

**Rekonstruktion der eiszeitlichen Verbreitung
und Artbildung vier alpiner Primeln
durch Artenverbreitungsmodelle und
Phylogeographie**

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Primula marginata Standort, ND de Clausius Richtung Col Blanchet, Cottische Alpen

Allgemeine Einleitung

Die Alpenaurikel sind eine kleine Pflanzengruppe, die in alten lateinisch verfassten Kräuterbüchern häufig unter der Bezeichnung „Auricula ursi“ (dt. Bärenöhrchen) bezeichnet wird. Sie ist die einzige rein europäische Primelgruppe (*Primula* Sektion *Auricula*), zu der auch die meisten europäischen Primelarten gehören. Die Gruppe ist wahrscheinlich ostasiatischen Ursprungs und umfasst 25 Arten, die in den europäischen Gebirgszügen endemisch sind. Vor etwa 2,4 Millionen Jahren entstanden aus dem gemeinsamen Vorfahren der Sektion zwei Teilgruppen (Zhang & Kadereit, 2004b). Diese sind in den westlichen (westliche Gruppe) bzw. östlichen (östliche Gruppe) europäischen Gebirgen verbreitet und geographisch weitgehend vikariant. Die Grenze zwischen den beiden Teilgruppen verläuft in den Alpen, in denen die meisten der Arten vorkommen. Zhang et al. (2004a) formulierten die Hypothese, dass diese erste Aufspaltung in die beiden Teilgruppen durch eine frühe Vergletscherung der Alpen ausgelöst wurde, die das Gesamtareal des Vorfahren in ein westliches und ein östliches Refugium aufteilte. Spätere Untersuchungen zeigten, dass die Aufspaltungsereignisse in der westlichen Gruppe mit den Klimaveränderungen im Quartär zusammenhängen und die meisten Arten wahrscheinlich während der Eiszeiten entstanden sind. In der östlichen Gruppe besteht dieser Zusammenhang allerdings nicht (Kadereit et al., 2004). Eine Konzentration von Artbildungsereignissen in den Eiszeiten des Quartärs impliziert, dass allopatrische Artbildung (Artbildung in geographisch isolierten Arealen) in eiszeitlichen Refugialgebieten der häufigste Artbildungsmodus in der westlichen Gruppe war.

In der vorliegenden Arbeit sollte diese Hypothese durch die Modellierung eiszeitlicher Refugialgebiete mit Artenverbreitungs-Modellen (engl. species distribution models, SDMs) einerseits, in Zusammenarbeit mit dem ‚Department of Ecology and Evolution‘ (Universität de Lausanne), und der Analyse der geographischen Verbreitung innerartlicher genetischer Variation andererseits untersucht werden. Potentielle Refugialgebiete alpiner Pflanzen wurden in frühen Studien durch Vergleiche rezenter Verbreitungsmuster und der geographischen Verteilung von floristischem Arten- und Endemitenreichtum ermittelt (z. B. (Brockmann-Jerosch & Brockmann-Jerosch, 1926, Merxmüller 1952, 1953 & 1954), zusammengefasst in (Stehlik, 2000). In neuerer Zeit werden molekulare Methoden verwendet und phylogeographische Muster von Alpenpflanzen mit potentiellen Refugialgebieten verglichen, die auf der Grundlage von Paläo-Umweltdaten postuliert werden (vgl. Schlußbetrachtungen Teil 2) (Stehlik 2003, Diadema et al., 2005, Schönswetter et al., 2005, Naciri & Gaudeul, 2007, Parisod & Besnard, 2007, Szövényi et al., 2009). Tribsch & Schönswetter (2003)

verglichen solche hypothetische Refugialgebiete mit der geographischen Verteilung von Endemitenreichtum *und* phylogeographischen Mustern verschiedener Alpenpflanzen in den Ostalpen. In neuester Zeit werden zunehmend SDMs benutzt, um auf der Grundlage heutiger klimatischer Nischen einzelner Pflanzen oder Tiere, oder den klimatischen Rahmenbedingungen ganzer Vegetationseinheiten eiszeitliche (oder holozäne) Verbreitungen zu rekonstruieren (Hugall et al., 2002, Carstens & Richards, 2007, Waltari et al., 2007, Carnaval & Moritz., 2008, Pearman et al., 2008b, Alsos et al., 2009, Cordellier & Pfenninger, 2009, Jakob et al., 2009). Diese modellierten eiszeitlichen (holozänen) Verbreitungen werden mit statistisch-phylogeographischen, phylogenetischen oder populationsgenetischen Analysen, oder mit Pollenfunden verglichen und evaluiert. In der vorliegenden Arbeit verwenden wir diese Methode zu unserem Wissen erstmals, um eiszeitlichen Refugien von europäischen Alpenpflanzen zu rekonstruieren und werden diskutieren ob, und welche neuen Einsichten die Einbeziehung dieser Datenquelle bringt.

Zur Untersuchung wurden zwei auf der Grundlage einer molekularen Phylogenie (internal transcribed spacer, ITS) statistisch relativ gut unterstützte Schwesterartenpaare, *P. hirsuta*/*P. daonensis* und *P. latifolia*/*P. marginata*, aus der westlichen Gruppe ausgewählt (Zhang & Kadereit, 2004b). Da diese Phylogenie nicht vollständig aufgelöst war, unternahmen wir in Zusammenarbeit mit S. Crema und G. Cristofolini (Universität Bologna) parallel zu dieser Arbeit Anstrengungen, um die Verwandtschaftsverhältnisse mit Hilfe weiterer molekularer Marker (cpDNA und AFLPs) aufzuklären (Crema et al., in prep.). Dabei wurden überraschenderweise die zuvor relativ gut gestützten Schwesterbeziehungen zwischen *P. marginata* und *P. latifolia* sowie *P. hirsuta* und *P. daonensis* nicht wiedergefunden. Die Arten beider Paare sind aber nach wie vor eng miteinander verwandt und stehen zusammen mit anderen Arten in phylogenetischen Polytomien. Generell konnten die Verwandtschaftsverhältnisse innerhalb der westlichen Teilgruppe trotz der zusätzlichen molekularen Marker nicht besser aufgelöst werden, und die phylogeographischen Ergebnisse deuten darauf hin, dass mehrere Arten simultan, d.h. während der selben Eiszeit/en aus dem gleichen Vorfahren hervorgegangen sind. Dies steht im Einklang mit der zuvor formulierten Hypothese, dass in der westlichen Gruppe Artbildung in geographisch isolierten Eiszeitrefugien ancestraler Arten stattgefunden hat.

P. latifolia und *P. marginata* können dennoch als Schwesterarten in einem weiteren Sinn betrachtet werden, da sie aus demselben Vorfahren hervorgegangen sind. Somit konnten im ersten Artikel der vorliegenden Arbeit die geographischen Umstände der Artbildung bei

P. marginata und *P. latifolia* untersucht werden. Im zweiten Artikel wird die eiszeitliche sowie die postglaziale Geschichte von *P. hirsuta* und *P. daonensis* untersucht und diskutiert.

1. Artikel

Exploring modes of speciation in Alpine *Primula* by combining paleodistribution models and phylogeographic approaches

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Introduction

There is a longstanding debate whether the Quaternary with its drastic successive climate oscillations and Ice Ages was a period of extinction or stasis, or rather a period speciation. Some authors argue that the Ice Ages were not important in terms of evolution, and their findings support evolutionary stasis (Coope, 2004). One possible reason for this finding is that, due to the repeated environmental changes during the Pleistocene, the divergence of fragmented populations during cold phases could have been lost during subsequent warm phases by hybridization of re-overlapping distribution ranges (Coope 1978, Bennett 1990). On the other hand, the hypothesis that the Quaternary Ice Ages were an important time for genetic diversification and speciation has become a research focus during the past decade. The steady accumulation of dated molecular phylogenies clearly shows that much speciation took place in different parts of the world during the Quaternary including *Primula* sect. *Auricula* in the European Alpine system (Knowles, 2001, Richardson et al., 2001, von Hagen & Kadereit, 2001, Veith et al., 2003, Richardson et al., 2004, Zhang et al., 2004a, Zhang et al., 2007).

The implementation of species distribution modeling (SDM) brings new insights into the ongoing debate about Pleistocene influences on the diversification of plants and animals (Graham et al., 2004, Knowles et al., 2007, Bell et al., 2007, Kozak et al., 2008, Jakob et al., 2009). Here we set out to investigate the geographical mode of speciation in one sister species pair (*Primula latifolia* and *P. marginata*) of the European mountain endemic *Primula* sect.

Auricula using a combination of species distribution modeling and phylogeographic approaches. Species distribution modeling, also called environmental niche modeling (ENM), is an approach to investigate Quaternary distribution and is especially useful in the absence of an extensive fossil record as typical for most mountain plants of the European Alps. The method is based on the relationship of species occurrence and environmental data (Guisan & Zimmermann, 2000) and it provides an estimate of the historical distribution based on current ecological niches of species. Its use has been pushed by the increasing availability of digital maps of recent and paleoclimate estimates. Paleodistribution maps are generated by projecting SDMs back in time using these paleoclimate estimates.

SDMs and georeferenced environmental data are increasingly applied to evolutionary questions (reviewed in Kozak et al., 2008 and Pearman et al., 2008b). Several authors (Peterson et al., 1999, Graham et al., 2004, Kozak et al., 2006, Carstens & Knowles, 2007, Jakob et al., 2007) for example used modeling approaches to investigate the evolutionary history of lineages. They located probable geographical areas involved in speciation, pointed out environmental parameters that correlate with speciation or explored the evolution of ecological niches along a phylogenetic tree. An important advantage of using paleodistribution models to infer the geography of speciation compared to phylogenetic approaches is that range shifts due to climate changes are taken into account, whereas using phylogenetic approaches, the assumption that current geographical distribution correlates strongly with the geographical distribution at the time of speciation must be met.

Molecular clock analyses revealed that *Primula* sect. *Auricula* originated around 3.6 million years ago and first diverged around 2.4 million years ago (Zhang et al., 2004a). This split created two lineages, which subsequently evolved during the Quaternary and which today principally occupy the western and eastern parts of the European mountain ranges respectively. The ‘western clade’ with 15 species mainly occupies the western parts of the Alps, the Pyrenees, the Apennines and the Cordillera Cantabrica. The ‘eastern clade’ with 10 species is mainly distributed in the eastern parts of the Alps, the Dinaric Alps, the Balkan Mountains, the Carpathians and Sudetan. The highest species diversity is found in the Alps. Zhang et al. (2004a) hypothesized that this initial split of the section might have been caused by an early glaciation of the Alps forcing the last common ancestor of the two clades into western and eastern refugia, respectively. Later, Kadereit et al., (2004) revealed that the most common mode of speciation in the ‘western clade’ probably is allopatric speciation in isolated glacial refugia.

In the present study we investigate this hypothesis in the sister species pair *P. latifolia* and *P. marginata*, which was one of few sister species pairs identified with good support in a nrITS phylogeny (Zhang & Kadereit, 2004b). The hypothesis that the two species originated in geographically isolated refugia implies that their common ancestor was forced into isolated refugia during a glacial phase, where evolutionary divergence took place, and that the resulting entities were reproductively isolated when getting into contact in the course of recolonization of formerly glaciated areas. To examine this scenario we use paleodistribution models to predict the location of climatically suitable refugial areas of the two species during the last glacial maximum (LGM), 21,000 years ago, and then analyze population genetic patterns to evaluate these predictions. The existence of geographically isolated glacial refugia predicted by the models and their spatial overlap with, e.g., areas of high genetic diversity will be interpreted as fitting the hypothesis of allopatric speciation in glacial refugia.

An intrinsic assumption of paleodistribution modeling is that species conserve their ecoclimatological niches through time. Niche conservatism is indeed found in many taxa over a time period from LGM to present (Martinez-Meyer et al., 2004, Martinez-Meyer et al., 2006, Pearman et al., 2008a, Pearman et al., 2008b, Peterson et al., 1999), while in other cases there is evidence for niche shifts (Hadly et al., 1998, Davis & Shaw, 2001, Broennimann et al., 2007, Pearman et al., 2008a, Pearman et al., 2008b). The combination of two approaches based on independent data allows us to explore this topic. Congruence of modeled paleodistribution areas with refugial areas as indicated by population genetic patterns can be interpreted as evidence for niche conservatism.

Material and Methods

Molecular methods

Sampling

We sampled 28 populations (353 individuals) of *P. marginata* and 15 populations (172 individuals) of *P. latifolia* (Table 2; Figure 1 A (species distribution) + B (sampled populations)) across the whole distribution range of *P. marginata*, and in *P. latifolia* with an emphasis on the western part of the disjunct distribution of the species where it overlaps with the range *P. marginata* (Figure 1 A). At two sampling sites (LAC and CFRE) the two grew

sympatrically and at four sites the two species were sampled less than three km apart from one another. Leaf material of c. 10 to 15 individuals per population was collected. Chosen individuals had a minimum distance of three meters to avoid collecting the same clone. Where this was not possible due to small population size, the individual AFLP fingerprints were compared to eventually exclude identical fingerprints. If possible, sampled individuals were evenly distributed across the populations' whole range. In very large populations the sampling was limited to an extent comparable to the other populations. Voucher specimens are deposited in the herbarium of the Institut für Spezielle Botanik, Mainz (MJG).

DNA Extraction

Total genomic DNA was extracted from silica-gel-dried leaf material (c. 20 mg dry weight) using the QUIAGEN DNeasy Plant Mini Kit following the standard protocol with the modification that 10 mM sodium meta disulfide was added to the AP1-buffer. DNA was eluted two times using 40 µl AE-buffer.

AFLP protocol

The AFLP protocol follows (Vos et al., 1995) with modifications. All reactions were performed in three steps. All Master Mixes were prepared at once for all individuals of the two species and aliquots for subsequent use were frozen at -20°C to ensure comparability. Total genomic DNA (c. 100 ng) was digested and ligated using 2 U of EcoRI and 0.8 U MseI (both from New England BioLabs, NEB) and 0.7 U T4-DNA ligase (GeneCraft) in a reaction volume of 10 µl containing 23.5 pmol MseI adapter (5'-GACGATGAGTCCTGAG-3'; 3'-TACTCAGGACTCAT-5') and 2.35 pmol EcoRI adapter (5'-CTCGTAGACTGCGTACC-3'; 3'-CTGACGCATGGTTAA-5') (both from Metabion), 0.95 µl 0.5M NaCl, 1 µg bovine serum albumin (NEB), 0.95 µl 10X T4 DNA ligasebuffer (GeneCraft) and DEPC treated water (Roth). Reactions were incubated at 23°C for 14 hours to ensure complete digestion and ligation. Products of the restriction-ligation reaction were diluted 10-fold, 2.5 µl were used as template in the preselective PCR at a reaction volume of 10 µl containing 5 µl 2X PCR Master Mix (Promega), 26 ng MseI+1 primer and 26.5 ng EcoRI+1 primer and DEPC treated water. The thermocycling profile started with 5 min at 65°C followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C. Products of the preselective PCR were diluted 20-fold and 5 µl were used as template in the selective PCR with a reaction volume of 15 µl. The Master Mix further contained 0.25 U BioTherm Taq-polymerase and 1.5 µl BioTherm 10X PCR-buffer (GeneCraft), 12.5 ng MseI+3 primer, 9 ng

labeled EcoRI+3 primer, 0.2 μ l 20 mM dNTP (GeneCraft), 1.13 μ l 50 mM MgCl₂ and DEPC treated water. A hotstart of 10 min at 95°C was followed by 36 cycles of 30 s at 94°C, 1 min at X°C and 2 min at 72°C, whereas in the first 13 cycles the annealing temperature was reduced by 1°C at each step starting with 65°C and ending with 56°C – with the exception that 64°C, 62°C and 58°C were repeated two times, and the last 23 cycles were continued at 56°C, completed by a terminal incubation of 10 min at 72°C.

Primers used in the preselective PCR were E01 (5'-GACTGCGTACCAATTCA-3') and M01 (5'-GATGAGTCCTGAGTAAC-3') and in the selective PCRs E38-Hex (5'Hex-GACTGCGTACCAATTCCTACT-3') combined with M52 (5'-GATGAGTCCTGAGTAACCC-3'), E38-6-Fam with M55 (5'-GATGAGTCCTGAGTAACGA-3') and E39-NED (5'-NED-GACTGCGTACCAATTCAGA-3') with M55 (all primers were from Metabion, except E39-NED from ABI). AFLP products were separated as multiplex of the three AFLP reactions using primers labeled with different fluorescent dyes (2.7 μ l 6-Fam, 3.3 μ l Hex, 4 μ l NED) on an ABI 3130xl Genetic Analyzer using POP-7™ Polymer (ABI). For capillary electrophoresis 2 μ l of this multiplex was combined with 7.8 μ l Hi-Di formamide (ABI) and 0.25 μ l ROX500 size standard (ABI).

Population genetic analyses

Scoring of AFLP fragments was performed with GeneMarker Version 1.5 (GeneMarker, SoftGenetics, LLC). We calculated mismatch error rates to evaluate the quality of our analyses as the 'number of genotype mismatches' divided by the 'number of replicate pairs' and the 'number of loci' (Paun et al., 2008). Genetic variation was quantified using several diversity measures. The allelic richness Br (ranging from 1 to 2) and the percentage of polymorphic loci with standardized sample sizes (PLP5, PLP1) were calculated with AFLPDiv1.0 (Coart et al., 2005) as comparable measures for genetic diversity inferred from AFLP data with an emphasis on the number of alleles. For populations with population sizes lower than the number of rarefactions used, the mean number of alleles per locus is given instead of the band richness. Nei's gene diversity H (Nei 1973) (also referred to as expected heterozygosity), which emphasizes the differences of allele frequencies, Shannon's information index I (Shannon & Weaver, 1949, Lewontin 1972) and the percentage of polymorphic loci PPL (regardless of allele frequencies) were calculated with Popgene 3.2 (Yeh & Boyle, 1997) assuming Hardy-Weinberg-equilibrium. The rarity index was calculated as the frequency of each marker in a population relative to the number of occurrences of the marker in the whole dataset, summed up for all markers and then divided by the number of

markers and multiplied with the number of individuals in the whole dataset (as described in Paun et al., 2008). Genetic structure of populations was analysed using ‘Bayesian Analysis of Population Structure’ (BAPS, vers. 5.1) (Corander et al., 2008). The procedure was run 10 times each for $K=2-10$ as the assumed maximum number of populations present in the sample. Admixture analyses (Corander & Marttinen, 2006) were run with 100 iterations to estimate admixture coefficients for individuals, 200 reference individuals from each population and 20 iterations to estimate admixture coefficients for reference individuals. To detect within-species admixture, the analyses were run based on mixture clustering, and to detect admixture between species, the analyses were run based on two predefined groups corresponding to the species. Analyses of molecular variance (AMOVA) were conducted using Arlequin vers. 3.1 (Excoffier et al., 2005). Principal coordinate analyses (PCoA) using Euclidean distances were conducted with PALaeontological STATistics (PAST, vers.1.62) (Ryan et al., 1995).

Reconstructing past species distribution

Principles of species distribution modeling (SDMs)

SDMs allow to generate maps of the expected distribution of a species using information on the environmental conditions where it is known to occur (many models also take the absence of species into account). Different modeling techniques exist determining the set of parameters which best predict the presence of species. In a further step, models of the extant species distribution can be projected to a different time using past climate models in order to generate paleodistribution maps.

Climate data

We used current climate data at a spatial resolution of 30’’ (c. 1 km) from the WorldClim database (Hijmans et al., 2005). These so-called bioclimatic variables proved meaningful to describe plant and animal species niches in several studies (Waltari et al., 2007) and include monthly temperature and precipitation variables describing annual trends, seasonal variability and extreme and potentially limiting environmental factors. The same variables for the last glacial maximum (LGM) climate data were drawn from general circulation model (GCM) simulations from two climate models: the Community Climate System Model (CCSM, version 3) (Collins et al., 2006) and the Model for Interdisciplinary Research on Climate (MIROC, version 3.2) (Hasumi & Emori, 2004). The downscaled climate surfaces at 2.5’ (c. 5

km) spatial resolution were provided by Robert Hijmans and derived from the original climate simulations as described in (Waltari et al., 2007).

