergence of the northernmost population 18 from the northern group in *C. morettiana* occurred by vicariance long before the LGM. The poor performance of the goodness-of-fit test (wide confidence intervals, deviation of the log-likelihood from its distribution) of the analysis with the southern group as potential
sister indicates that the population pair probably does not fit true divergence (i.e., the true divergence occurred between the northern group and population 18). Similarly inconclusive results were obtained for the northernmost population 12 of *P. tyrolensis* and for the northeasternmost population 10 of *S. facchinii*. Populations 11 and 12–13 of *S. facchinii* diverged via vicariance events in strict isolation from the northern group before the LGM.

The number of SNPs used for the Stairway plot simulations of the $N_e$ are reported in Table S4. The results revealed increases towards the present in all studied populations of *C. morettiana* and *P. tyrolensis*, with exception of the northernmost populations 18 of *C. morettiana* and 12 of *P. tyrolensis*, which showed no significant changes and a narrow confidence interval (Figure 3a, b). These populations had particularly small population sizes, which could negatively impact the estimations, and the results are therefore considered spurious (see Discussion, Table S1, Liu & Fu, 2015). These $N_e$ increases occurred at different times before the LGM, in most cases early enough to accommodate for the uncertainty introduced by imprecise mutation rates and generation times. *Saxifraga facchinii* showed an ancestral $N_e$, which either suffered a temporal drop followed by recovery (southern and northern group), or gradually decreased towards the present (northernmost populations 10–13 and central populations 3–4; Figure 3c).

SDMs for current and LGM climatic conditions

Model evaluation under current climatic conditions indicated a good model performance for all modeling techniques and species (Table S5, Rota et al., 2022). For *C. morettiana* and *P. tyrolensis*, current distribution areas were well covered by the SDM predictions for current climatic conditions (Figures S9, S10), but many areas predicted as suitable are currently not occupied by the species (Table S6). The actual distribution area of *S. facchinii* was well covered by the SDM projections for current climatic conditions; large areas were not climatically suitable (Table S6, Figure S11). At the LGM, the habitat suitability (HS) for *C. morettiana* was higher in the central and northern Dolomites, where the species currently occurs (Figure S12). In contrast, the extent of putative suitable areas was lower than at present, because many cells were covered by glaciers (Table S6, Figures 2, S9, S12). For *P. tyrolensis*, the extent of putative suitable areas was smaller at the LGM than at present (Table S6, Figures 2, S10, S13). At the LGM, *S. facchinii* showed high habitat suitability within the continuous ice sheet (Figure 2, Figure S14). HS was high across most of the area, except for the lowlands southerly adjacent to the Dolomites. At the LGM the suitable area was larger than today (Table S6, Figures 2, S11, S14). For all species, high habitat suitability (HS) at the LGM was detected at the southern and periphery of the Dolomites and the Carnic Preamals, where currently only *P. tyrolensis* locally occurs (Figures S12–S14).

Discussion

The Piave Valley separates peripheral refugia in the Dolomites and the Carnic Preamals

Biogeographers have, since the origin of the discipline, identified barriers all over the world, which define distribution limits across different organisms. The Wallace line between Asia and the Indo-Australian Archipelago is among the best-known examples (Wallace, 1863). Barriers coinciding with deep valleys have been identified in many mountainous regions of the world, including the “Western Andean Portal” splitting the northern and central Andes (Antonelli et al., 2009) or the Neretva Valley subdividing the Dinaric Mountains (Śpaniel & Rešetnik, 2022). At the intraspecific level, these valleys define genetic splits, which are congruent across codistributed species (Antonelli et al., 2009; Qiu et al., 2011; Śpaniel & Rešetnik, 2022). The role of major valleys as barriers has been highlighted at the scale of the European Alps (Gugerli et al., 2023; Thiel-Egenter et al., 2011; Tribsch, 2004), with the Aosta Valley and the “Brenner Line” emerging as the most prominent barriers.