We chose a subset of eight bioclimatic variables by conducting preliminary model runs using all 19 available variables and identifying those with high variable importance. We additionally identified redundant variables by correlating 5000 random points distributed over the whole study range (3.5°-17°E, 43°-49°N). The following variables which correlated less than 0.8 among themselves were chosen as predictors: annual mean temperature (bio1), mean diurnal temperature range (bio2), temperature seasonality (bio4), mean temperature of wettest quarter (bio8), mean temperature of driest quarter (bio9), annual precipitation (bio12), precipitation seasonality (bio15) and precipitation of warmest quarter (bio18).

Fitting current species distribution models and projecting to the last glacial maximum (LGM)

Several recent studies advise to combine multiple modeling techniques (“consensus approach” or “ensemble forecasting”) (Thuiller, 2004, Pearson et al., 2006, Araujo & New, 2007, Pearson et al., 2007). This seems particularly appealing when models are used to project the distribution of species into independent situations, which is the case here where species distribution are projected to the LGM, a period in which the climate strongly differed from recent climate. This might reduce the predictive power of some techniques or cause discrepancies between different modeling techniques, making the choice of the appropriate model difficult. Consensus models highlight the areas, which are predicted by several modeling techniques making them more reliable.

We applied the following four modeling techniques using the latest release of the BIOMOD tool (Thuiller, 2003) implemented in R software (R Development Core Team, 2005): generalized linear models (GLM), generalized additive models (GAM), generalized boosting models (GBM) and random forests (RF). These techniques take the occurrences and absences of species into account. Occurrences were generated by combining several data sources (‘SOPHY’ (Ruffray et al., 2000-2009), ‘ZDSF’, data of local surveys from Alberto Selvaggi from the “Istituto per le Piante da Legno e l’Ambiente s.p.a.c.so Casale”, Torino, Italia and Filippo Prosser from the “Museo Civico de Rovereto”, Italia) and the localization of herbarium specimen from several collections (M, MSB, Z, ZT, RUEB, MJG). As no absences could be inferred from these data, we created 5000 pseudo-absences, which were randomly distributed over the entire study range as recommended in (Elith et al., 2006), and weighted to equal the number of presences per species. Continuous model predictions (ranging from 0 to

1) were transformed into binary predictions using the cut-off threshold maximizing AUC statistic (Area Under the Curve, meaning the Relative Operating Characteristic (ROC) curve). Binary predictions of the four techniques were cumulated. Using CCSM3 climate resulted in more inter-model variability regarding the distribution areas than did the use of MIROC3.2 climate. However, the consensus distribution areas obtained with both climates respectively are widely overlapping. We consequently also cumulated the consensus maps derived with the two climates separately to create distribution maps which outline those areas predicted by most modeling techniques under both climates. In *P. latifolia* the paleodistribution maps using GLM & GAM with CCSM3 climate failed to predict the LGM distribution of the species and were omitted. Consequently, the consensus models of *P. marginata* comprise eight models and those of *P. latifolia* six models.

Model evaluation

The predictive performance of the models was evaluated by 100-fold cross-validation, each time using a random subset (70%) of the total dataset to calibrate the models, while the remaining 30% were used to evaluate the model, calculating three different measures, namely the ROC curve, the True Skill Statistic (TSS) and the Kappa statistic.

Postglacially colonized areas

In order to identify regions that were colonized during the Holocene, we overlaid the present distribution maps with the paleodistribution maps. This was done for each of the eight models (four modeling techniques and two climates) separately and the resulting eight maps showing colonized areas were cumulated in a next step. In *P. latifolia* we omitted the paleodistribution maps using GLM & GAM with CCSM3 climate, because these models failed to predict the LGM distribution of the species. We consequently cumulated the six remaining models. To do so, the resolution of the present distribution maps was transformed from a 1 km grid into a 5 km grid (corresponding to the past prediction maps) using the minimum criterion, i.e. each new grid cell was considered occupied, when at least one of the 25 small grid cells within a new grid cell was occupied by the species. This procedure probably causes an overestimation of the postglacially colonized areas but ensures that no predicted present distribution areas are omitted due to the change in resolution.

Overlapping LGM distribution

The overlap of paleodistribution ranges of *P. marginata* and *P. latifolia* during the last glacial maximum was quantified using the percentage of overlapping area from the whole paleodistribution ranges of both species. For this, paleodistribution derived from consensus models using two thresholds were considered: a) areas that were predicted by the majority of modeling techniques (4-6 in *P. latifolia*, 5-8 in *P. marginata*) and b) areas that were predicted by at least half of the modeling techniques (3-6 in *P. latifolia*, 4-8 in *P. marginata*).

Combination of the approaches

Evaluation of the modeled last glacial maximum (LGM) refugia using spatial patterns of genetic diversity and rarity

Populations were classified into two types regarding their population history as inferred from the paleodistribution models, 1) populations located in last LGM distribution areas and 2) populations located in colonized areas. These classifications were done on the basis of the consensus maps in such a way that populations were assigned to the type which is indicated by the majority of models. If equal numbers of models indicate one *or* the other type, the population was not taken into account. In Figure 2, the population types are indicated with “*” for refugial populations, “-” for colonized populations and “?” for populations which cannot be assigned to a type. In *P. latifolia* the consensus of only six models was used to classify because using CCSM climate, GAM and GLM failed to predict the LGM distribution. We used randomization tests to compare the means of various diversity measures as well as rarity and the number of private alleles of LGM populations versus colonized populations, applying 10,000 Monte Carlo runs using Poptools 3.0 (Hood, 2008). Additionally, the number of alleles private to all refugial populations (considered as one group) was compared to the number of alleles private to all colonized populations (considered as another group).

Results

Modeled glacial refugia and colonized areas

In both species the paleodistribution models predict reductions in range size during the colder and drier climate of the LGM (Figure 1 A+B) and, by comparing them to the current distribution models, surprisingly little range shifts. According to the consensus models, *P. marginata* was restricted to the foothills of the Alpi Marittime at both sides of the glacier and the Préalpes Provencales (Figure 1 B above), and post-glacially expanded into the high mountains of the Alpi Marittime and to the North into the Alpi Cozie (Figure 1 C above).

For *P. latifolia* the paleodistribution models predict a restriction in range size to southern parts of the Alpi Graie and larger areas of the Alpi Cozie and the Alpi Marittime, and additionally to small areas in the Berner Alpen and Walliser Alpen (Figure 1 B below). All refugial areas which are outlined in *P. latifolia* are situated within the maximum extend of Alpine glacier on peripheral nunataks (outside the snowline) as well as on central nunataks (within the last glacial maximum snowline). *P. latifolia* post-glacially colonized the Walliser Alpen, the northern area in the Alpi Graie (Gran Paradiso), the western parts of the Alpi Cozie as well as the Eastern parts of the Massif du Pelvoux (west of the Alpi Cozie) and the foothills of the Alpi Marittime as well as parts of the Alpes Provencales (Figure 1 C below). Surprisingly, the areas outside the maximum extension of the LGM glacier, namely the eastern foothills of the Western Alps and the southwestern Préalpes, seem to have been post-glacially colonized rather than having served as refugia.

While the consensus model predicted glacial distribution of *P. latifolia* is predominantly situated in areas within the ice-shield where only high mountain tops surmounting the glacier (nunataks) were inhabitable, the predicted glacial distribution of *P. marginata* is located west and east of the main glacier.

Figure 1 A Predicted present distribution and occurrences

Above: *Primula marginata*, below: *P. latifolia*

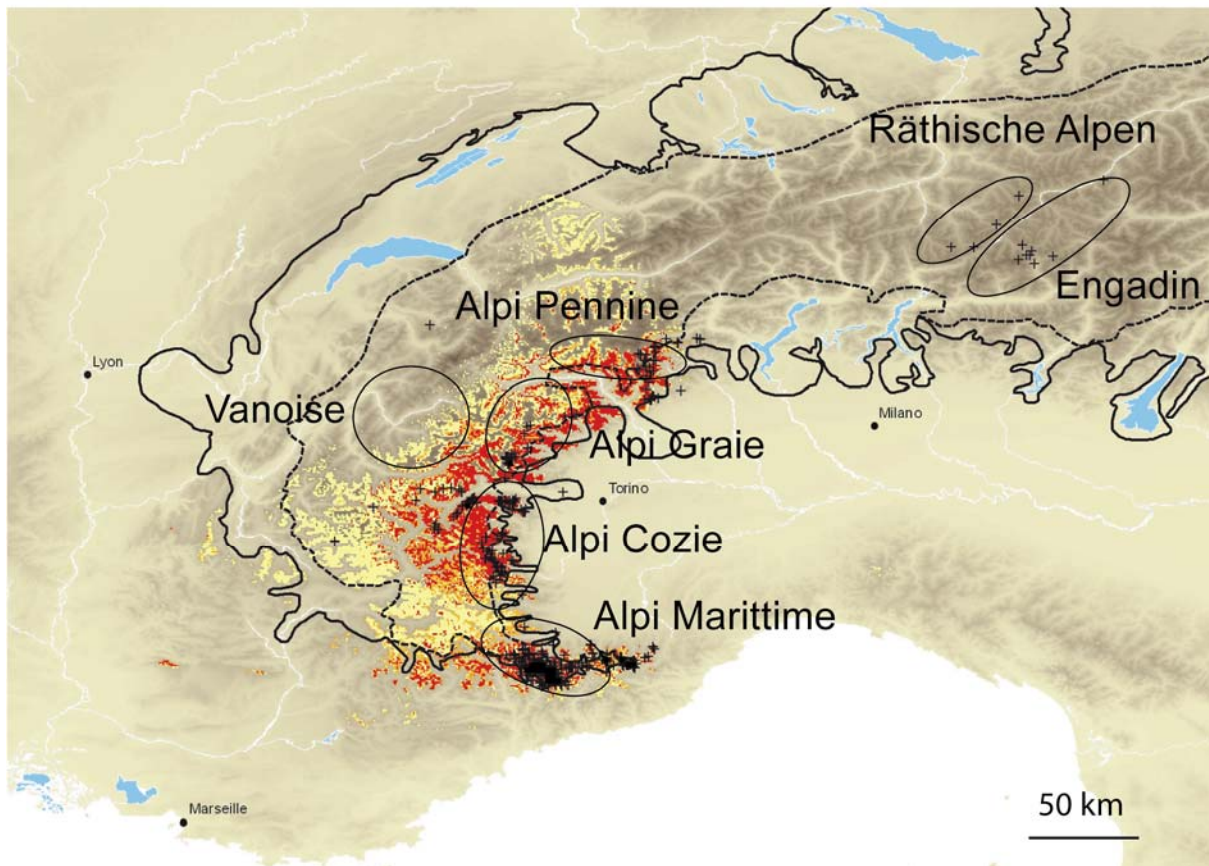
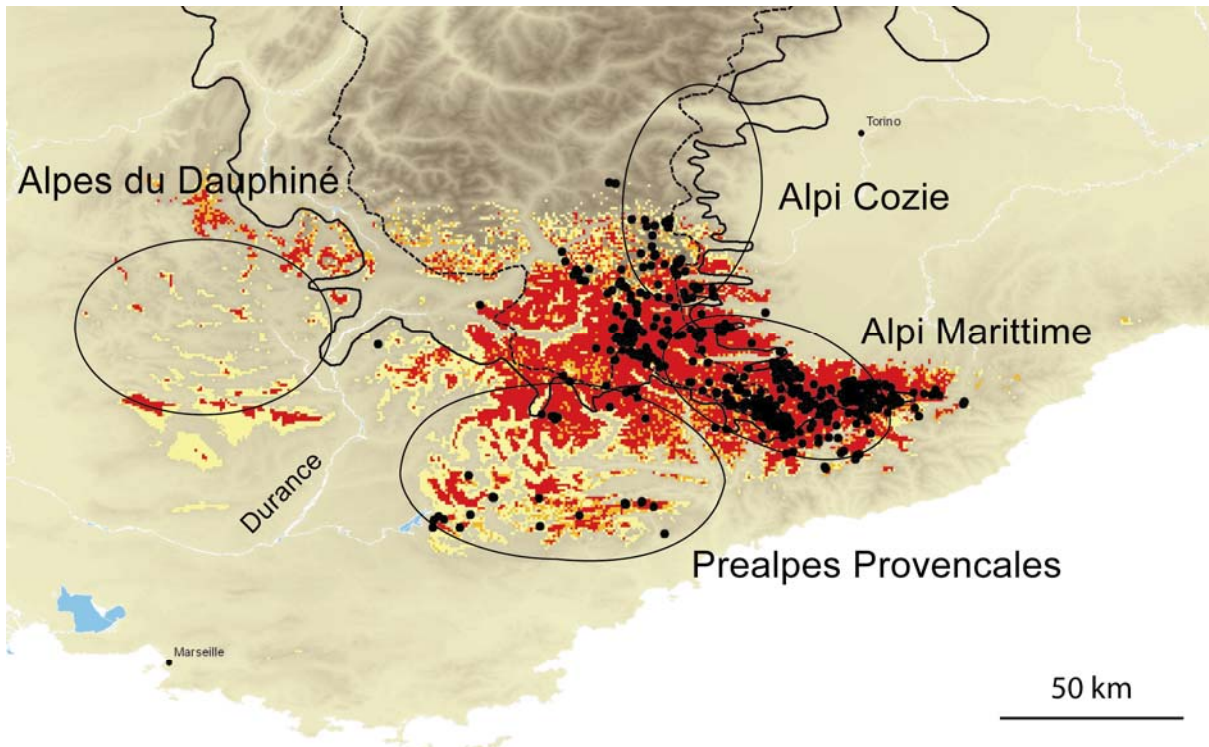


Figure 1 B Predicted LGM distribution (21 kyr) and sampled populations
Above: *Primula marginata*, below: *P. latifolia*

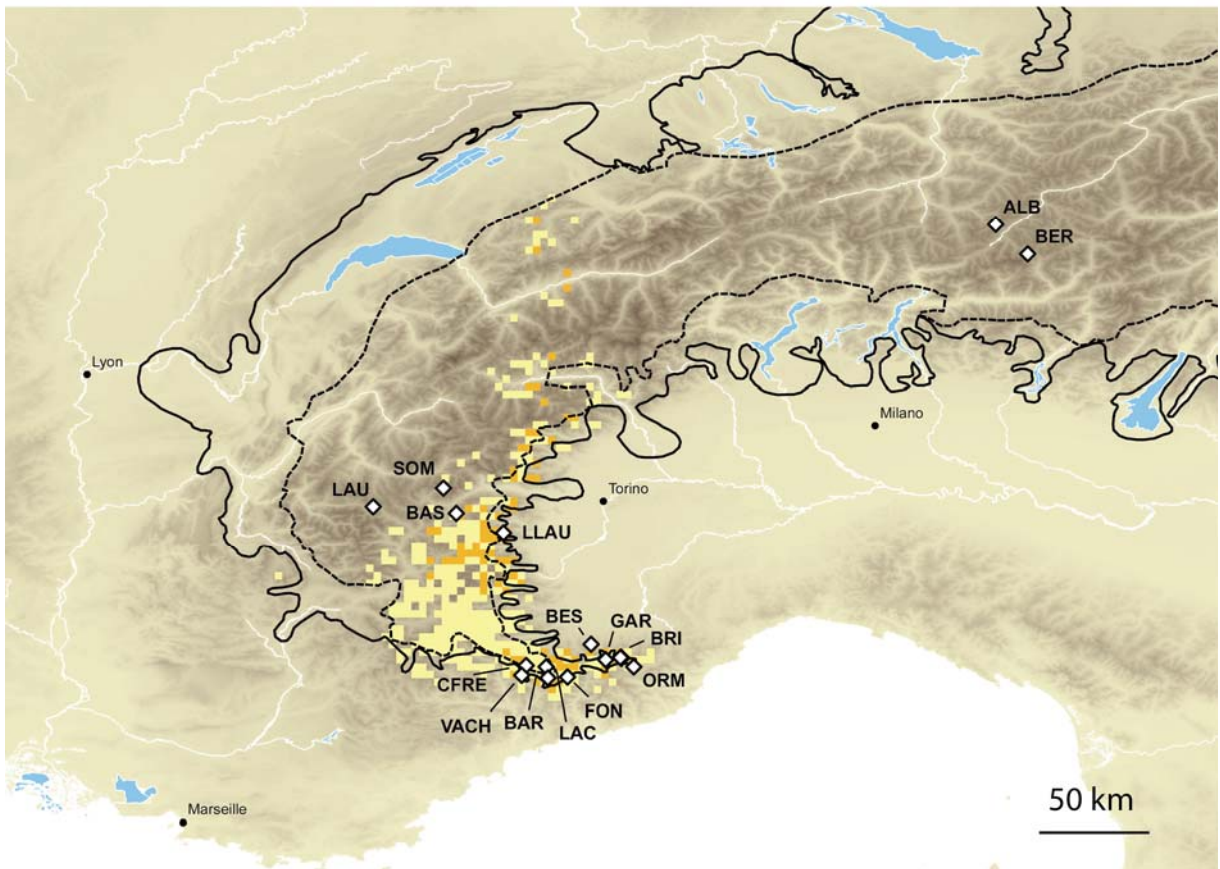
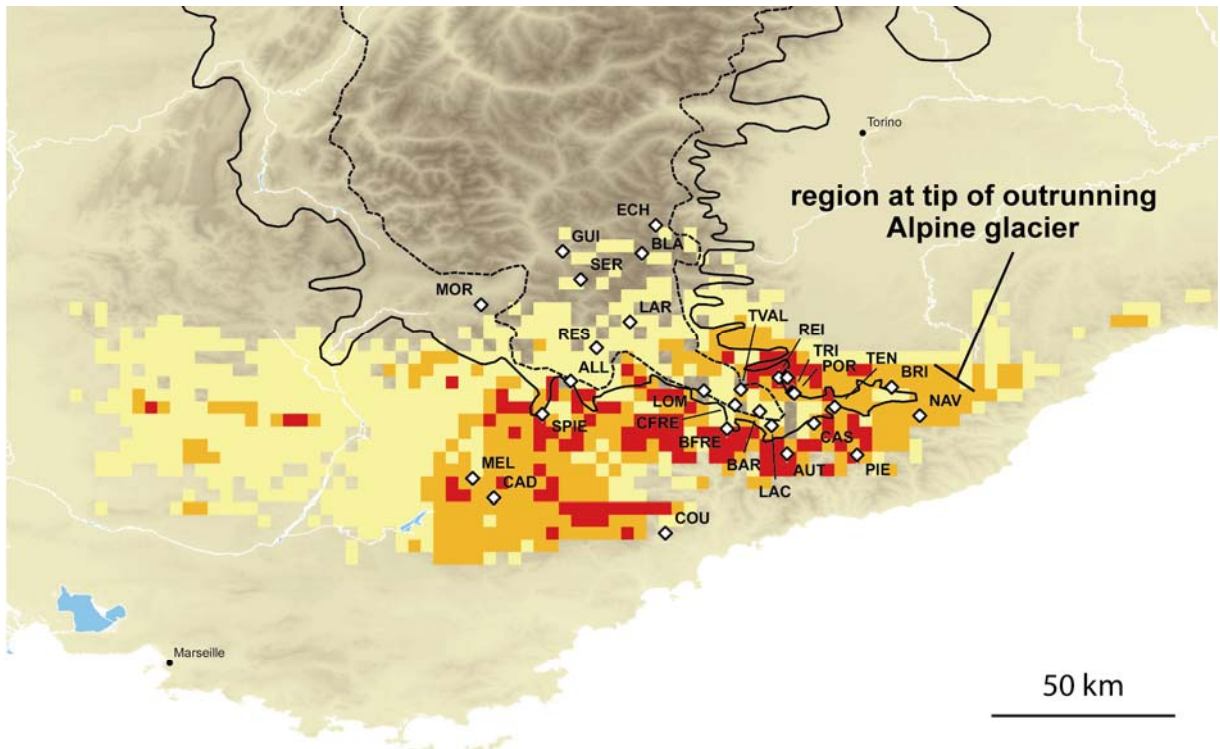


Figure 1 C Predicted colonized areas
Above: *Primula marginata*, below: *P. latifolia*

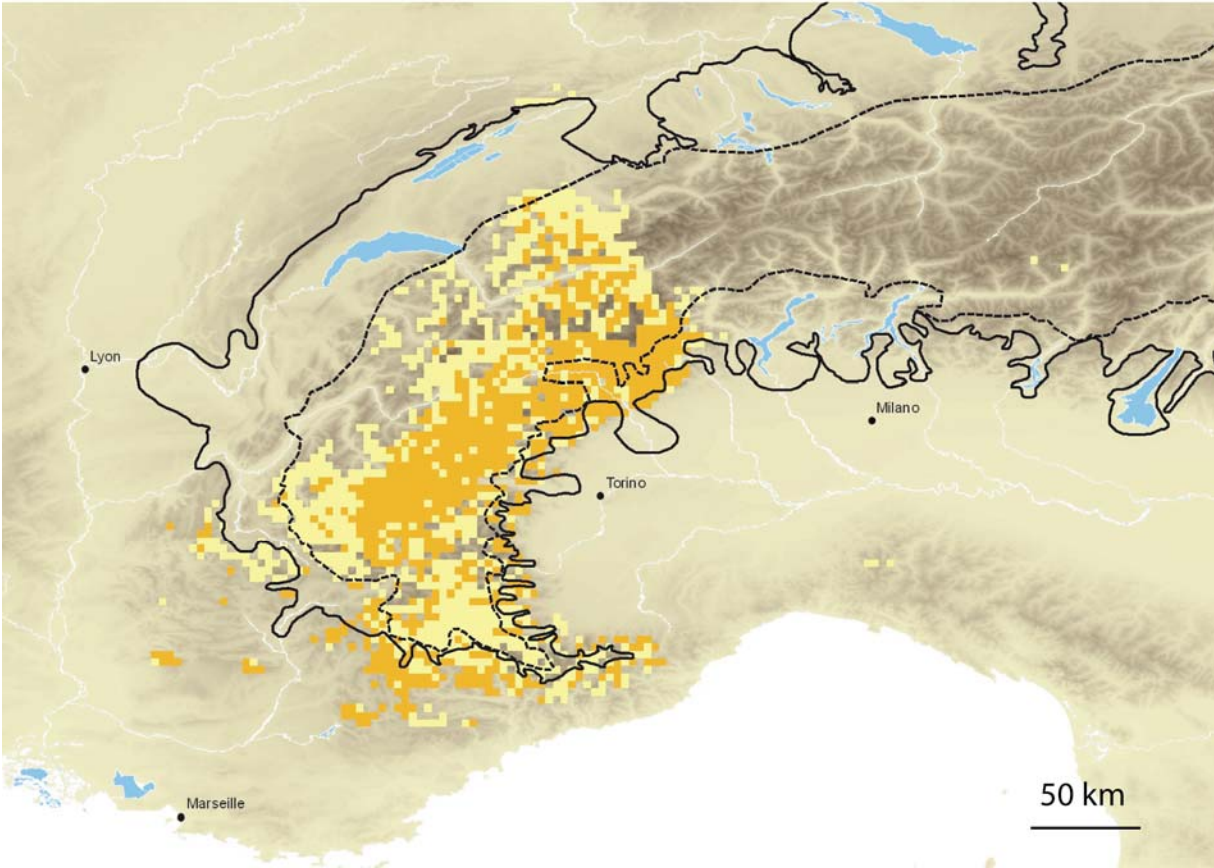
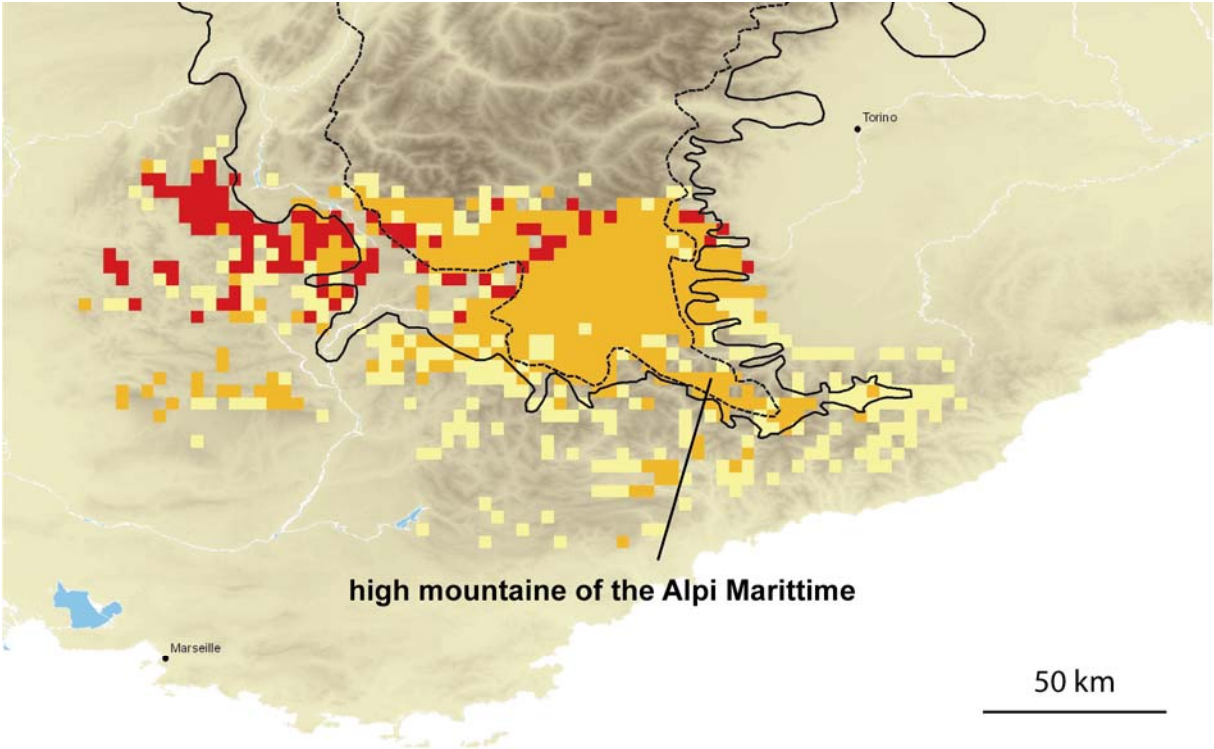


Figure 1 Legend



Figure 1 Species distribution models from consensus modeling for *Primula marginata* (above) and *P. latifolia* (below) for today (A) and the last glacial maximum, 21,000 years ago (B), and post-glacially colonized areas (C) derived by combining the two predictions. Species occurrences are shown in A, sampled populations are shown in B.

Evaluation of model predictions

Under current climate conditions AUC values indicate ‘good to excellent’ model performance across methods following Swets scale (Swets 1988), TSS indicates ‘excellent or high’ performance, whereas Kappa indicates ‘fair’ to ‘good’ model performance for *P. latifolia* and ‘good’ model performance for *P. marginata* following (Thuiller et al., 2008) (Table 1); the predictions for *P. marginata* are generally better.

The realized current distribution are generally well reflected by the models (Figure 1 A) with one major exception: The distribution models fail to predict the eastern part of the current distribution of *P. latifolia* in the Engadin and Rätische Alpen (Figure 1 A below) which may as well cause an under-prediction of the LGM distribution area (Figure 1 B below). This shortcoming is the result of an underrepresentation of the geographical distribution range of the species by the occurrence records, because no occurrence points available for this area were precise enough to use them for building the models. The climatic niche in the Engadin and Rätische Alpen obviously differs from the SW part of *P. latifolia*’s distribution area and hence this area can not be represented here.

In both species there are small areas which represent over-predictions of the current distribution. Small areas in the Walliser Alpen for *P. latifolia* and habitats east of the Durance for *P. marginata* (Figure 1 A). Different floras report *P. marginata* from the Alpes du Dauphiné (Pignatti 1982, Bock, 2000-2009, Ruffray et al., 2000-2009) which would correspond to the latter predicted areas, however, it remains unclear whether the species occurs there or not.

Table 1 Evaluation of single modeling techniques for the two species.

	AUC	Kappa	TSS
<i>P. marginata</i>			
GLM	0.977	0.720	0.926
GBM	0.982	0.752	0.930
GAM	0.975	0.716	0.920
RF	0.984	0.740	0.923
<i>P. latifolia</i>			
GLM	0.959	0.514	0.858
GBM	0.971	0.621	0.880
GAM	0.956	0.494	0.844
RF	0.969	0.635	0.859

AFLP-patterns

With the three primer combinations used, 247 polymorphic AFLP-fragments could be scored unambiguously. Of those, 223 were found in *P. marginata* (28 populations, 352 individuals and 185 in *P. latifolia* (15 populations, 172 individuals). This means that 25.1% (62 fragments) were private to *P. marginata* and 9.7% (24 fragments) to *P. latifolia*. The number of AFLP-fragments per individual varied from 41 to 78 in *P. marginata* and from 36 to 69 in *P. latifolia*. The mismatch error was 3.8% comparing 19 replicate pairs within the AFLP run.

Geography of within-species genetic groups

BAPS analyses revealed five genetic groups in *P. marginata* and four in *P. latifolia* (Figure 2 A; Table 2) (probability of the number of clusters 0.999 for *P. marginata* and 1 for *P. latifolia*). They occupy geographically separated areas which barely overlap (SE, SW and N) and one distinct population exists in both species, which is located at the margin of the distribution area, and is not mixed with other groups (ORM/MEL).

In *P. marginata* the two SE-groups occupy the eastern part of the Alpi Marittime at the Italian and French sides of the main mountain ridge and the Alpi Liguri W of Colle di Tenda, SE1 is distributed westerly and SE2 easterly, whereby population BRI is divided between the two, the N-group ranges from the northern part of the Alpi Marittime to the Alpi Cozie, the SW-group includes populations from the southern margin of the Alpi Marittime at the French side and the Préalpes Provençales, and the population at Clue de la Melle (MEL) in the Préalpes Provençales forms another distinct group. Populations from the SW-group are only mixed (i.e. contain individuals which belong to another genetic group than most of the populations' individuals) with the SE-groups, and populations of the N-group are only mixed with the SW-group, while populations from the SE-groups are only mixed between SE1 and SE2. A principle coordinate analysis shows a clear split between the SE-groups and the remaining three groups and a minimum spanning tree reveals that the SE-groups are closest to the SW-group (Figure 3 A+C). The position of the MEL-group is intermediate between the SW-group and the N-group. Each of the genetic groups in *P. marginata* comprises refugial populations and colonized populations (Figure 2 A left).

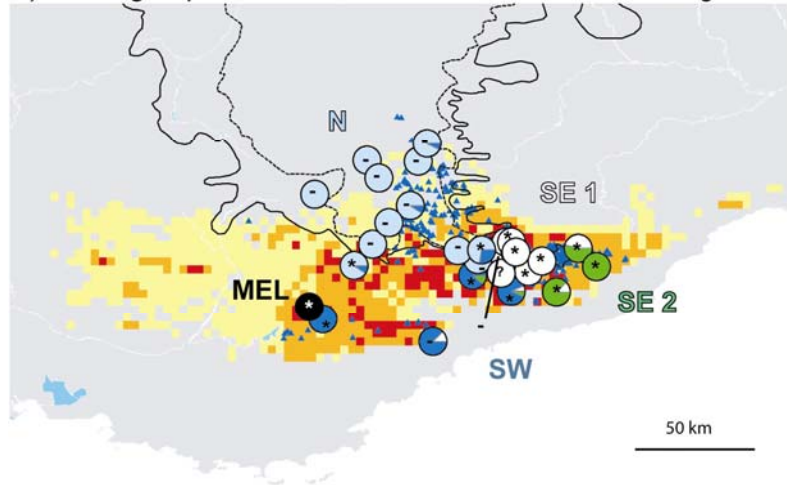
In *P. latifolia* the SW-group occupies the central part of the Alpi Marittime and both sides of the main mountain ridge which separates France and Italy (Figure 2 A right), the SE-group occupies the easternmost part of the Alpi Marittime/Alpi Ligurie west of Colle di Tenda at the Italian side and includes the population LLAU at the eastern foothills of the Alpi Cozie, the population ORM at the southeastern foothills of the Alpi Marittime forms a third

group, and the N-group comprises populations in the Swiss Engadin as well as the Northern part of the Alpi Cozie and thus connects populations of the two separated parts of *P. latifolia*'s disjunct Alpine distribution range. Mixed populations only occur in the SW-group, which is mixed with the SE-group. The N-group is clearly separated from the other BAPS-groups in a principal coordinate analyses and according to the minimum spanning tree most closely related to the SE-group (Figure 3 B+D). The ORM-group shows the closest relationship with the SE-group, which is also geographically closest.

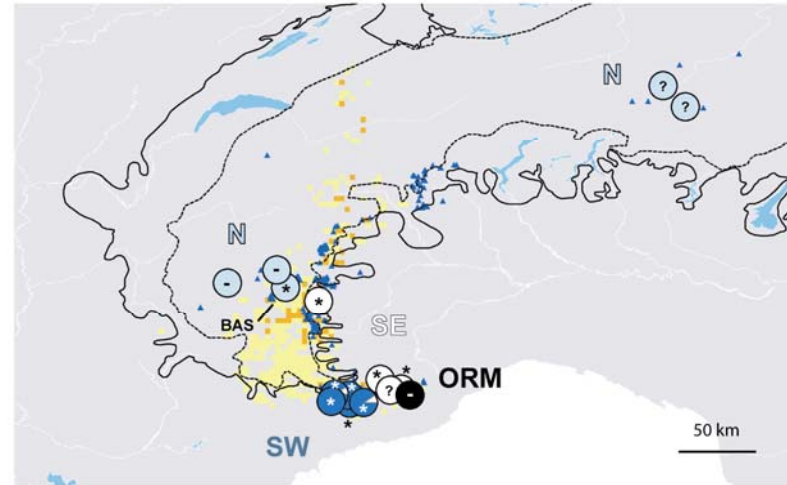
P. marginata, which has a rather narrow distribution range, shows less variation between those different genetic groups than *P. latifolia*, which is widespread across the Alps and divided into three subranges in the Pyrenees, the Western Alps and the Central Alps (Table 3). In *P. marginata* hierarchical AMOVAs attributed 15.9% of the overall variation to variation among the BAPS-groups, 11.45% to variation among the populations within the groups, and 73.46% to variation within populations. In *P. latifolia* 20.84% of the overall variation is attributed to variation among BAPS-groups, 10.79% to variation among the populations within the groups, and 68.37% to variation within populations.

A) BAPS-groups

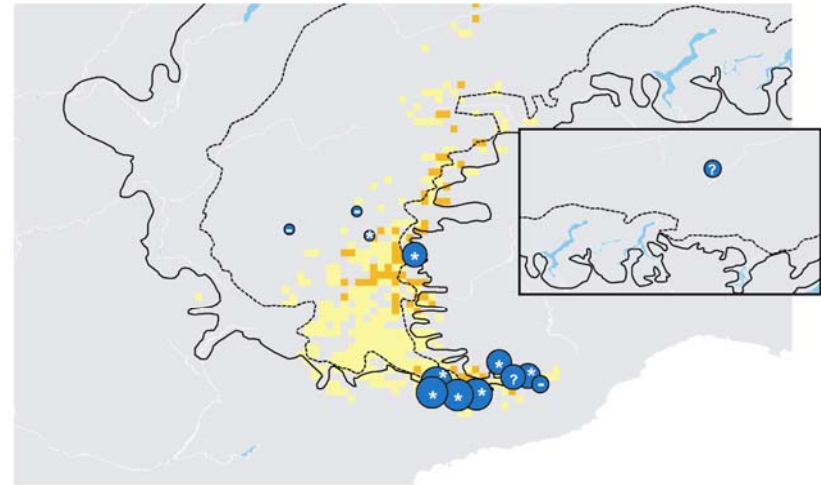
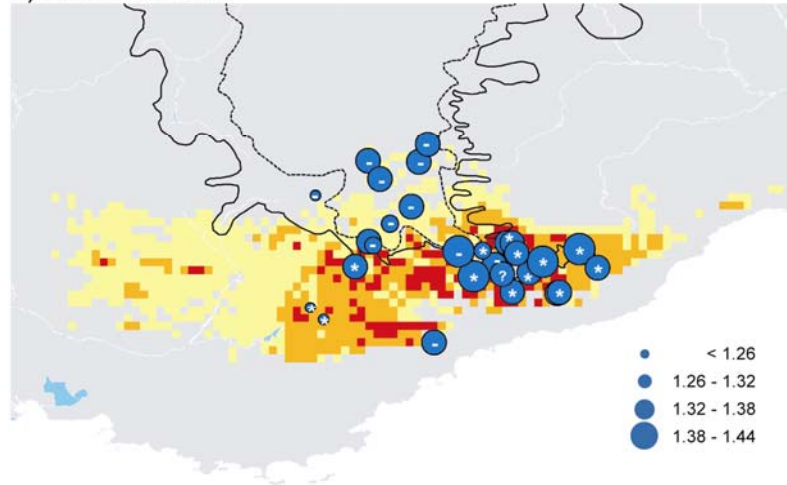
P. marginata



P. latifolia



B) Allelic richness



Climate suitability from consensus models at LGM (21 kyr):

- 3-4 models
- 5-6 models
- 7-8 models

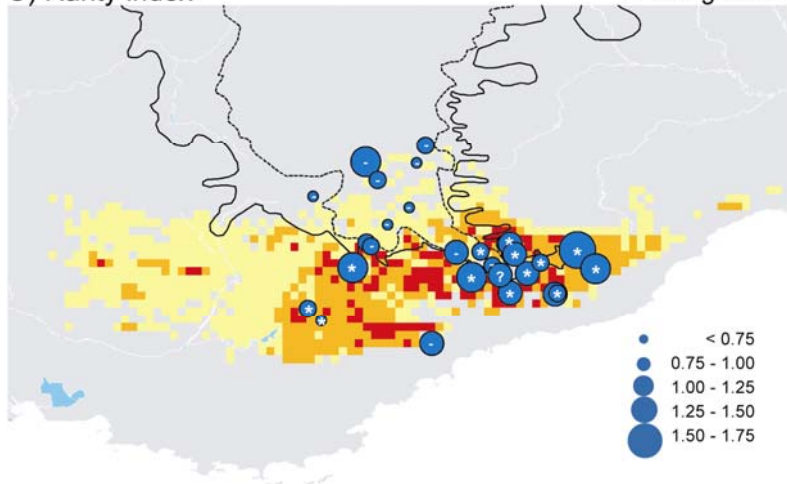
Population characterisation:

- * Refugial population
- Colonized population
- ? Unclear population history

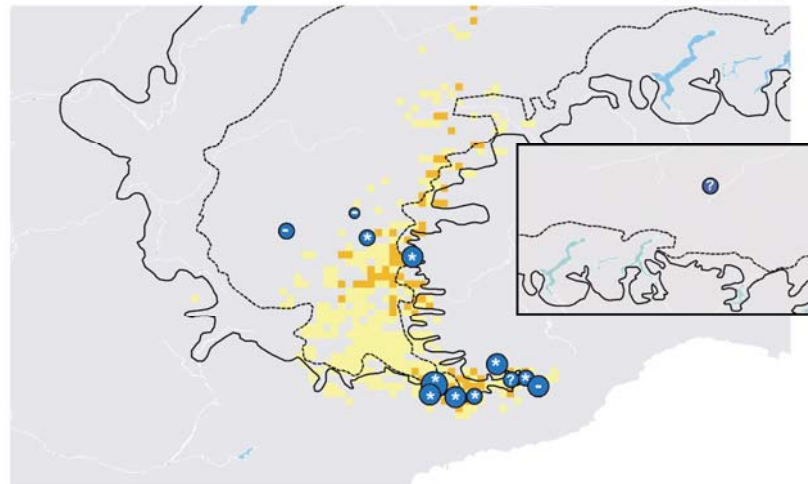
- Species occurrences
- Lakes and rivers
- Maximum extent of LGM glacier
- LGM snowline

C) Rarity index

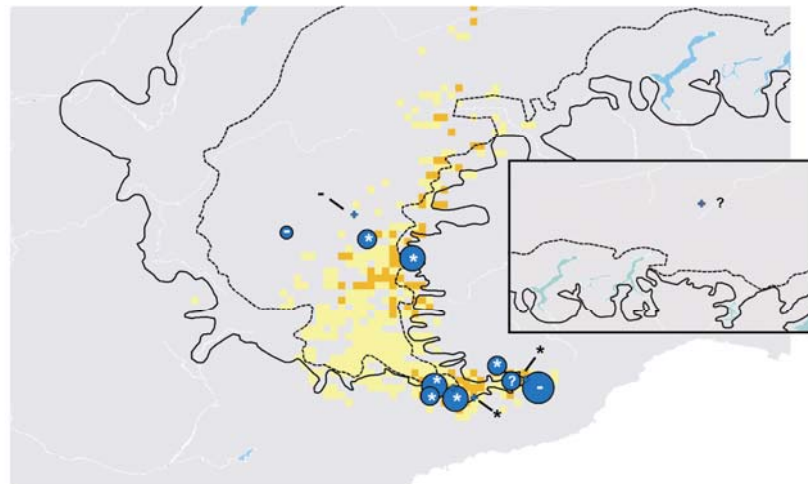
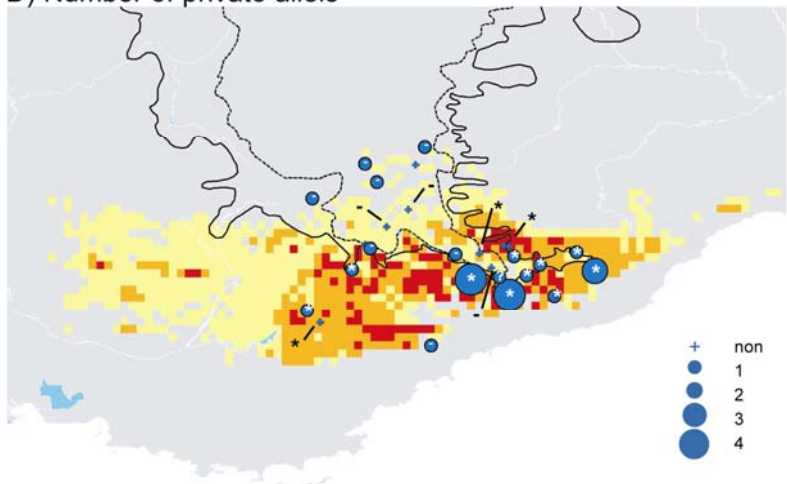
P. marginata



P. latifolia



D) Number of private alleles



Climate suitability from consensus models at LGM (21 kyr):



Population characterisation:

- * Refugial population
- Colonized population
- ? Unclear population history

- Lakes and rivers
- Maximum extent of LGM glacier
- LGM snowline

Figure 2 Population structure (A) and population genetic indices, namely B) allelic richness (Pb), C) rarity and D) the number of private alleles for *P. marginata* (left) and *P. latifolia* (right) overlaid onto consensus maps of glacial refugial areas predicted with paleodistribution models for LGM, 21,000 years ago. The ‘population characterization’ as refugial population or colonized population is based on the present and past consensus models.