Within the Southern Limestone Alps, the Piave Valley was identified as an important biogeographic barrier. Indeed, the main genetic break in *C. morettiana* and *P. tyrolensis*, defining the split between the two main intraspecific genetic groups, follows the Piave Valley, the geographic border between the Dolomites and the Carnic Preamals (Figure 1). This strong differentiation is reflected in clusters in the genetic structure analyses, main groups in the Neighbor-Nets and the main phylogenetic split in *C. morettiana* (Figures 2, S3–S5). The
Piave Valley roughly coincides with major genetic breaks in the (sub)alpine species *Campanula barbata* L., *Hypochaeris uniflora* Vill. and *Phyteuma betonicifolium* Vill. (Thiel-Egenter et al., 2011), as well as for the rupicolous snail *Charpentiera itala* (Xu & Hausdorf, 2021). Marked genetic differentiation is interpreted as old divergence events, and is considered, together with the observed high levels of genetic diversity (\( \pi \), Table S1) and high numbers of private alleles (Figure 2, Table S1), indicative for independent peripheral glacial refugia (e.g., Carnicero et al., 2022; Schönswetter et al., 2005; Westergaard et al., 2019; Záveská et al., 2021).

We used a demographic modeling approach to test the existence of glacial refugia east and west of the Piave Valley, which supported divergence times between the groups largely predating the LGM (Figure 3a, b; Figure S1). These results are the first molecular-based evidence of a refugium for calcicolous species in the Dolomites and the southwestern Carnic Prealps. To date, only the siliceous parts of the southern Dolomites were identified as glacial refugium for silicicolous mountain plants (Schönswetter et al., 2005; Ronikier et al., 2008; Escobar García et al., 2012; György et al., 2018). We observed clear patterns of substructure within the Dolomites (see next section) only for *Campanula morettiana*, and no subdivision was evident in the Carnic Prealps (Figure 2). However, the strongly uneven sizes of the partial distribution ranges west and east of the Piave Valley preclude further interpretations.

The estimated climatic suitability for the two species was high in the southwestern Carnic Prealps both at present (Rota et al., 2022) and during the LGM (Figures S9, S10, S12, S13). This is indicative for stable suitable conditions across the Pleistocene climatic fluctuations, further supporting the refugium east of the Piave Valley. In contrast, the valley floor was unsuitable for alpine species, either due to presence of a glacier tongue during glacials, or expansion of forests during interglacials (Magri et al., 2015; Seguinot et al., 2018). However, the moderate levels of admixture observed and the selection of demographic models with migration indicate a certain permeability of the barrier (Figures 2, 3). For lower-elevation species linked to forests such as *Aposeris foetida* and *Helleborus niger* or the amphibian *Salamandra atra*, the Piave Valley delimited no major intraspecific genetic groups (Bonato et al., 2018; Voisin et al., in prep; Záveská et al., 2021). In line with this finding, previous studies (e.g., Strait of Gibraltar: Nieto Feliner, 2014; the Andes: Antonelli & Sanmartín, 2011) have identified the same geographic feature as either barrier or corridor for species with different ecological preferences.

Evidence of multiple glacial refugia within the Dolomites

The retrieved genetic structure of the study species within the Dolomites rejected the hypothesized recent expansion from a southern refugium for *Campanula morettiana*; for *Phyteuma tyrolensis* the results were ambiguous. Specifically, in *Campanula morettiana* a marked genetic structure was evident, albeit with significant levels of admixture (Figure 2a). The three main groups identified corresponded to geographic units, whereas the northernmost population 18 formed an additional single-population group at higher values of \( K \) (discussed below). Additionally, high values of habitat suitability were recorded for *Campanula morettiana* throughout its range at the LGM (Figure 2a). In *Phyteuma tyrolensis*, the weak genetic structure and the star-shaped Neighbor-Net along with low habitat suitability towards the north during the LGM (Figure 2c, d) rather support a recent-expansion scenario. Given the lack of a clear grouping of *Phyteuma tyrolensis* populations within the Dolomites in the genetic structure analyses, no demographic modeling was conducted at the level of the entire Dolomites. The differentiation of the northernmost population 12 as a unique genetic group at \( K = 4 \) is likely postglacial (discussed below).