Table 2 Locations of the sampled populations of *P. marginata* and *P. latifolia* and affiliation to genetically defined groups inferred with BAPS (BAPS-group) as well as the number of individuals, which are admixed between species (adm). For localities where several ‘populations’ were sampled at a minimum distance of a few 100 m, the number of sampled individuals per location is given in brackets and differing altitudes of the subsamples are specified by small letters. In mixed populations the different BAPS-groups are separated by a slash; brackets indicate that only one of the populations’ individuals belongs to the specified BAPS-group.

code	locality, country	altitude (m)	lat/long	n° ind.	BAPS-group	adm.
<i>P. marginata</i> (28 populations)						
ALL	Lacs d'Allos, F	1880 (a), 2200 (b)	6.707°/44.234°	22 (12+10)	N	
BLA	Col Blanchet, F	2630	6.944°/44.663°	10	N	
CFRE	Colle di Fremamorta, I/F	c. 2650	7.254°/44.155°	2	N	
ECH	Echalp, F	c. 1750	6.990°/44.756°	13	N/(SW)	
GUI	Guillestre, F	c. 1150	6.680°/44.668°	10	N	1
LAR	Col de Larche, F	c. 2200	6.904°/44.433°	11	N/(SW)	
LOM	Col de Lombard, F	c. 2400	7.149°/44.202°	8	N	
MOR	Cirque de Morgon, F	c. 1900	6.408°/44.491°	16	N	
RES	Col de Restefond, F	c. 2450	6.793°/44.345°	10	N	
SER	Col de Serenne, F	c. 2600	6.741°/44.575°	14	N	
SPIE	Gorges St Pierre, F	c. 1900	6.613°/44.124°	11	N/(SW)	
TVAL	Therme di Valdieri, I	c. 1300	7.272°/44.206°	11	N/SW	
AUT	Cirque de l'Authion, F	c. 1900	7.426°/43.993°	15	SW/(SE1)/(SE2)	
BFRE	Baus de la Frema, I	c. 1850	7.226°/44.075°	9	SW/(SE2)	
CAD	Les Cadières, F	1330	6.450°/43.847°	19 (14+5)	SW	
COU	Pic de Courmettes, F	c. 1200	7.021°/43.729°	8	SW/(SE)	
BAR	Vallone delle Barra, I	c. 2100-2200	7.335°/44.133°	12	SE1	
CAS	Vallone Casterino, I	1560	7.516°/44.094°	17	SE1	
ENT	Entraque, I	c. 1000	7.399°/44.246°	11	SE1	
LAC	Baisse des Cinq Lacs, F	c. 2400	7.374°/44.085°	13	SE1	
POR	Titti Porcera, I	1150	7.444°/44.197°	10	SE1	
REI	Gorgo della Reina, I	1140 (a), c. 1200 (b)	7.427°/44.246°	15 (13+2)	SE1	
TEN	Colle di Tenda, I	1900	7.584°/44.149°	12	SE1	1
TRI	Titti Trinita, I	1260	7.450°/44.193°	10	SE1	
BRI	Lago di Brignola, I	c. 1850	7.773°/44.214°	13 (10+3)	SE2/SE1	
NAV	Ponte di Nava, I	c. 800	7.868°/44.118°	13	SE2	
PIE	Monte Pietravecchia, I	2020 (a) 1670 (b)	7.659°/43.988°	25 (15+10)	SE2/SE1	
MEL	Clue de la Melle, F	1060	6.380°/43.910°	12	MEL	
<i>P. latifolia</i> (15 populations)						
ALB	Albulapass, CH	2315-2350	9.83°/46.58°	8	N	1
BER	Berninapass, CH	2250	10.005°/46.415°	1	N	
BAS	Colle Basset, I	c. 2350	6.872°/44.994°	15	N	
LAU	Col du Lautaret, F	2050-2100	6.415°/45.031°	15	N	
SOM	Col Sommeiller, F	c. 2200	6.8°/45.132°	10	N	
BES	Monte Besimauda, I	2130	7.611°/44.271°	14	SE	
BRI	Lago di Brignola, I	2000 (c), 2140 (d+e)	7.775°/44.194°	17 (3+13+1)	SE	
LLAU	Lago Lausun, I	c. 2100	7.129°/44.887°	11	SE	
GAR	Rifugio Garelli, I	2040	7.692°/44.186°	10	SE	
BAR	Vallone delle Barra, I	1870	7.363°/44.147°	4	SW	
CFRE	Colle di Fremamorta, I/F	2570 (a), c. 2300 (b)	7.254°/44.155°	11 (9+2)	SW/(SE)	1
FON	Vallone de Fontanalba, F	1920	7.482°/44.09°	11	SW/(SE)	
LAC	Baisse des Cinq Lacs, F	c. 2400	7.374°/44.085°	15	SW/(SE)	
VACH	Vacherie Andue, F	c. 1800	7.229°/44.101°	15	SW	
ORM	Pizzo d'Ormea, I	1840	7.845°/44.147°	15	ORM	

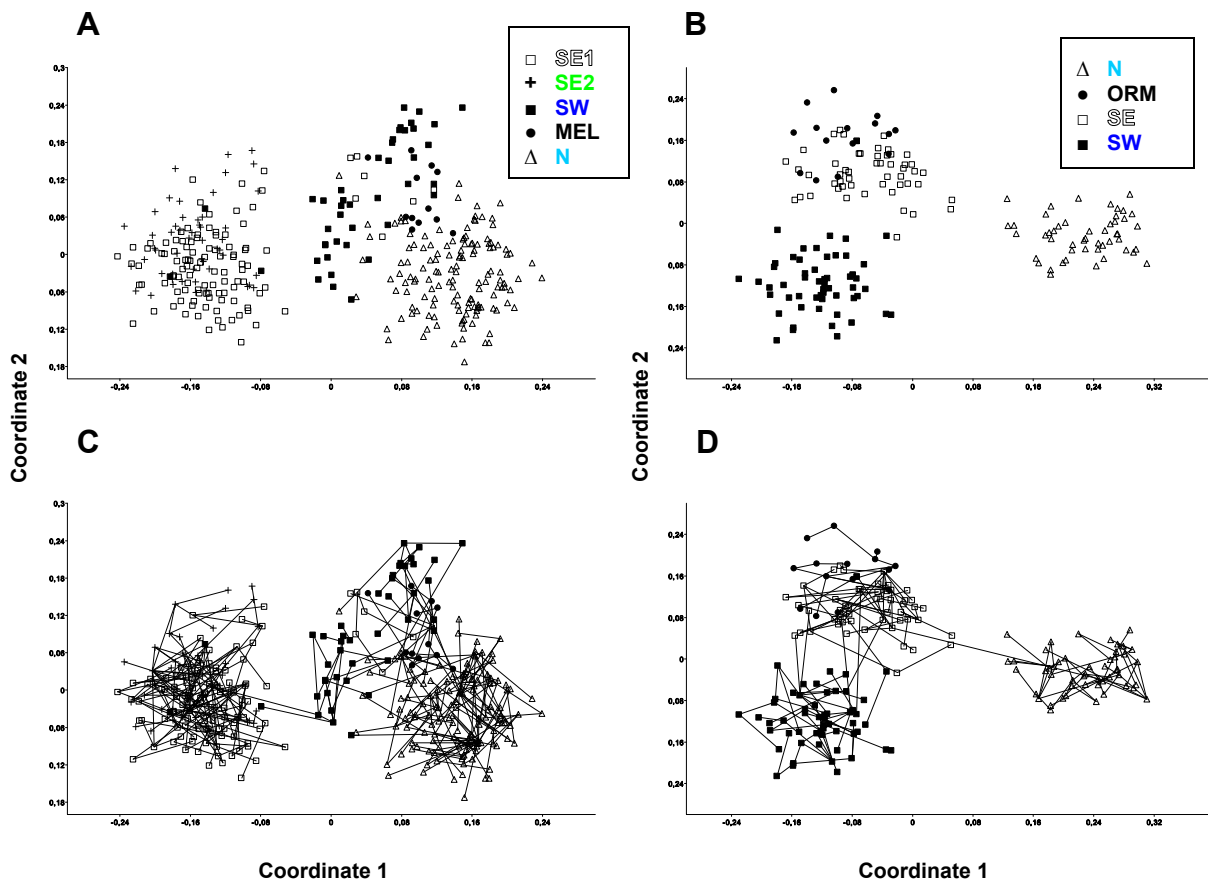


Figure 3 Principle coordinate analyses of *P. marginata* (A, C)/*P. latifolia* (B, D) with minimum spanning tree using Euclidean distances among 352 AFLP multilocus phenotypes from 28 populations/172 AFLP multilocus phenotypes from 15 populations respectively. Symbols indicate the genetic groups derived with BAPS; to visualize individuals that genetically belong to a different BAPS-groups than the majority of individuals of their population, all individuals from a population are assigned to the BAPS group, where most of the population's individuals belong to; colors of group names correspond to colors of BAPS-groups in Figure 2. Coordinates explain 10.55%, 3.28%/12.2%, 6.2% respectively; axes are scaled using the square root of the eigenvalues.

Table 3 Analyses of molecular variance (AMOVA) for AFLP phenotypes of the 28 populations of *Primula marginata* and *P. latifolia* using 1,000 permutations.

Source of variation	d.f.	Sum of squares	Variance components*	% of variation	F _{ST} *
<i>P. marginata</i>					
Among populations	27	2129.49	5.01	23.69	0.24
Within populations	324	5231.80	16.15	76.31	
Among BAPS-groups (N, SW, SE1, SE2, MEL)	4	1052.16	3.32	15.09	0.27
Among populations within BAPS-groups	23	1077.33	2.52	11.45	
Within populations	324	5231.80	16.15	73.46	
<i>P. latifolia</i>					
Among populations	14	1014.81	5.22	28.23	0.28
Within populations	157	2082.66	13.27	71.77	
Among BAPS-groups (N, SW, SE, ORM)	3	621.46	4.04	20.84	0.32
Among populations within BAPS-groups	11	393.35	2.09	10.79	
Within populations	157	2082.66	13.27	68.37	

*All p-values < 0.001

Percentage of overlap of paleodistribution

The LGM distribution of the two species overlap at the easternmost part of the Alpi Marittime in a zone along the border of the LGM glacier limit (Figure 4). Using paleodistribution that were predicted by at least half of the modeling techniques (3-6 in *P. latifolia*, 4-8 in *P. marginata*), the overlapping area represents 10.5% of the paleodistribution ranges of both species. Using LGM distribution predicted by the majority of modeling techniques used (4-6 in *P. latifolia*, 5-8 in *P. marginata*), 6.3% of overlap is found.

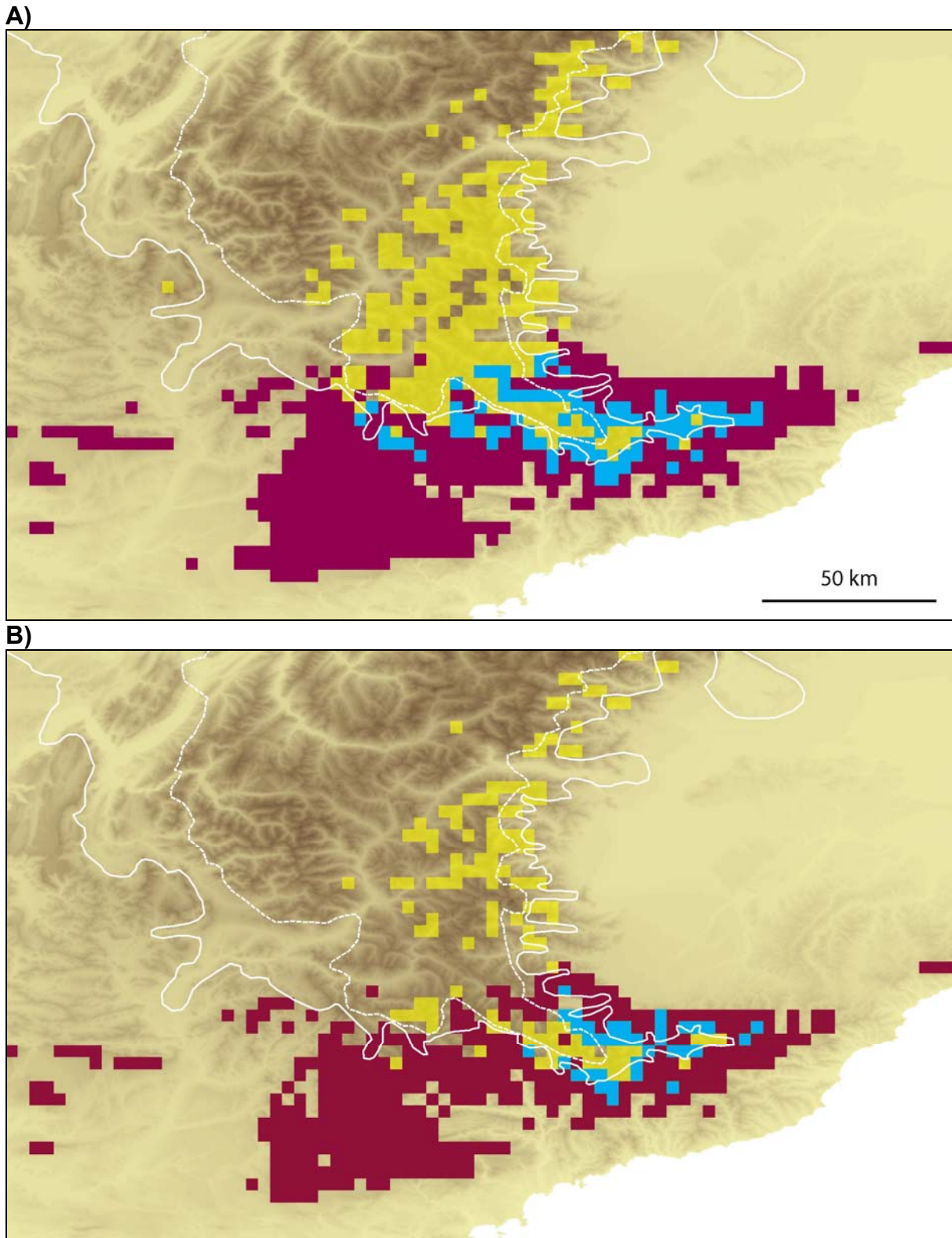


Figure 4 Overlapping zone (blue) of glacial refugial areas in the Alpi Marittime of *P. latifolia* (yellow) and *P. marginata* (violet) derived from consensus modeling. The areas shown are predicted by A) at least half of the models (3-6 in *P. latifolia*, 4-8 in *P. marginata*) and B) the majority of models (4-6 in *P. latifolia*, 5-8 in *P. marginata*) For further descriptions see Figure 1 or 2.

Evaluation of modeled refugia with population genetic patterns

In both species we find large congruence between modeled glacial distribution and population genetic patterns. There is a clear trend over all calculated indices for higher diversity and rarity in populations located in model-predicted refugial areas compared to colonized areas (Figure 2 B+C), and varying indices in the two species show significant differences using randomization tests (Figure 5) In *P. latifolia*, for five of the six diversity indices the relation is significant and in *P. marginata*, for two of the six diversity indices and rarity. The number of private alleles is not different between the two groups but the highest numbers are found in model-predicted refugial areas (Table 4; Figure 2 D) with only one exception, the *P. latifolia* population on Pizzo d'Ormea (ORM), which is located in a colonized area, but harbors four private alleles (see Figure 1 B below for names of sampled populations).

Furthermore, modeled refugial areas harbor more private alleles than colonized areas: All refugial populations (113 individuals) of *P. latifolia* taken together share 59 fragments which do not occur in colonized populations, whereas only seven fragments were exclusively found in colonized areas (40 individuals). In *P. marginata* 34 fragments were private to populations in glacial refugial areas (203 individuals), while only 10 were exclusively found in colonized areas (136 individuals).

Table 4 Population genetic indices for the investigated populations of *P. marginata* and *P. latifolia*. Sampling sites which are located in a few hundred meters distance are underlined; out of those, the sampling sites which were not used to evaluate the paleodistribution models, are listed at the end of the table. ‘Type of pop.’ specifies whether paleodistribution models indicate, that the population is situated in colonized area (C) or in refugial area (R). ‘Size of pop.’ = number of individuals per population, ‘N° frag.’ = number of AFLP-fragments, h = Nei’s gene diversity, I = Shannon index, NPL = number of polymorphic loci, PPL = percentage of polymorphic loci, Pb7 = band richness with rarefaction sample size 7, PPL1/PPL5 = percentage polymorphic loci at the 1%/5% level, only calculated if ‘size of pop.’ > 6, ‘priv. Al.’ = number of private alleles.