Divergence times between the main genetic groups in *Campanula morettiana* largely predated the LGM (Figures 3a, S1), which is a clear indication for at least three glacial refugia in the southern (populations 2–9), western (populations 10–12) and northern Dolomites (populations 13–18). These findings reject the hypothesized postglacial northward migration from a peripheral refugium and related founder events in this species. The southern and western glacial refugia were probably more favorable than the northern one, where a recent bottleneck was observed, not estimated for the two other groups (Figure 3a). This pattern correlates with the elevation of the glacier surface, which was higher in the central Dolomites than towards their southern and western margin (van Husen, 1987) but cannot be observed as decreased habitat suitability during the LGM in our SDMs (Figure 2a).
For subnival *S. facchinii* several glacial refugia were expected, which was confirmed by the strong genetic structure observed (Figure 2e) and by high suitability values at the LGM within the current distribution range (Figure S14). Further, our models indicate that the strong admixture in the central populations 3 and 4 results from secondary contact of the southern and northern groups with the central populations, and not from a recent admixed origin (Figure 3c). This, therefore, suggests the presence of at least three refugia in the north, center and south of the current distribution area. Interestingly, the three northernmost massifs hosting populations 10–13 constituted divergent unique genetic groups at higher Ks.

Indeed, a congruent pattern among the three study species is the divergence of the northernmost populations at high Ks (population 18 of *C. morettiana*; population 12 of *P. tyrolensis*; populations 10–13 of *S. facchinii*; Figure 2). The divergence of single populations is often the result of strong genetic drift, which may result from different demographic histories (Lawson et al., 2018). Demographic modeling allows distinguishing between two relevant scenarios. Specifically, divergent populations may be old populations (i.e., predating the LGM), which strongly drifted due to small population size and lack of gene flow with neighboring groups. Alternatively, divergence may be caused by strong drift due to bottlenecks related to recent founder events (i.e., postglacial colonization). In population 18 of *C. morettiana* and populations 11–13 of *S. facchinii*, the old divergence times allow concluding that these populations diverged before the LGM (Figure 3, Figure S1), and therefore survived at least the LGM in nunatak refugia (Holderegger & Thiel-Egenter, 2009). In contrast, models were inconclusive for the northernmost population of *P. tyrolensis* and population 10 of *S. facchinii*.

While evidence for peripheral glacial refugia clearly prevails (Schonswetter et al., 2005), nunatak survival has been invoked in several studies on alpine or arctic species as the most parsimonious hypothesis to explain observed genetic structure patterns (Bettin et al., 2007; Lohse et al., 2011; Schonswetter & Schneeweiss, 2019; Stehlik et al., 2001; Stehlik et al., 2002; Westergaard et al., 2011). To date, the most unequivocal support for nunatak survival came from range-wide studies unraveling unique divergent genotypes in areas with strong LGM glaciation, while widespread genotypes dominated in potential peripheral refugia (Stehlik et al., 2002; Escobar Garcia et al., 2012). Recently, the application of spatially explicit demographic models has provided independent support for nunatak survival in different geographic settings (*Carex chalciolepis* Holm.: Massatti & Knowles, 2014; *Pedicularis asplenifolia* Florke ex Willd.: Pan et al., 2020).

Disentangling artifacts from the true demographic history

The inclusion of goodness-of-fit tests in our demographic modeling approach proved highly useful. Amongst others, they allowed us to approximate confidence intervals around divergence times estimates. Most importantly, they provided a double-check of model accuracy (Barratt et al., 2018); often, the best model selected based on the AIC showed a deficient fit and inability to estimate the parameters of the model (Figures S1, S7, S8). While the exact reason for the inaccuracy of the selected models cannot be ascertained, some factors may increase the probability of a misleading model selection (Adams & Huson, 2004; Robinson et al., 2014). A central one is lack of statistical power, understood here as the ability to detect the true model. It results from lacking data to inform the model selection, either due to a low number of sampled individuals per population, insufficient genomic information (i.e., number of SNPs), or both.

Previous simulation studies have highlighted the importance of sufficient sample size for selecting the correct model and estimating the parameters (Adams & Huson, 2004; Liu & Fu, 2015; Robinson et al., 2014). While old and pronounced demographic events were accurately modeled in these studies based on a sampling of three to five individuals per genetic group, larger sample sizes were necessary for correctly describing recent and more moderate events. Moreover, these simulations were performed based on a number of SNPs greater than 10,000, much higher than here and in most RADseq-based publications. In these cases, a higher sample size (Robinson et al., 2014; Lou et al., 2021) should be considered to achieve satisfactory results. In our case, the demographic analyses of single northernmost populations do clearly not meet these thresholds as sample sizes range between four and six individuals. Additionally, the bias of AIC towards more complex models is well-known (Cavanaugh & Neath, 2019), and it is therefore recommended to compare the best model with models with fewer parameters. In cases braving the edge of recommended sampling size and
number of SNPs, we strongly advocate a careful and conservative interpretation of the results, application of additional tests such as the goodness-of-fit, and visual inspection of simulated and empirical SFSs.

Altitudinal segregation largely determines glaciation-induced demographic fluctuations

Differences in ecological preferences can determine divergent demographic histories in codistributed species (Carnicero et al., 2022; Massatti & Knowles, 2014). In mountain regions, ecological differences are often reflected in altitudinal segregation, which may confer survival in different glacial refugia ‘displacement refugia model’; Kropf et al., 2003; Surina et al., 2011). Here, we suggest that altitudinal segregation of our study species is causing the major differences in the temporal fluctuation of effective population size across species. On the one hand, C. morettiana and P. tyrolensis showed reduced effective population sizes in the past, followed by an increase towards the present (Figure 3a, b), which we interpret as a recovery of population size after unsuitable conditions during glacial periods. These results are in line with the climatic SDM projections for P. tyrolensis, that show higher values of habitat suitability and large suitable areas at the LGM in southeastern peripheral areas, but not within the ice sheet. For C. morettiana, in contrast, northern areas within the ice sheet were climatically suitable at the LGM, which together with the above-mentioned results (section ‘Evidence of multiple glacial refugia within the Dolomites’) suggests in-situ survival under harsh conditions as illustrated by the decrease in population sizes for the northern populations.

In contrast, S. facchinii exhibited similar or higher effective population sizes in the past than at present for all main genetic groups (Figure 3c). Accordingly, S. facchinii is showing both higher values of habitat suitability and larger suitable areas at the LGM within the ice sheet as compared to the present. This pattern suggests the availability of sufficiently large, climatically suitable areas for the species’ survival during cold periods, in some cases even allowing for larger effective population sizes than at present (populations 3–4, 10, and 12–13; Figure 3c). However, the marked, short-time bottlenecks for the northern (5–9) and southern (1, 2) populations indicate a short period of reduced climatic suitability, probably corresponding to particularly harsh conditions at the LGM. We suggest that the plateau morphology of the mountains harboring these particular populations might have favored formation of permanent snowfields and ice caps during the LGM, which strongly reduced the available area for S. facchinii during a relatively short period.

Taken together, the different demographic trajectories reconstructed for the three study species can be largely explained by their altitudinal segregation and the landscape of the Dolomites during glacial periods. Then, glaciers covered all main valleys, leaving ice-free areas above them (Van Husen, 1987) with harsh conditions, likely comparable to those of the nival belt at present (Korner, 2021). There, S. facchinii might have found enough suitable habitat to maintain relatively large populations, whereas C. morettiana and P. tyrolensis likely survived in smaller suitable patches. The stable effective population sizes with a narrow confidence interval observed in the northernmost population 18 of C. morettiana and population 12 P. tyrolensis, as well as in population 11 of S. facchinii, are interpreted as artifacts, likely resulting from the above-discussed lack of statistical power to accurately model the populations’ demographic history (Liu & Fu, 2015).

Conclusions

We provide the first evidence for two refugia for calcicolous species in the Dolomites and the southwestern Carnic Prealps, which reflect the area’s geography as well as previously identified areas of endemism (Tribisch 2004). We additionally highlight the importance of nunatak survival in the Dolomites in addition to peripheral refugia (Holderegger & Thiel-Egenter, 2009; Pan et al., 2020). Glacial survival patterns strongly depend on ecological preferences of the study species with nunatak survival in S. facchinii, nunatak and peripheral refugia for C. morettiana, and no strong evidence for nunatak survival for P. tyrolensis. Altitudinal segregation strongly influenced the species’ demographic fluctuations, with C. morettiana and P. tyrolensis showing higher effective population sizes at present than during glacial periods, and S. facchinii showing the opposite trend, in congruence with warm-stage refugia for cold-adapted species (Gentili et al., 2015). While it has been argued that cold-adapted species can face warming through short shifts into topographical micro-refugia maintaining cold conditions (Korner & Hiltbrunner, 2021), we show how warming since the
LGM had a negative impact on effective population size of several populations of *S. facchinii*. Even if these cold-adapted species can survive in micro-refugia the next few decades, the decrease of effective population size could reach a critical point, resulting in an increase of inbreeding and accumulation of deleterious mutations, eventually leading to extinction (Dullinger et al., 2012b; Kardos et al., 2021).