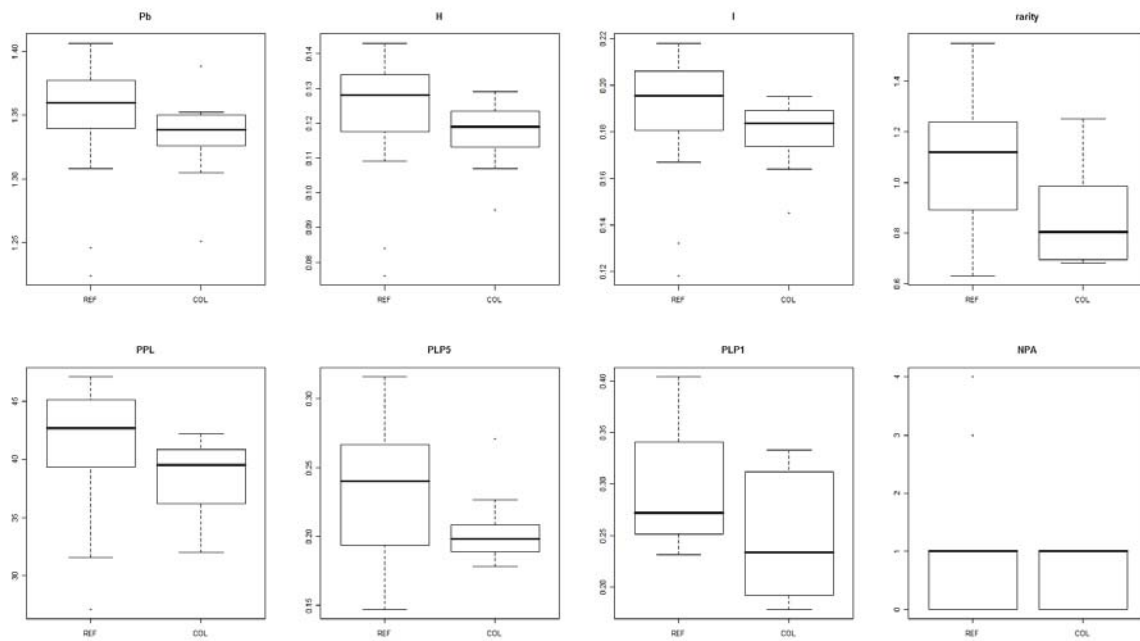
Code	Type of pop.	Size of pop.	N° frag.	h	I	NPL	PPL	Pb7	PLP5	PLP1	Rarity	Priv. Al.
<i>P. marginata</i>												
<u>ALLa</u>	C	12	113	0.12	0.18	94	41.78	1.35	0.20	0.33	0.81	0
<u>ALLb</u>	C	10	98	0.12	0.18	81	36.00	1.32	0.19	0.19	0.93	1
AUT	R	15	121	0.12	0.19	105	46.67	1.35	0.29	0.35	1.24	4
BAR	C	12	114	0.13	0.19	91	40.44	1.34	0.19	0.32	0.82	0
BFRE	R	9	119	0.13	0.20	101	44.89	1.40	0.19	0.26	1.42	4
BLA	C	10	104	0.12	0.18	85	37.78	1.33	0.20	0.20	0.69	0
<u>BR1a*</u>	R	10	125	0.14	0.21	98	43.56	1.39	0.25	0.25	1.55	1
<u>CADa</u>	R	14	93	0.08	0.13	71	31.56	1.25	0.20	0.28	0.63	0
CAS	R	17	130	0.13	0.21	106	47.11	1.38	0.28	0.40	1.16	1
CFRE	C	2	64	0.03	0.04	15	6.67	1.07*	-	-	0.65	0
COU	C	8	105	0.11	0.17	82	36.44	1.34	0.18	0.18	1.21	1
ECH	C	13	108	0.13	0.20	95	42.22	1.35	0.27	0.31	0.96	1
ENT	R	11	111	0.12	0.18	84	37.33	1.34	0.16	0.25	0.81	0
GUI	C	10	107	0.12	0.19	88	39.11	1.35	0.18	0.18	1.33	1
LAC	?	13	122	0.13	0.19	94	41.78	1.35	0.25	0.32	1.08	1
LAR	C	11	107	0.12	0.19	90	40.00	1.34	0.21	0.28	0.70	0
LOM	C	8	109	0.12	0.19	93	41.33	1.39	0.20	0.20	1.05	1
MEL	R	12	87	0.08	0.12	61	27.11	1.22	0.15	0.23	0.85	1
MOR	C	16	96	0.10	0.15	72	32.00	1.25	0.20	0.27	0.70	1
NAV	R	13	118	0.13	0.20	98	43.56	1.36	0.27	0.33	1.39	3
<u>PIEa</u>	R	15	123	0.13	0.21	102	45.33	1.36	0.32	0.36	1.11	1
<u>PIEb</u>	R	10	117	0.13	0.19	94	41.78	1.36	0.27	0.27	0.95	0
POR	R	10	117	0.13	0.20	93	41.33	1.36	0.23	0.23	1.23	0
<u>RE1a</u>	R	13	121	0.14	0.22	100	44.44	1.37	0.24	0.31	1.06	0
RES	C	10	101	0.11	0.16	78	34.67	1.31	0.20	0.20	0.71	0
SER	C	14	105	0.13	0.19	91	40.44	1.33	0.23	0.32	0.77	1
SPIE	R	11	111	0.12	0.18	93	41.33	1.34	0.24	0.30	1.29	1
TEN	R	12	126	0.14	0.22	106	47.11	1.41	0.22	0.38	0.98	1
TRI	R	10	119	0.13	0.19	94	41.78	1.36	0.25	0.25	1.18	1
TVAL	R	11	100	0.11	0.17	79	35.11	1.31	0.17	0.24	0.78	0
<i>P. latifolia</i>												
BAR	R	4	80	0.10	0.15	50	0.27	1.27*	-	-	0.83	0
BES	R	14	103	0.14	0.21	86	0.46	1.38	0.26	0.37	1.05	2
<u>BR1d</u>	R	13	104	0.13	0.20	87	0.47	1.38	0.30	0.36	0.81	0
FON	R	11	103	0.14	0.21	85	0.46	1.40	0.24	0.32	0.88	0
LLAU	R	11	103	0.13	0.20	83	0.45	1.37	0.25	0.31	1.23	3
GAR	?	10	91	0.11	0.17	69	0.37	1.33	0.22	0.22	0.99	2
LAU	C	15	83	0.09	0.14	60	0.32	1.25	0.22	0.25	0.76	1
ORM	C	15	101	0.10	0.16	73	0.39	1.29	0.27	0.31	1.19	4
SOM	C	10	67	0.07	0.11	42	0.23	1.21	0.09	0.09	0.58	0
ALB	?	8	82	0.08	0.13	60	0.32	1.30	0.18	0.18	0.89	0
BAS	R	15	81	0.09	0.14	61	0.33	1.25	0.23	0.27	0.79	2

Table 4 continued

Code	Type of pop.	Size of pop.	No frag.	h	l	NPL	PPL	Pb7	PLP5	PLP1	Rarity	Priv. Al.
BER	?	1	40	-	-	-	-	-	-	-	-	0
<u>CFREa</u>	R	9	114	0.14	0.22	91	0.49	1.44	0.2	0.32	1.50	3
LAC	R	15	120	0.15	0.23	103	0.56	1.44	0.40	0.46	1.14	3
VACH	R	15	110	0.14	0.21	93	0.50	1.39	0.37	0.43	1.07	2
<u>ALLa+b</u>	C	22	120	0.13	0.20	106	47.11	1.35	0.32	0.42	0.87	1
<u>BRla+b*</u>	R	13	134	0.15	0.23	113	50.22	1.41	0.35	0.38	1.48	1
<u>BRlb</u>	R	3	93	0.08	0.12	48	21.33	1.21*	-	-	1.24	0
<u>CADa+b</u>	R	19	102	0.09	0.14	82	36.44	1.26	0.25	0.32	0.68	1
<u>CADb</u>	R	5	77	0.07	0.11	50	22.22	1.22*	-	-	0.81	1
<u>PIE</u>	R	25	138	0.14	0.22	121	53.78	1.37	0.40	0.49	1.04	1
<u>REI</u>	R	15	123	0.14	0.22	102	45.33	1.38	0.28	0.35	1.01	0
<u>RElb</u>	R	2	76	0.07	0.10	36	16.00	1.16*	-	-	0.71	0
<u>BRlc+d+e</u>	R	17	112	0.15	0.23	98	0.53	1.40	0.35	0.44	1.01	2
<u>BRlc</u>	R	3	82	0.10	0.15	49	0.26	1.26*	-	-	1.71	2
<u>BRle</u>	R	1	58	-	-	-	-	-	-	-	-	0
<u>CFREa+b</u>	R	11	115	0.14	0.22	94	0.51	1.44	0.28	0.37	1.42	3
<u>CFREb</u>	R	2	70	0.07	0.10	30	0.16	1.16*	-	-	1.08	0

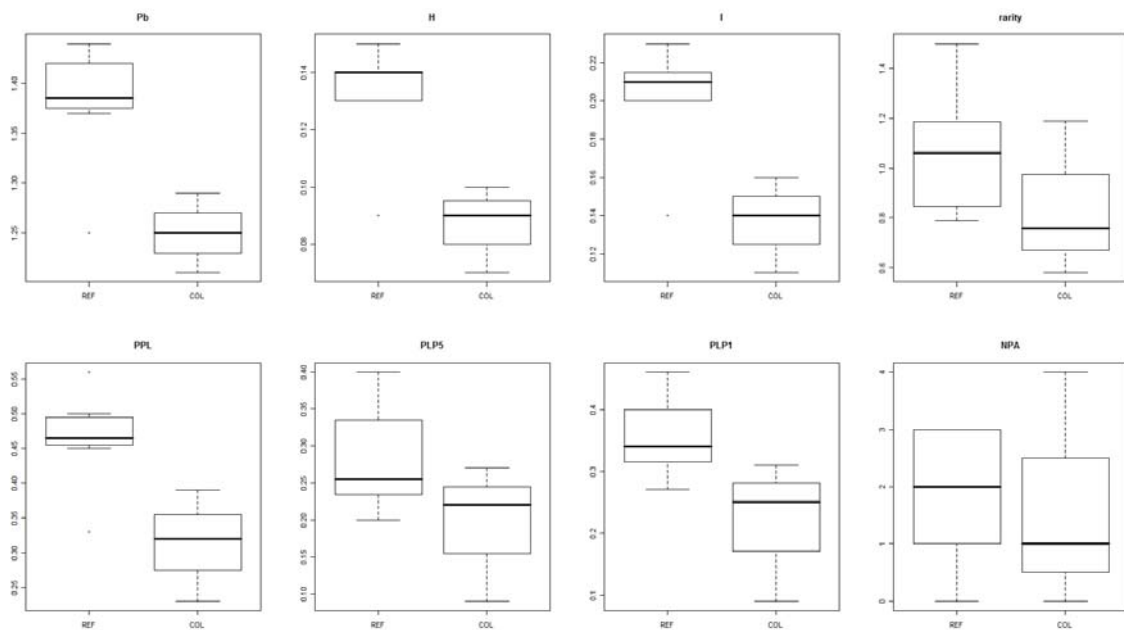
* calculated without rarefaction, instead the mean number of alleles is given

A *P. marginata*



PB7	H	I	RARITY	PPL	PLP5	PLP1	NPA
0.2044	0.2125	0.1709	0.014*	0.077	0.035*	0.021*	0.144

B *P. latifolia*



Pb7 ¹	H	I	RARITY	PPL	PLP5 ¹	PLP1 ¹	PRIVAL
0.011*	0.019*	0.017*	0.135	0.018*	0.070	0.017*	0.518

Figure 5 Boxplots of various genetic indices of *P. marginata* (A) and *P. latifolia* (B) comparing populations situated in glacial refugia (“REF”: *P. latifolia*: BES, BRId, FON, LLAU, BAS, CFREa, LAC, VACH; *P. marginata*: AUT, BFRE, BRIa, CADa, CAS, ENT, MEL, NAV, PIEa, PIEb, POR, REIa, SPIE, TEN, TRI, TVAL) versus colonized areas (“COL”: *P. latifolia*: LAU, ORM, SOM; *P. marginata*: ALLa, ALLb, BAR, BLA, COU, ECH, GUI, LAR, LOM, MOR, RES, SER) as indicated by SDMs and paleodistribution models. Whiskers extend to the most extreme data point within the interquartile range from the box. P-values from one-tailed randomization tests using 10,000 Monte Carlo iterations comparing the two groups are given below the boxplots, significant differences are marked with an asterisk.

Admixture between species

Despite the large overlap of the species present distribution ranges, negligible admixture is found between species. An admixture analysis (using BAPS) based on two predefined groups corresponding to the two species revealed that of 524 individuals only two individuals per species are significantly admixed with the other species (Table 2). They belong to populations ALB and CFRE in *P. latifolia* (with admixture coefficients 0.16 and 0.28 respectively) and to GUI and TEN in *P. marginata* (with admixture coefficients 0.11 and 0.23 respectively). CFRE and TEN are located in the Alpi Marittime, where current distribution overlap and possibly also the refugial areas, whereas the other two populations are located in areas, where current distribution do not overlap.

Discussion

Glacial and postglacial history of *Primula marginata*

Most of the models (7-8) indicate glacial survival of *P. marginata* in refugia west and east of the ice-shield in the Alpi Marittime (Figure 1 B above; see Figure 1 A above for names of mountain ranges). In both areas calcareous and siliceous bedrock alternate and Schönswetter et al., (2005) proposed various potential peripheral refugial areas there for calcareous alpine plants. Fewer models (5-6) indicate that these refugia were connected at the tip of the outrunning Alpine glacier. However, the clear divergence of the SE-groups from the other genetic groups (SW, N, MEL) (Figure 3 A+C) indicates that the refugial areas west (southwestern refugium) and east (southeastern refugium) of the former glacier were not connected. Supporting this, both groups comprise refugial populations and colonized populations (Figure 2 A left). The area at the tip of the outrunning glacier predominantly consists of siliceous bedrock. This may have served as a dispersal barrier between the southwestern and the southeastern refugium during the last glacial maximum (LGM), but this remains unclear since *P. marginata*'s extant distribution is not limited to calcareous bedrock. Survival in two different refugia is also supported by the relatively high genetic structuring found, which likely arose from genetic drift in isolated glacial populations. Five groups are detected, which are characteristic of a defined geographic area (Figure 2 A left), and more variation is distributed among these genetic groups than among the populations within the groups. Additionally, the F_{st} values are similar to *P. hirsuta* where survival in different refugia is also proposed (Table 3; second article Table 3).

A paleoecological study suggested that the southern part of the Maritime and Ligurian Alps was a major glacial refugium, as this region was somewhat sheltered from the impact of Pleistocene glaciations (Nicole-Pichard & Dubar, 1998). In the few phylogeographic studies that have been done in the Southwest Alps to infer the glacial and postglacial history of alpine plants, refugial areas similar to the ones identified here have been proposed. The two predicted refugia of *P. marginata* overlap with five different peripheral refugial areas proposed for the strictly calcareous *Gentiana ligustica* on the basis of ITS sequence variation, Pleistocene glaciation, topography and geology (Diadema et al., 2005). Naiciri & Gaudeul (2007) also found strong evidence for a southwestern refugium of *Eryngium alpinum*, based on cpDNA variation. They propose a large area at the periphery of the Southwestern Alps overlapping with the southwestern refugium and partly the southeastern refugium of *P. marginata*.

The northern parts of the extant distribution of *P. marginata* were presumably colonized from the southwestern refugium, which is indicated by the close relation of the N-group and the SW-group (Figure 3 A+C), and by the fact that many of the northern populations are mixed with the SW-group (Figure 2 A left; Table 2). The highest mountains in the inner parts of the Alpi Marittime, however, were probably colonized from the southeastern refugium, since the SE1-group today occupies both areas (Figure 1 C above; Figure 2 A left).

To summarize, based on SDMs and population genetic patterns, *P. marginata* presumably survived the LGM in peripheral refugia in the Alpi Marittime, which has also been proposed for other alpine species. These refugia were located west and east of the former glacier and probably separated.

Glacial and postglacial history of *Primula latifolia*

In *P. latifolia*, most of the paleodistribution models (5-6) indicate survival in the Alpi Graie, the Alpi Cozie and the Alpi Marittime, both on peripheral nunataks (at the periphery of the glacier, but outside the snowline) and on central nunataks (inside the snowline) (Figure 1 B below; see Figure 1 A below for names of mountain ranges). The peripheral nunatak areas in the Alpi Cozie, the Alpi Graie and the Alpi Marittime overlap with potential peripheral refugia for silicicolous plants based on geological and paleoenvironmental data (Schönswetter et al., 2005). Additionally, the paleodistribution models include several nunatak areas in the highest mountains of the Alpi Marittime, the predominantly siliceous Massif du Mercantour (Diadema et al., 2005). One of these nunataks (above the upper Tinée valley) has also been proposed for *Saxifraga florulenta*, a rare endemic of the Alpi Marittime which is restricted to siliceous substrates, based on AFLP variation, geology and the maximum glacial extent (Szövényi et al., 2009). Most probably, nunataks were also located in the Alpi Graie and the Alpi Cozie, where refugia are predicted for *P. latifolia*.

The N-group is distributed in both, the southwestern and the central Alpine (Engadin) subrange of *P. latifolias*' disjunct distribution (Figure 2 A right). The clear divergence between the N-group and the other groups (Figure 3 B+D), even though the N-group is geographically adjacent to the SE-group in the Alpi Cozie, indicates that this split has been caused by glacial survival in different refugial areas. However, from our data it is difficult to infer the location of a putative refugium of the N-group: Regarding the species distribution models (SDMs) (Figure 1 B below; see Figure 1 A below for names of mountain ranges), the refugium of the N-group presumably has been situated on a nunatak in the Alpi Cozie, and the

Engadin was consequently colonized from there. But this can not be confirmed with the population genetic data, since only one of the collected populations (BAS) in the Alpi Cozie represents a refugial population (population BAS; Figure 2 A right; see Figure 1 B below for locations of populations). Additionally, our data do not allow us to reject the possibility that a refugium was located in or close to the Engadin, and that the populations colonized the southwestern Alps from this central Alpine refugium. This is because the SDMs failed to predict the extant distribution in the Engadin (Figure 1 A below), probably caused by the low number of occurrences available from this area to fit the models, and consequently may also have failed to predict a glacial refugium there (Figure 1 B below). On the basis of the population genetic patterns it is difficult to distinguish between these two scenarios, survival on nunataks in the Alpi Cozie *or* in the Engadin, because only one population was sampled with several individuals in the Engadin. The populations in the Alpi Cozie and west of them (LAU, SOM, BAS; see Figure 1 B below for locations of populations) all have low diversities which could have been caused by a bottleneck due to long distance dispersal from a putative refugium in or close to the Engadin (Figure 2 B right). A central Alpine refugial area in the Engadin is conceivable since the Engadin is one of the floristically rich nunatak areas proposed by Brockmann-Jerosch & Brockmann-Jerosch (1926), reviewed and mapped in (Stehlik, 2000), and it has been proposed for *Eritrichium nanum* based on phylogeographic evidence (Stehlik et al., 2001, Stehlik et al., 2002a, Stehlik, 2003).

Other refugial areas were presumably located in the Alpi Marittime and/or along the southeastern periphery from the Alpi Marittime to the Alpi Cozie, where today three different genetic groups (SW, SE, ORM) are located (Figure 2 A right). Populations in the Alpi Marittime, especially at the southwestern margin, and populations at the southeastern periphery of the Alpi Marittime and the Alpi Cozie have high diversities (Figure 2 B right), and also harbour two or three private fragments (Figure 2 D right; Table 4; see Figure 1 B below for locations of populations). Survival in several refugia is supported by the relatively high genetic structuring: more variation is distributed among the genetic groups than between populations within the genetic groups (Table 3). Additionally, the F_{st} values are even higher than in *P. hirsuta* (second article Table 3), which is also widespread throughout the Alps, and in *P. marginata* (Table 4), where survival in different refugia is also proposed for both species.

To summarize, based on SDMs and population genetic patterns, as well as potential peripheral refugia based on paleoenvironmental and geologic evidence, *P. latifolia* presumably survived the last glacial maximum in several refugia on peripheral nunataks

(outside the snowline but inside the glacier) and on central nunataks. One refugial area was located in the Alpi Marittime, which has also been proposed for *Saxifraga florulenta*. The southeastern periphery of the Alpi Marittime and the Alpi Cozie presumably also served as a refugial area. Central nunataks in the Alpi Cozie are likely to have served as refugial area for the N-group, but it can not be excluded that the Engadin or areas close to it have served as a central Alpine refugia.

Congruence of population genetics and paleodistribution models indicate niche conservatism

It is often expected and has been shown that populations in refugial areas exhibit higher rarity and/or higher diversity (Hewitt 1996, Comps et al., 2001, Widmer & Lexer, 2001, Stehlik et al., 2002a, Stehlik et al., 2002b, Tribsch et al., 2002, Paun et al., 2008). Congruency of diversity indices, rarity and populations with higher numbers of private fragments with glacial refugia predicted by the species distribution models (SDMs) (Figure 2 B-D; Figure 5; Table 4) indicate correct predictions of the glacial refugia which are based on the extant ecoclimatological niches of the species. This is a clear argument in favour of niche conservatism for the time period from the last glacial maximum (LGM, 21,000 years ago) to present. The finding that much more fragments were private to the refugial populations (considered as one group) than to the colonized populations (considered as one group) supports this. It has to be noted, however, that the number of individuals from colonized populations was lower than the number of individuals from refugial populations.

Niche conservatism in evolutionary time has also been shown for North American plants and animals using different approaches. Peterson et al. (1999) examined sister taxon pairs of birds, mammals and butterflies and tested the degree to which ecological characteristics of one taxon were able to predict the geographic distribution of its putative sister taxon and vice versa. Martinez-Meyer & Peterson (2006) and Martinez-Meyer et al. (2004) assessed the ability of SDMs based on the extant ecological niches of trees, shrubs and mammals to predict geographic distribution during the Pleistocene and vice versa. Model predictions were evaluated by comparing them to pollen records and the extant distribution respectively. Waltari et al., (2007) compared locations of North American Pleistocene refugia of vertebrate species, derived by paleodistribution modeling on the one hand and phylogeographic methods on the other hand. All studies found evidence for niche conservatism over at least short evolutionary time periods.

Geography of speciation

Although, considering their genetic divergence, based on phylogenetic evidence (Zhang & Kadereit, 2004b), *P. latifolia* and *P. marginata* are not likely to have originated in the last glacial maximum (LGM, 21,000 years ago), but rather in an earlier cold phase, we assume that the paleoclimate from the LGM can be used to represent Quaternary cold phases climate in general. This means that the refugial areas identified for LGM are assumed to be representative of glacial refugia during earlier cold phases where speciation took place.

As explained in the introduction, the finding of isolated glacial refugia during the last glacial maximum would fit the hypothesis of allopatric speciation during a glacial phase. In support of this hypothesis, the results from paleodistribution models, which are congruent with population genetic patterns, reveal different spatial distribution of suitable areas during the LGM for the two species respectively.

However, the glacial distribution of the two species overlaps along a small zone at the border of the former ice-shield in the Alpi Marittime (Figure 4). This zone of overlap becomes smaller when a higher threshold is used to define the glacial distribution of the two species from the consensus models (at least half of the modeling techniques/the majority of modeling techniques). This trend shows that the geographical distribution of the climatic niches of both species diverge. Presumably, the overlap is caused by the relatively low spatial resolution (c. 5 km) of the paleoclimate models used. This probably leads to a lack of resolution of the altitudinal differences between the species' glacial distribution: while the refugial areas of *P. latifolia* in the Alpi Marittime are situated on the high mountain ridges, the refugia of *P. marginata* are situated in the lower foothills of the Alpi Marittime.

The position of the glacial ice sheet in the Alpi Marittime, which is not incorporated in the paleodistribution maps, restricts *P. latifolia* mainly to central nunataks within the glacier and to small discrete areas outside the glacier, and *P. marginata* to peripheral refugia outside the glacier. The zone of overlap of the two species' glacial distribution is consequently reduced to small discrete areas outside the glacier. This overlap, even without considering the ice sheet, is rather small, c. 11% and c. 6% of the total distribution of both species respectively, depending on the threshold used to define the glacial distribution from the consensus models (at least half of the models/the majority of models). Using phylogenetic comparative methods, which represent a different approach to investigate the geographical mode of speciation, different thresholds for the amount of overlap are used to distinguish between geographical modes of speciation, depending on the authors (Losos & Glor, 2003). Lynch (1989) for example, does not consider an overlap of <20% of the distribution ranges of

sister taxa to be indicative for sympatric speciation. To summarize, the overlap of glacial distribution between *P. latifolia* and *P. marginata* presumably was very small and restricted to small discrete areas outside the glacier and probably does not indicate sympatric speciation. The larger geographically separate glacial refugial areas of the two species rather support allopatric speciation.

Although *P. latifolia* and *P. marginata* were the only sister species pair identified with good support in Zhang & Kadereit (2004b) based on nrITS (internal transcribed spacer) sequences, continuous efforts to improve the phylogenetic resolution in the ‘western clade’ could not confirm this sister group relationship. Instead, the analysis of AFLP, nrITS and cpDNA variation indicate a burst of simultaneous speciation events (Crema et al., in prep.), which is in accordance with our model of speciation in isolated glacial refugial areas proposed in (Kadereit et al., 2004). According to these results, *P. latifolia* and *P. marginata* can be regarded as sisters in a broader sense, since they originated from the same ancestor and have a partly overlapping distribution. Consequently, the evidence for allopatric speciation found here is still significant.

Assumptions of the approach

Reconstructions of the geographic mode of speciation are only useful if the underlying assumptions of the methods, which are being used, are met. We here assume that the range of the ancestor of *P. latifolia* and *P. marginata* at the time of speciation can be inferred from the current distribution of the species. Specifically we assume that (i) the climate models of the last glacial maximum (LGM) are representative for the climate during earlier glacial phases in which speciation took place and that (ii) the climatic niches of the two species did not change since their origin. If these assumptions do not hold, the species distribution models (SDMs) do not correctly predict the glacial refugia the species occupied during the speciation process and our conclusions on the geographic mode of speciation may be wrong. These two assumptions are being discussed.

(i) No estimates of the exact time of speciation are available to us. The dates inferred by Crema (2009) using an AFLP clock developed by Kropf et al., (2009) seem too young compared to the estimations of (Zhang et al., 2004a) based on an ITS phylogeny. Estimates using the AFLP clock indicate that all speciation events within the ‘western clade’ of *Primula* section *Auricula* took place during the LGM while estimates of the molecular clock derived from the ITS phylogeny date the age of the ‘western clade’ to the beginning of the Pleistocene (c. 2.4 million years ago). Therefore, we do not have any means to say whether the climate

models used are representative for the climate at the time of speciation. Additionally, climate models for the use in SDMs do not exist for previous glacials.

(ii) As indicated by the congruence of SDMs and population genetic patterns, the ecoclimatic niche of the species did not change during the last 21,000 years. This may be an indication for the stability of the ecological niche over several previous glacial - interglacial cycles, but it essentially remains unknown whether the species changed their ecological niches after their origin or not. However, our approach has the advantage that climatically induced range shifts – between the time of speciation and today – are taken into account. In contrast, comparisons of the extant distribution of sister species to infer the geography of speciation assumes that the extant distribution correlates strongly with the geographical distribution at the time of speciation. Furthermore to infer the geographical distribution of ancestral taxa using ancestral area analyses (e.g. Cabrera, 2007), one must assume that distribution are so static that ancestral geographical ranges can be inferred from the ranges of their descendants.

Recently, increasing efforts have been made to investigate niche conservatism, which is often assumed when discussing geographical modes of speciation. A review of the studies of niche conservatism or shift during the last decade is given in Pearman et al., (2008a). Several approaches combined SDMs and phylogenetic methods to explore niche evolution (Peterson et al., 1999, Peterson & Holt, 2003, Stockman et al., 2008, Warren et al., 2008). However, neither niche stasis nor rapid niche shift prevail in current studies and no particular ecological conditions, morphological characteristics or evolutionary histories were associated with the frequency of niche shift or conservatism. That means that the current state of knowledge offers no generality of niche dynamics in evolutionary time scales.

2. Artikel

Integrating species distribution models (SDMs) into a phylogeographic framework to investigate Pleistocene species history in two Alpine *Primula*

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Introduction

Fossil evidence suggests that the climatic oscillations of the Quaternary had a strong influence on the geographical distribution of plants (Lang 1994). Analyses of DNA sequence data and fingerprints have provided new insights into the Quaternary distribution of plants and animals in the northern hemisphere, and the locations of glacial refugia. For example, so-called cryptic northern refugia are now identified for various plant and animal taxa in the northern hemisphere, and it seems increasingly likely that, besides the well-known southern refugia, they played an important role for glacial survival (Stewart & Lister, 2001, Bhagwat & Willis, 2008, Birks & Willis, 2008, Provan & Bennett, 2008). Further, the importance of Mediterranean refugia for the recolonization of northern parts of Europe seems smaller than previously assumed (birch: Maliouchenko et al., 2007, beech: Magri et al., 2006, Norway spruce: Heuertz et al., 2006).

For the glacial survival of alpine plants, two contrasting possibilities have been discussed since the beginning of the 20th century, i) glacial survival at the periphery of the Alpine ice shield and subsequent re-colonization of vacant areas after the retreat of glaciers

(tabula rasa hypothesis) or ii) glacial survival *in situ* within glaciated areas on ice-free locations above the glacier (nunatak hypothesis) (reviewed in Stehlik 2000 and Brockmann-Jerosch & Brockmann-Jerosch, 1926). Since the paucity of pollen records for most mountain plants of the European Alps during the last glacial maximum (LGM) has rendered it impossible to locate refugia, molecular methods are being used and are complemented by analyses of patterns of endemism and biogeography to refine our picture of persistence scenarios. Many recent molecular studies of different plant taxa support one or the other scenario of glacial survival, or a combination of both (nunatak survival or both scenarios: (Ravazzi 1997, Fürchter et al., 2001, Stehlik et al., 2001, Stehlik, 2002, Stehlik et al., 2002a, Schönswetter et al., 2003b, Parisod & Besnard, 2007); survival in peripheral refugia: (Schönswetter et al., 2002, Tribsch et al., 2002, Schönswetter et al., 2003a, Schönswetter et al., 2004, Naciri & Gaudeul, 2007, Paun et al., 2008). Most of these studies compared phylogeographic patterns with hypothetical refugial areas based on floristic and biogeographic evidence (Stehlik, 2000), patterns of endemism (Tribsch & Schönswetter, 2003, Tribsch 2004) or paleoenvironmental data (limits of last glacial maximum ice shield and snowline, position of never glaciated areas and nunataks) and geology (Schönswetter et al., 2005), or on a combination of those.

However, to our knowledge no studies of mountain plants of the European Alps have been using species distribution models (SDM) to infer the glacial distribution of single species while studies with such approaches of European trees accumulate slowly (e.g. Alsos et al., 2009). SDMs are based on the relationship of species occurrence and environmental data (Guisan & Zimmermann, 2000), and provide an estimate of the historical distribution based on the current ecological niche of species. It is clear from the pollen record and simulation models that each species responded individually to the climate changes by tracking their particular set of environmental requirements (Hewitt 1996). Therefore, using SDMs to locate glacial refugia clearly represents an advantage compared to the approach explained above since it models the individual response of a species to climate changes. In cases where it remained impossible to reconstruct the glacial history, e.g. *Saxifraga oppositifolia* (Stehlik, 2003), this approach may provide new insights.

We here combine SDM with phylogeographic methods to locate glacial refugia and reconstruct past range dynamics in two closely related *Primula* species of section *Auricula* (*Primula hirsuta* and *P. daonensis*). Similar approaches are increasingly used and help to understand the causes that influence current population genetic structure, diversity and divergence. This informs us on the response of species to environmental changes, which is

crucial to predicting future plant responses to climatic changes (Hugall et al., 2002, Carstens & Richards, 2007, Knowles et al., 2007, Richards et al., 2007, Pearman et al., 2008b, Jakob et al., 2009, Moussalli et al., 2009). *Primula daonensis* and *P. hirsuta* are closely related predominantly silicicolous species, which originated during the Pleistocene, presumably in isolated glacial refugia (Kadereit et al., 2004, Zhang et al., 2004a, Crema, 2009). These species were first chosen as a relatively well supported sister species pair based on an ITS phylogeny (Zhang & Kadereit, 2004b) in order to investigate the role of glacial refugia for speciation. However, new results based on AFLPs, nrITS and cpDNA indicate that they are not sister species but presumably originated simultaneously together with one or several other species during an early cold phase (Crema et al., in prep.). Regardless of their phylogenetic relationship, these two parapatrically distributed species represent an interesting study system to investigate glacial survival and re-colonization patterns: *P. hirsuta* is widespread across the Alps (and also occurs in the Pyrenees) which may indicate survival on central nunataks. The distribution of *P. daonensis*, however, is restricted to few mountain ranges in the southern Central Alps and is connected to the Alpine periphery, which might indicate peripheral survival.

The aim of this study is to locate the glacial refugia of the two species differing in the size and position of their extant distribution ranges, to identify whether nunatak survival or rather peripheral survival or a combination of both applies to them, and to reconstruct postglacial re-immigration. Nunatak survival should lead to a patchy distribution of different genotypes – exhibiting variation among them – throughout the formerly glaciated Inner Alps. In contrast, survival in peripheral refugia and consequent colonization of formerly glaciated areas should result in large areas occupied by closely related genotypes without exhibiting much variation between populations. Thereby populations of peripheral refugia and the Central Alps should belong to the same gene pool. A major interest is to point out if and how a combined approach using SDMs and population genetic analyses supports or modifies the predictions that can be made on the bases of phylogeographic methods alone, or compared to hypothetical refugia based on biological, geological and/or paleoenvironmental evidence.

In this study we use species distribution models to predict the glacial distribution of *Primula hirsuta* and *P. daonensis* during the LGM, 21,000 years ago, and then analyze population genetic patterns to evaluate these predictions. We assume, that populations in refugial areas are characterized by higher genetic diversity and rarity than populations, that colonized previously glaciated areas, as has been shown for other taxa (Hewitt 1996, Comps

et al., 2001, Widmer & Lexer, 2001, Stehlik et al., 2002a, Stehlik et al., 2002b, Tribsch et al., 2002, Paun et al., 2008), and use this information to evaluate the predictions of the SDMs.

Material and Methods

Molecular methods

Sampling

Eight populations of *P. daonensis* and 21 populations of *P. hirsuta* were sampled in 2005 and 2006, covering nearly the entire range of the two species (Table 1; Figure 1 A+C (species distribution) and B+D (sampled populations)). Leaf material of c. 10 to 15 individuals per population was collected. Chosen individuals had a minimum distance of three meters to avoid collecting the same clone. Where this was not possible due to small population size, individual AFLP fingerprints were compared to eventually exclude identical fingerprints. If possible, sampled individuals were evenly distributed across the populations' whole range. In very large populations the sampling was limited to an extend comparable to the other populations. Voucher specimens are deposited in the herbarium of the Institut für Spezielle Botanik, Mainz (MJG).

DNA Extraction

Total genomic DNA was extracted from silica-gel-dried leaf material (c. 20 mg dry weight) using the QUIAGEN DNeasy Plant Mini Kit following the standard protocol with the modification that 10 mM sodium meta disulfide was added to the AP1-buffer. DNA was eluted two times using 40 µl AE-buffer.

Table 1 Locations of the sampled populations of *P. daonensis* and *P. hirsuta* affiliation to genetically defined groups inferred with BAPS (BAPS-group) as well as the number of individuals admixed between the species (adm). For localities where several ‘populations’ were sampled at a minimum distance of a few 100 m, the number of sampled individuals per location is given in brackets and differing altitudes of the subsamples are specified by small letters. In mixed populations the different BAPS-groups are separated by a slash; brackets indicate that only one of the populations’ individuals belongs to the specified BAPS-group.

code	locality, country	altitude (m)	lat/long	n° ind.	BAPS-group	adm.
<i>P. daonensis</i>						
VIV	Passo Vivione, I	c. 1900	10.201/46.035	24 (12+12)	W	
GAV	Passo Gavia, I	2570 (a), c. 2700 (b)	10.485/46.339	13 (8+5)	W/E/(N)	
UMB	Passo Stelvio, I	2560	10.439/46.535	13	W/N/(E)	
MUR	Alp Muraunza, CH	c. 2200	10.433/46.558	24 (5+4+6+9)	W/N/E	
SCO	Monte Scorluzzo, I	3050	10.442/46.521	8	N	
GOL	Goletto di Crocette, I	2111	10.376/45.859	12	E	
LCAS	Lago Casinei, I	2090	10.513/45.947	9	E	
PED	Cascada Pedruc, Val Genova, I	1530	10.623/46.196	10	E/W/(N)	
<i>P. hirsuta</i>						
LAU	Col du Lautaret, F	2050-2100	6.415/45.031	12	SW	
OLA	Ref. d'Olan, Valgaudemar, F	c. 1690 (a), 2320-2400 (b)	6.204/44.842	9 (1+8)	SW	
GRAE	Grächen, M Mattertal, CH	2100	7.868/46.208	9	W	
GRUE	Grütschalp, Lauterbrunnental, CH	2050	7.948/46.579	11	W/(C1)	
ARC	Arcegno, CH	450	8.744/46.158	11	C1/C2	
FLU	Flüelapass, CH	c. 2400	9.933/46.767	12	C1/(C2)	
GAL	Galzig, AU	2180	10.227/47.132	9	C1	
GAU	Gauertal, AU	c. 2200	9.818/47.056	20 (11+9)	C1	
IND	Indemini, I	980	8.816/46.095	8	C1/(C2)	
OB	Oberalp pass, CH	c. 1850	8.686/46.650	12	C1	
SBER	San Bernadino, CH	1804	9.197/46.471	10	C1	
SEL	Selva, CH	c. 1540	8.714/46.655	10	C1	
TIR1	Neustift im Stubaital, AU	1870	11.185/47.092	9	C1/(W)	
TIR2	Vals im Valsertal, AU	1440	11.585/47.043	9	C1	
TIR3	Gries im Venntal, AU	1530	11.536/47.013	7	C1	
ZEI	Zeinisjoch, AU	c. 1850	10.127/46.978	12	C1	
BER	Berninapass, CH	2250	10.005/46.415	12	C2	
MAL	Malojapass, CH	1840	9.694/46.399	9	C2	
GRI	Grigna settentrionale, I	1930	9.384/45.963	11	GRI	3
VAL	Mte Nudo in Valcuvia, I	1080	8.674/45.925	10	IND	
SUL	Suldental, I	c. 2360-2450	10.575/46.520	22 (9+10+3)	SUL	18

AFLP protocol

The AFLP protocol follows (Vos et al., 1995) with modifications. The restriction-ligation was performed at once for all individuals of one species respectively. Total genomic DNA (c. 100 ng) was digested and ligated simultaneously using 2 U of EcoRI and 0.8 U MseI (both from New England BioLabs, NEB) and 0.7 WU T4-DNA ligase (GeneCraft) in a reaction volume of 10 µl containing 23.5 pmol MseI adapter (5'-gacgatgagctctgag-3'; 3'-TACTCAGGACTCAT-5') and 2.35 pmol EcoRI adapter (5'-CTCGTAGACTGCGTACC-3'; 3'-CTGACGCATGGTTAA-5') (both from Metabion), 0.96 µl 0.5m NaCl, 1 µg bovine serum albumin (NEB), 0.96 µl 10X T4 DNA ligasebuffer (GeneCraft) and DEPC treated water (Roth). Reactions were incubated at 37°C for 2 h and 15°C for 8 h to ensure complete digestion and ligation. Products of the restriction-ligation reaction were diluted 4-fold, 5 µl were used as template in the preselective PCR at a reaction volume of 25 µl containing 0.5 U BioTherm Taq-polymerase and 2.5 µl of BioTherm 10X PCR-buffer (GeneCraft), 1.56 µl 50 mM MgCl₂, 0.33 µl of 20 mM dNTPs, 12.5 ng MseI+1 primer and 12.5 ng EcoRI+1 primer and DEPC treated water. The thermocycling profile started with 2 min at 72°C followed by 30 cycles of 20 s at 94°C, 30 s at 56°C, 3 min at 72°C and terminated with 10 min 72°C. Products of the preselective PCR were diluted 20-fold and 5 µl were used as template in the selective PCR with a reaction volume of 15 µl. The Master Mix further contained 0.25 U BioTherm Taq-polymerase and 1.5 µl BioTherm 10X PCR-buffer 12.5 ng MseI+3 primer, 9 ng labeled EcoRI+3 primer, 0.2 µl 20mM dNTPs, 1.13 µl 50mM MgCl₂ and DEPC treated water. A hotstart of 10 min at 95°C was followed by 36 cycles of 30 s at 94°C, 1 min at X°C and 2 min at 72°C, whereas in the first 13 cycles the annealing temperature was reduced by 1°C at each step starting with 65°C and ending with 56°C – with the exception that 64°C, 62°C and 58°C were repeated two times, and the last 23 cycles were continued at 56°C, completed by a terminal incubation of 10 min at 72°C.

Primers used in the preselective PCR were E01 (5'-GACTGCGTACCAATTCA-3') and M01 (5'-GATGAGTCCTGAGTAAC-3') and in the selective PCRs E38-Hex (5'-Hex-GACTGCGTACCAATTC-3') combined with M52 (5'-GATGAGTCCTGAGTAACCC-3'), E38-6-Fam with M55 (5'-GATGAGTCCTGAGTAACGA-3') and E39-NED (5'-NED-GACTGCGTACCAATTCAGA-3') with M55 (all primers were from Metabion, except E39-NED from ABI). The three AFLP reactions using primers labeled with 6-Fam, Hex and NED were multiplexed and purified using Sephadex (Millipore). AFLP fragments were separated using ABI 3730 DNA Analyzer and ROX500 size standard (ABI).