References


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Data Accessibility

Raw sequence reads are deposited in the SRA (BioProject XXX, will be provided after eventual acceptance)

Author Contributions


Figures
Figure 1 Study area and investigated species.  

a) View of the Dolomites from Piz Lavarella (Conturines massif) towards the southwest, showing the typical geomorphology of the Dolomites, i.e., Triassic coral reefs, which now form sky-islands. 

b) Density of occurrence along the elevation gradient for all known populations of each species. 

c) Distribution ranges of _Campanula morettiana_ (blue dots and solid line), _Primula tyrolensis_ (pink dots and short-dashed line) and _Saxifraga facchinii_ (red dots and long-dashed line). White triangles highlight the Piave Valley. Green-colored areas represent carbonate bedrock, which is suitable for the study species. The hillshade represents the elevation. Maps were generated with UTM 32 N projection, with datum WGS84. Photo of landscape, _C. morettiana_ and _S. facchinii_ by F. Rota, photo of _P. tyrolensis_ by J. Nascimbene.
Figure 2 Habitat suitability at the Last Glacial Maximum (LGM) and genetic structure of *Campanula morettiana* (a–b), *Primula tyrolensis* (c–d) and *Saxifraga facchinii* (e–f). In the maps, white and black dots represent sampled and unsampled populations, respectively; numbers are population identifiers. Pie charts indicate admixture proportions (at \( K = 5 \) for *C. morettiana* and at \( K = 4 \) for *P. tyrolensis* and *S. facchinii* ) derived from STRUCTURE analyses. Sizes of pie charts reflect the number of private alleles (PA). Light-blue areas indicate the maximum extent of glaciers during the LGM 21,000 years ago (based on Ehlers et al., 2011, Van Husen 1987). The climatic habitat suitability (HS) at the LGM ranges from 0 (light yellow) to 1 (red). Areas with siliceous bedrock, which is unsuitable for the study species, are grey shaded, lowland areas below 150 m above sea level are white. Maps were generated with UTM 32 N projection, with datum WGS84. The STRUCTURE bar plots depict the cluster memberships of each individual at the two best Ks. In the Neighbor-Nets (b, d, f), populations are colour-coded according to the most likely clustering solution in STRUCTURE analyses. Populations with <70% assignment to a group are in black depending on the genetic cluster.
Figure 3 Demographic modeling analyses of (a) *Campanula morettiana*, (b) *Primula tyrolensis* and (c) *Saxifraga facchinii*. The upper panels illustrate the geographic configuration of the tested scenarios. White and black dots represent sampled and unsampled populations, respectively; numbers are population identifiers. The base map is a digital elevation model overlaid with the extension of glaciers at the Last Glacial Maximum (LGM). Coloured outlines indicate the population groups used in the different demographic comparisons, which are illustrated with arrows equipped with letters. The lower panels show the demographic modeling results; color coding of population groups and letters match those in the upper panels. The left column shows a graphical representation of the selected two- or three-population models in $\delta^{2}\alpha$; “2nd best model” indicates that the depicted model does not correspond to the best model according to the Akaike Information Criterion (see the main text for an explanation). The central column shows the distribution of the estimated divergence time after the goodness-of-fit test with 100 replicates. The histogram represents the distribution, the blue curve shows the density distribution, and the red line represents the LGM (20 ka). In case of three-population models in (c), the goodness-of-fit test could not be performed, instead a graphical representation of the second-best three-population model is shown. The right column shows the change of the effective population size ($N_e$) over time modeled with the software Stairway plot. The coloured curves indicate the fluctuation of $N_e$ of the analyzed population groups, 95% confidence intervals are shown as dotted lines.