Population genetic analyses

Scoring of AFLP fragments was performed with GeneMarker Version 1.75 (GeneMarker, SoftGenetics, LLC). We calculated mismatch error rates to evaluate the quality of our analyses as the ‘number of genotype mismatches’ divided by the ‘number of replicate pairs’ and the ‘number of loci’ (Paun et al., 2008). Genetic variation was quantified using several diversity measures. The allelic richness P_b (ranging from 1 to 2) and the percentage of polymorphic loci with standardized sample sizes (PLP5, PLP1) were calculated with AFLPDiv1.0 (Coart et al., 2005) as comparable measures for genetic diversity inferred from AFLP data with an emphasis on the number of alleles. For populations with population sizes lower than the number of rarefactions used, the mean number of alleles per locus is given instead of the band richness. Nei's gene diversity H (Nei 1973) (also referred to as expected heterozygosity), which emphasizes the differences of allele frequencies, Shannon's information index I (Shannon & Weaver, 1949, Lewontin 1972) and the percentage of polymorphic loci PPL (regardless of allele frequencies) were calculated with Popgene 3.2 (Yeh & Boyle, 1997) assuming Hardy-Weinberg-equilibrium. The rarity index was calculated as the frequency of each marker in a population relative to the number of occurrences of the marker in the whole dataset, summed up for all markers and then divided by the number of markers and multiplied with the number of individuals in the whole dataset (as described in Paun et al., 2008). Genetic structure of populations was analysed using ‘Bayesian Analysis of Population Structure’ (BAPS, vers. 5.1) (Corander et al., 2008). The procedure was run 10 times each for $K=2-10$ as the assumed maximum number of populations present in the sample. Admixture analyses (Corander & Marttinen, 2006) were run with 100 iterations to estimate admixture coefficients for individuals, 200 reference individuals from each population and 20 iterations to estimate admixture coefficients for reference individuals. To detect within-species admixture, the analyses were run based on mixture clustering, and to detect admixture between species, the analyses were run based on two predefined groups corresponding to the species. Analyses of molecular variance (AMOVA) were conducted using Arlequin vers. 3.1 (Excoffier et al., 2005). Principal coordinate analyses (PCoA) using Dice (Sorensen) similarity were conducted with PAleontological STatistics (PAST, vers.1.62) (Ryan et al., 1995).

Reconstructing past species distribution

Principles of species distribution modeling (SDMs)

Species distribution models allow to generate maps of the expected distribution of a species using information on the environmental conditions where it is known to occur (many models also take the absence of species into account). Different modeling techniques exist determining the set of parameters which best predict the presence of species. In a further step, models of the extant species distribution can be projected to a different time using past climate models in order to generate paleodistribution maps.

Climate data

We used current climate data at a spatial resolution of 30'' (c. 1 km) from the WorldClim database (Hijmans et al., 2005). These so-called bioclimatic variables proved meaningful to describe plant and animal species niches in several studies (Waltari et al., 2007) and include monthly temperature and precipitation variables describing annual trends, seasonal variability and extreme and potentially limiting environmental factors. The same variables for LGM climate data were drawn from general circulation model (GCM) simulations from two climate models: the Community Climate System Model (CCSM, version 3) (Collins et al., 2006) and the Model for Interdisciplinary Research on Climate (MIROC, version 3.2) (Hasumi & Emori, 2004). The downscaled climate surfaces at 2.5' (c. 5 km) spatial resolution were provided by Robert Hijmans and derived from the original climate simulations as described in (Waltari et al., 2007).

We chose a subset of eight bioclimatic variables by conducting preliminary model runs using all 19 available variables and identifying those with high variable importance. We additionally identified redundant variables by correlating 5000 random points distributed over the whole study range (3.5°-17°E, 43°-49°N). The following variables which correlated less than 0.8 among themselves were chosen as predictors: annual mean temperature (bio1), mean diurnal temperature range (bio2), temperature seasonality (bio4), mean temperature of wettest quarter (bio8), mean temperature of driest quarter (bio9), annual precipitation (bio12), precipitation seasonality (bio15) and precipitation of warmest quarter (bio18).

Fitting current species distribution models and projecting to the last glacial maximum (LGM)

Several recent studies advise to combine multiple modeling techniques (“consensus approach” or “ensemble forecasting”) (Thuiller, 2004, Pearson et al., 2006, Araujo & New, 2007, Pearson et al., 2007). This seems particularly appealing when models are used to project the distribution of species into independent situations, which is the case here where species distribution are projected to the LGM, a period in which the climate strongly differed from recent climate. This might reduce the predictive power of some techniques or cause discrepancies between different modeling techniques, making the choice of the appropriate model difficult. Consensus models highlight the areas, which are predicted by several modeling techniques making them more reliable.

We applied the following four modeling techniques using the latest release of the BIOMOD tool (Thuiller, 2003) implemented in R software (R Development Core Team, 2005): generalized linear models (GLM), generalized additive models (GAM), generalized boosting models (GBM) and random forests (RF). These techniques take the occurrences and absences of species into account. Occurrences were generated by combining several data sources (‘SOPHY’ (Ruffray et al., 2000-2009), ‘ZDSF’, data of local surveys from Alberto Selvaggi from the “Istituto per le Piante da Legno e l'Ambiente s.p.a.c.so Casale”, Torino, Italia and Filippo Prosser from the “Museo Civico de Rovereto”, Italia) and the localization of herbarium specimen from several collections (M, MSB, Z, ZT, RUEB, MJG). As no absences could be inferred from these data, we created 5000 pseudo-absences, which were randomly distributed over the entire study range as recommended in (Elith et al., 2006), and weighted to equal the number of presences per species. Continuous model predictions (ranging from 0 to 1) were transformed into binary predictions using the cut-off threshold maximizing AUC statistic (Area Under the Curve, meaning the Relative Operating Characteristic (ROC) curve). Binary predictions of the four techniques were cumulated. Using CCSM3 climate resulted in more inter-model variability regarding the distribution areas than did the use of MIROC3.2 climate. But the consensus distribution areas obtained with both climates respectively are widely overlapping. We consequently also cumulated the consensus maps derived with the two climates separately to create distribution maps which outline those areas predicted by most modeling techniques under both climates.

Model evaluation

The predictive performance of the models was evaluated by 100-fold cross-validation, each time using a random subset (70%) of the total dataset to calibrate the models, while the remaining 30% were used to evaluate the model, calculating three different measures, namely the ROC curve, the True Skill Statistic (TSS) and the Kappa statistic.

Range shifts during Holocene

In order to identify regions that did not serve as refugia but were colonized during Holocene, we overlaid the present distribution maps with the paleodistribution maps. We overlaid those areas where the majority of models predict present and past distribution (3-4 at present and 5-8 at the last glacial maximum). Stable areas, where both, present and past model-predicted distribution overlap, can be distinguished from areas, where only present species distribution is predicted (postglacially colonized areas) and areas, where only past distribution is predicted (refugial areas, which were postglacially abandoned).

Combination of the approaches

Evaluation of the modeled last glacial maximum (LGM) refugia using spatial patterns of genetic diversity and rarity

Populations were classified into two types regarding their population history as inferred from the paleodistribution models, 1) populations located in LGM distribution areas and 2) populations located in colonized areas. These classifications were done on the basis of the consensus maps in such a way that populations were assigned to the type which is indicated by the majority of models. If equal numbers of models indicate one *or* the other type, the population was not taken into account. In Figure 3, the population types are indicated with “*” for refugial populations, “-” for colonized populations and “?” for populations which cannot be assigned to a type. We used randomization tests to compare the means of various diversity measures as well as rarity and the number of private alleles of LGM populations versus colonized populations, applying 10,000 Monte Carlo runs using Poptools 3.0 (Hood, 2008). Additionally, the number of alleles private to refugial populations (considered as one group) was compared to the number of alleles private to colonized populations (considered as another group).

Results

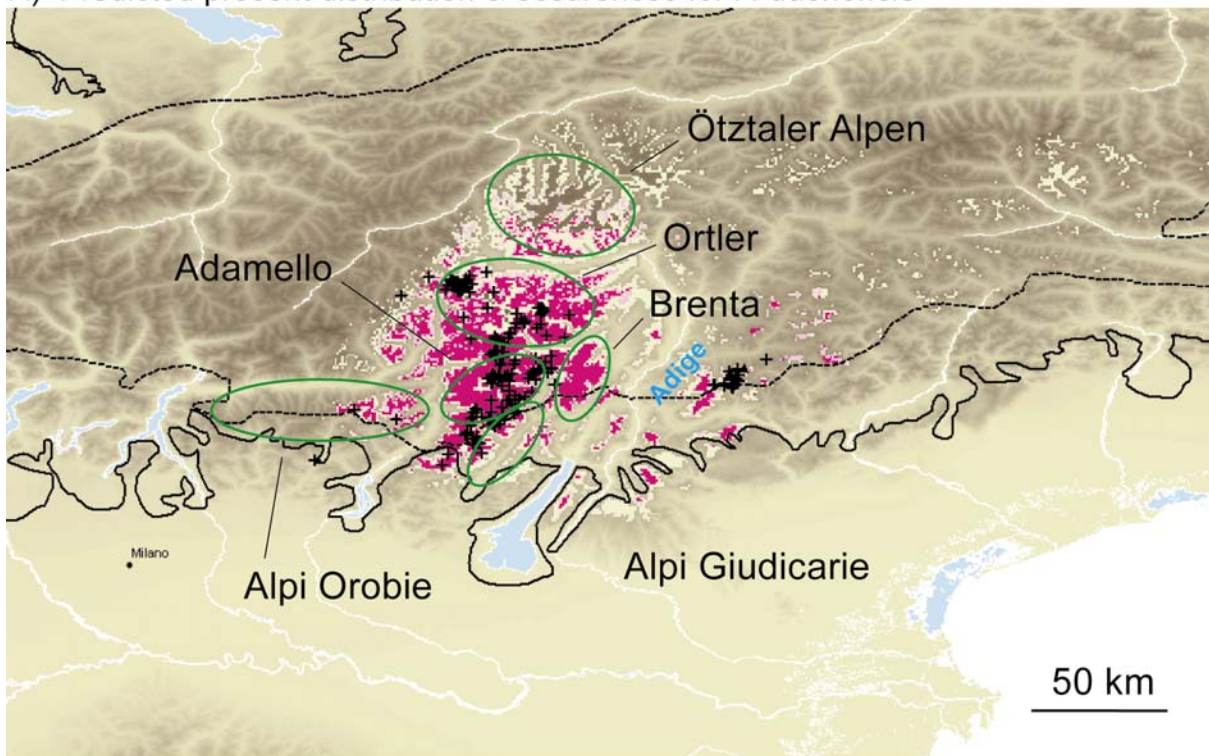
Modeled glacial refugia and colonized areas

The present ranges of *P. hirsuta* and *P. daonensis* are well reflected by the consensus models (Figure 1 A+C). One exception is that the southwestern part of *P. hirsuta*'s distribution is not predicted (from the Valle d'Aoste to the Massif du Pelvoux). This is presumably a consequence of the sparse number of occurrences that were available from this part of *P. hirsuta*'s extant distribution to fit the models. In *P. daonensis*, the models were able to predict areas east of the Adige in Langorai (porphyry bedrock) which are occupied by *P. daonensis*, but were not included to fit the models. The Brenta Massif is predicted as suitable habitat for *P. daonensis* today, but it is not populated, probably due to the predominantly calcareous bedrock. The niche projections on ecoclimatic layers for the last glacial maximum predict reductions in range during the drier and cooler conditions of this period. In *P. daonensis* most of the models (6-7) predicted refugial areas in Brenta and the Alpi Giudicarie (which both predominantly consist of calcareous rock) and eastern Adamello (siliceous rock) (Figure 1 B) and additionally small discrete refugia east of Lago di Garda on Monte Baldo (calcareous rock). South-western parts of the Ortler (siliceous rock) and southern parts of the Ötztaler Alpen (siliceous rock) as well as small discrete areas in Monte Lessini (calcareous rock) are also predicted by at least half of the models (5-7). The colonized areas, which are inferred by combining present and past consensus models, reveal a major range shift from eastern refugia (Figure 2 A, red and green) to the west and northwest (yellow).

In *P. hirsuta*, too, consensus models predominantly predict refugial areas within the major ice-shield (Figure 1 D) which overlap with glacial-morphologically reconstructed ice-free areas (Hess et al., 1967) and nunataks (Jäckli 1962) (Figure 5). In the Central Alps only small-scale range shifts are indicated by the models and modeled past and present distribution overlap widely (Figure 2 B, green). The eastern part of the distribution range, east of a line from the river Rhein to the Lago di Como, did not represent an ecoclimatologically suitable habitat during LGM and is consequently indicated as colonized area (yellow). In summary, the distribution of *P. hirsuta* slightly shifted from a more westerly distribution during the last glacial maximum to a more easterly distribution today. This is different to the pattern found in *P. daonensis*.

Figure 1 *Primula daonensis*

A) Predicted present distribution & occurrences for *P. daonensis*



B) Predicted LGM distribution (21 kyr) for *P. daonensis*

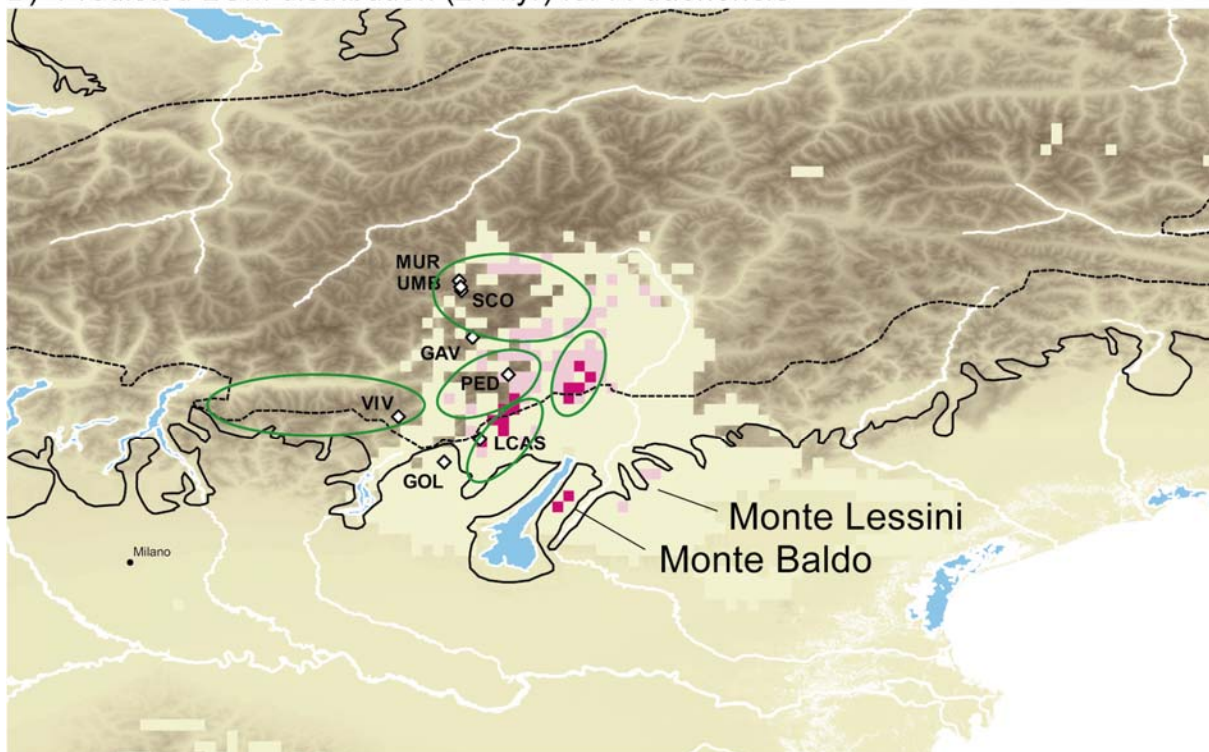
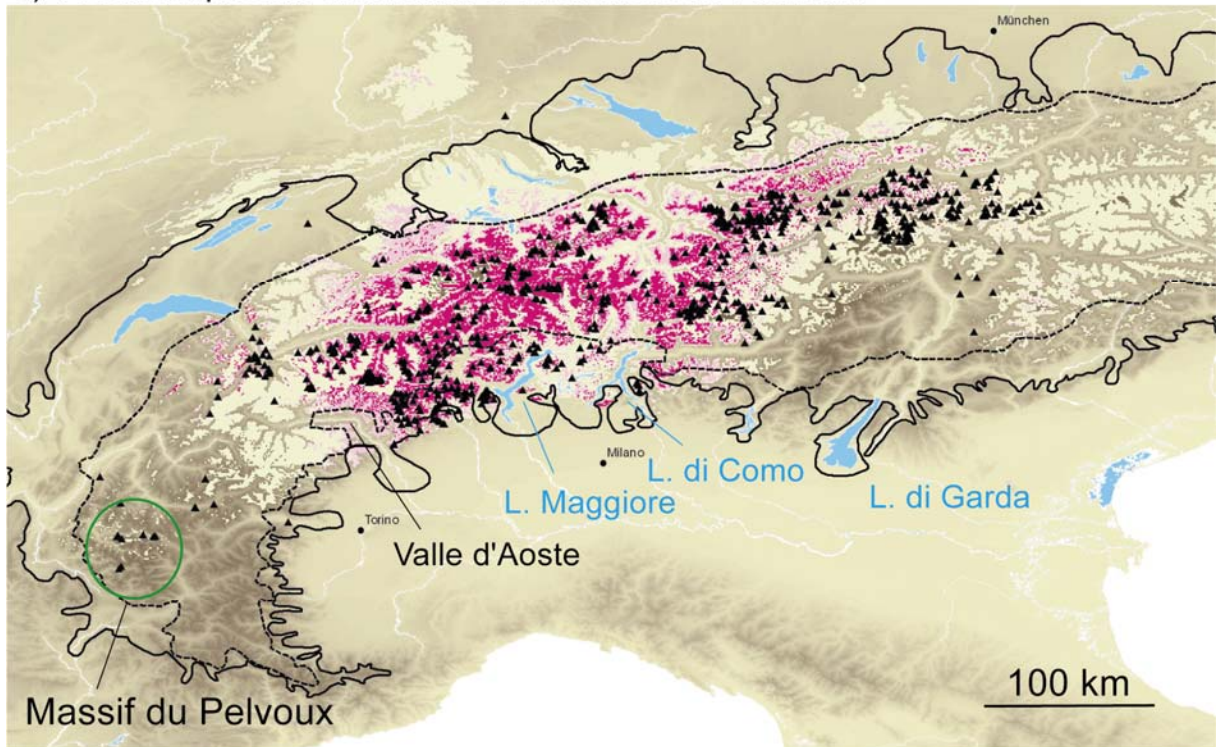


Figure 1 cont. *Primula hirsuta*

C) Predicted present distribution & occurrences for *P. hirsuta*



D) Predicted LGM distribution (21 kyr) for *P. hirsuta*

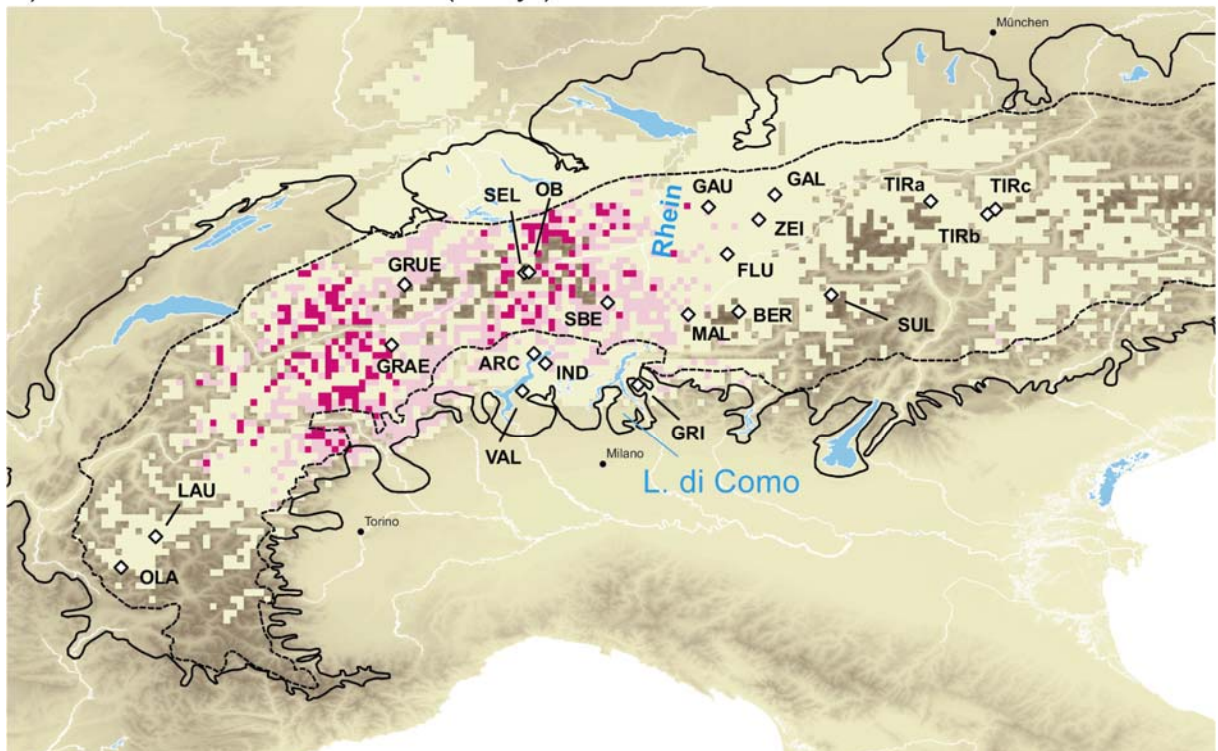


Figure 1 Legend

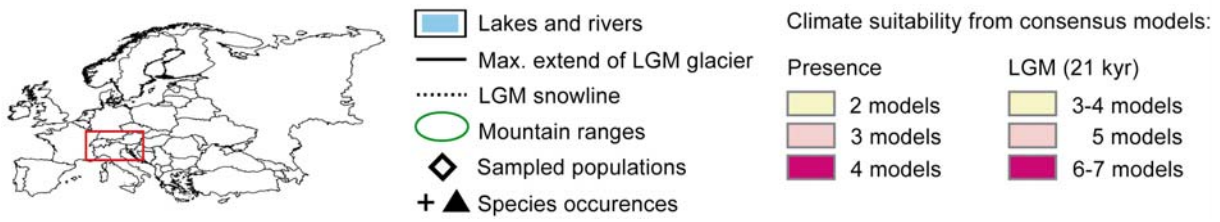


Figure 1 Species distribution models from consensus modeling for *P. daonensis* (A,B) and *P. hirsuta* (C,D) for today (A+C) and the last glacial maximum, 21,000 years ago (B+D). Species occurrences are shown in A and C, sampled populations are shown in B and D.

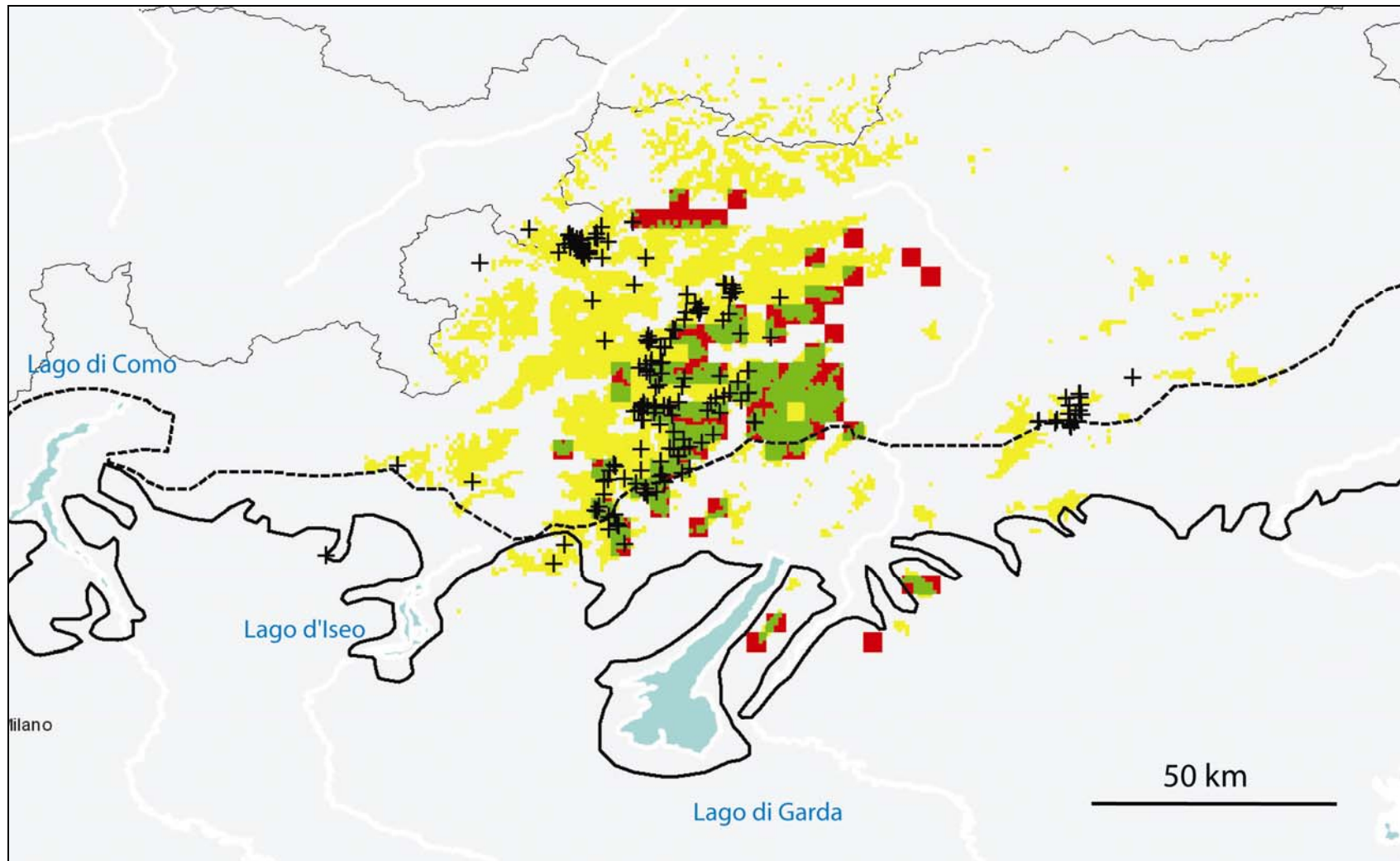


Figure 2 A Species distribution of *P. daonensis* predicted by the majority of modeling techniques (3-4 models for today and 5-7 models for LGM) for today (yellow) and the last glacial maximum (red); stable areas are areas, where present and past distributions overlap (green). For further descriptions see Figure 1 or 3.

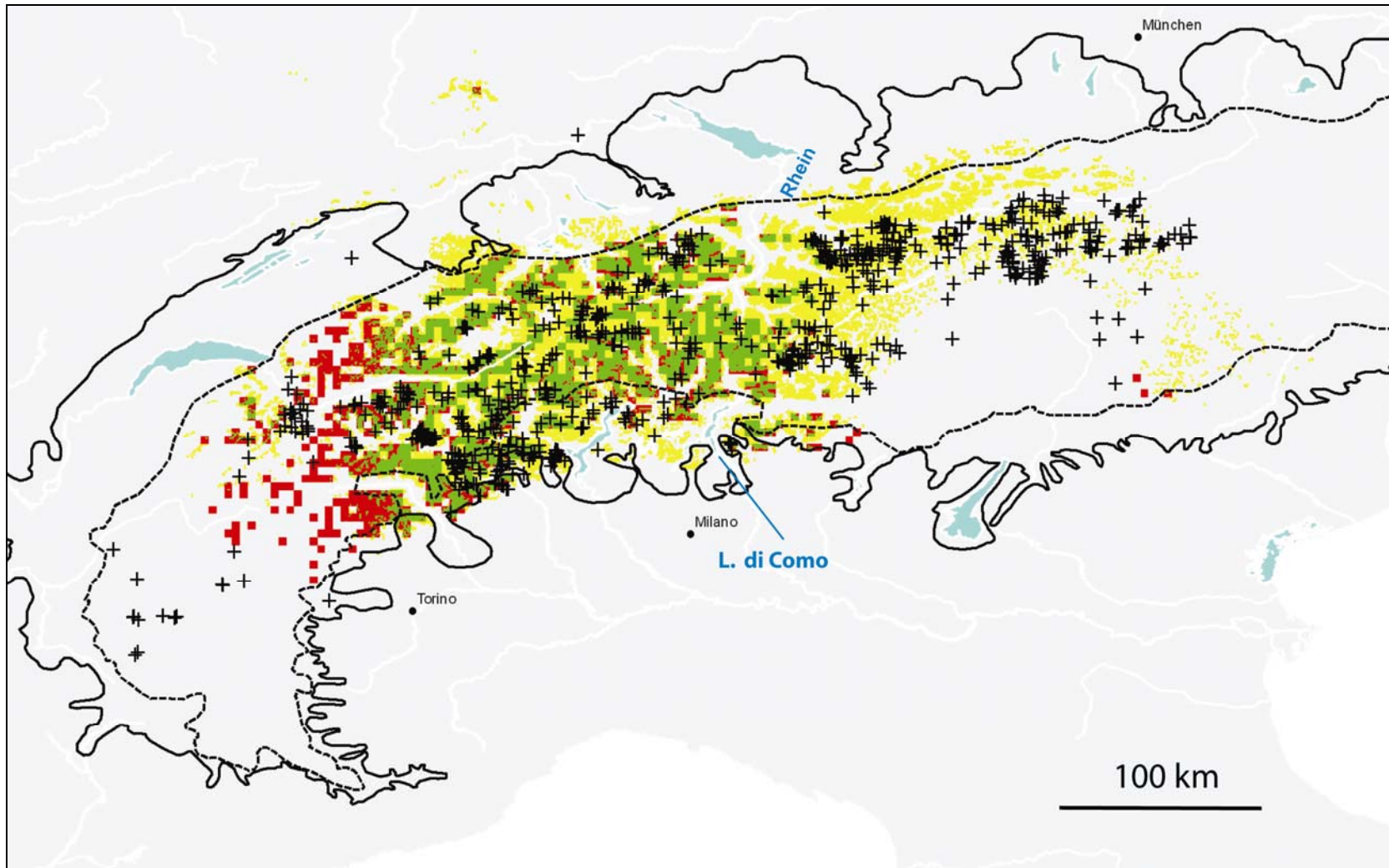


Figure 2 B Species distribution of *P. hirsuta* predicted by the majority of models (3-4 models for today and 5-7 models for LGM) for today (yellow) and the last glacial maximum (red); stable areas are areas, where present and past distributions overlap (green). For further descriptions see Figure 1 or 3.

Evaluation of model predictions

Under current climate conditions AUC values indicate ‘good to excellent’ model performance for both species across modeling techniques following Swets scale (Swets 1988) (Table 2). TSS indicates for *P. hirsuta* ‘good’ to ‘high or excellent’ performance, whereas Kappa indicates ‘fair’ model performance; for *P. daonensis* TSS and Kappa indicate ‘high or excellent’ model performance following (Thuiller et al., 2008). The predictions for *P. daonensis* are generally better.

Table 2 Evaluation of single modeling techniques for the two species.

	AUC	Kappa	TSS
<i>P. daonensis</i>			
GLM	0.996	0.854	0.982
GBM	0.996	0.866	0.975
GAM	0.996	0.872	0.983
RF	0.997	0.877	0.980
<i>P. hirsuta</i>			
GLM	0.938	0.482	0.772
GBM	0.957	0.561	0.841
GAM	0.930	0.402	0.750
RF	0.963	0.582	0.855

AFLP-patterns

A total of 133 polymorphic fragments were scored unambiguously in *P. hirsuta* and *P. daonensis*, 97 of these were found in *P. daonensis* (8 populations, 113 individuals) and 130 in *P. hirsuta* (21 populations, 234 individuals). Only three of these fragments (2.3%) were private to *P. daonensis* and 36 (27.1%) to *P. hirsuta*. The mismatch error was 4.5% comparing 150 replicate pairs within and between three different AFLP runs.

Population structure of *P. daonensis* and *P. hirsuta*

BAPS analysis with *P. daonensis* detects three genetic groups (probability for number of clusters is 1) which are distributed over wide areas of the species distribution range and overlap geographically (Figure 3 A left; Table 1). The N-group occurs in the Ortler and the Northern Adamello, the W-group occurs in the Alpi Orobie, the Ortler and the northern Adamello and the E-group occurs in the Alpi Giudicarie, the Adamello and the Ortler. While the three southernmost populations (VIV, GOL & LCAS), and also SCO in the Ortler only comprise one genetic group, the more northern populations comprise all three genetic groups. No admixture was found between individuals.

P. hirsuta is divided into seven groups (probability for number of clusters is 1) which correspond to geographic regions (Figure 3 A right; Table 1). The SW-group occurs in the Massif du Pelvoux in the Southwestern Alps, the W-group occurs in the Walliser and Berner Alpen, three populations on the southern margin of the species distribution range form distinct groups respectively, namely VAL east of Lago Maggiore, GRI east of Lago di Como and SUL in the Ortler, which is geographically close to the distribution range of *P. daonensis*. GRI and VAL are populations from two southern Alpine sites, namely the Grigna Meridionale east of Lago di Como and mountains around the valley Valcuvia east of Lago Maggiore. Plants from these two localities have been described as local endemic taxa divergent from *P. hirsuta*: *Primula grignensis* (Moser 1998), re-included in *P. hirsuta* by (Zhang & Kadereit, 2004b), and *P. hirsuta* ssp. *valcuvianensis* (Jeßen & Lehmann, 2005). The remaining populations in the central Alps are split into two groups: C1 is distributed from the Alpi Lepontine to the Tuxer and Zillertaler Alpen in Austria and C2 occurs in the Engadiner Alpen. Mixture between groups (i.e. individuals of a population are assigned to different genetic groups) only occurs between C1, C2 and W: three populations of C1 are mixed with C2 and one is mixed with W, and in W one of the two populations is mixed with C1.

P. daonensis, which has a regionally restricted distribution range, shows less variation between different regional groups than *P. hirsuta* (Table 3). Hierarchical AMOVAs attribute only 7.72% of the overall variation to variation among the three major mountain ranges where the species is distributed, 9.16% to variation among populations within the mountain ranges and most of the variation (83.12%) to variation within populations. This is also reflected by a PCoA (Figure 4 A) which lacks any structure. In *P. hirsuta* 16.17% of the overall variation is attributed to variation among BAPS-groups, 11.88% to variation among populations within BAPS-groups and 71.94% to variation within populations. The BAPS-groups GRI, VAL,

SUL and SW can be clearly distinguished in a PCoA (Figure 4 B) from C1, C2 and W which are closer to each other. As expected for outcrossing species with a self-incompatibility system, most variation is found within populations, 84.94% in *P. daonensis* and 75.66% in *P. hirsuta*, whereas 15.06% and 24.34% respectively are found among populations (Table 3).

Admixture between species

An admixture analysis using two predefined groups corresponding to the two species reveals significant admixture in SUL (18 of 22 individuals are significantly admixed, $p \leq 0.05$) and GRI (three of 11 are significantly admixed) (Table 1). In a PCoA the first coordinate which explains 23% of the overall variation separates the two species. The populations SUL and GRI have an intermediate position but are much closer to *P. hirsuta* (Figure 4 C). A minimum spanning tree connects *P. daonensis* to SUL.

Table 3 Analyses of molecular variance (AMOVA) for AFLP phenotypes of 8 populations of *Primula daonensis* and 21 populations of *P. hirsuta* using 10,000 permutations.

Source of variation	d.f.	Sum of squares	Variance components*	% of variation	F _{ST} [†]
<i>P. daonensis</i>					
Among populations	7	192.57	1.42	15.06	0.15
Within populations	105	839.36	7.99	84.94	
Among mountain ranges (VIV-GOL, LCAS, PED-GAV, SCO, UMB, MUR)	2	98.27	0.74	7.72	0.17
Among populations within mountain ranges	5	94.30	0.88	9.16	
Within populations	105	839.36	7.99	83.12	
<i>P. hirsuta</i>					
Among populations	20	1018.47	3.59	24.34	0.24
Within populations	213	2375.17	11.15	75.66	
Among BAPS-groups (SW, W, C1, C2, VAL, GRI, SUL)	6	590.17	2.51	16.17	0.28
Among populations within BAPS-groups	14	28.30	1.84	11.88	
Within populations	213	2375.17	11.15	71.94	

*All p-values < 0.001

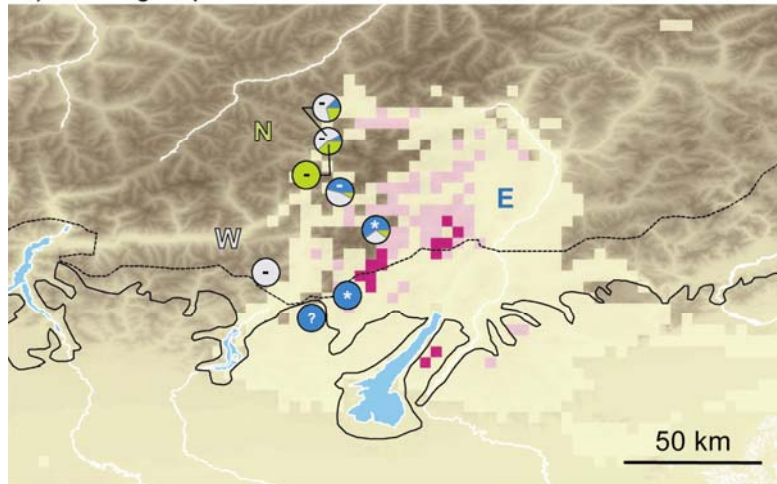
Table 4 Population genetic indices for the investigated populations of *Primula hirsuta* and *P. daonensis*. Sampling sites which are located in a few hundred meters distance are underlined; those sampling sites which were not used to evaluate the paleodistribution models are listed at the end of the table. ‘Type of pop.’ specifies whether paleodistribution models indicate, that the population is situated in colonized area (C) or in refugial area (R). ‘Size of pop.’ = number of individuals per population, ‘N° frag.’ = number of AFLP-fragments, h = Nei’s gene diversity, I = Shannon index, NPL = number of polymorphic loci, PPL =percentage of polymorphic loci, Pb7 = band richness with rarefaction sample size 7, PPL1/PPL5 = percentage polymorphic loci at the 1%/5% level (only calculated if ‘size of pop.’ > 6), ‘priv. Al.’ = number of private alleles.

Code	Type of pop.	Size of pop.	N° frag.	h	I	NPL	PPL	Pb6	PLP5	PLP1	Rarity	Priv. Al.
<i>P. daonensis</i>												
<u>GAVa+b</u>	C	13	70	0.16	0.24	48	49.48	1.40	0.31	0.37	1.07	1
GOL	?	12	60	0.12	0.19	35	36.08	1.32	0.13	0.28	0.79	0
LCAS	R	9	58	0.11	0.17	31	31.96	1.30	0.08	0.19	0.79	1
<u>MURc</u>	C	9	71	0.13	0.21	43	44.33	1.40	0.20	0.28	1.23	4
PED	R	10	62	0.12	0.17	34	35.05	1.31	0.19	0.19	0.71	0
SCO	C	8	63	0.15	0.22	40	41.24	1.39	0.14	0.14	1.06	0
UMB	C	13	70	0.17	0.26	48	49.48	1.41	0.26	0.32	0.96	0
<u>VIVa</u>	C	12	75	0.18	0.27	54	55.67	1.43	0.33	0.50	1.28	2
<u>VIVb</u>	C	12	70	0.16	0.24	47	48.45	1.38	0.30	0.42	0.99	0
GAVa	C	8	64	0.13	0.20	38	39.18	1.37	0.16	0.16	1.06	1
GAVb	C	5	61	0.12	0.18	30	30.93	1.31*	-	-	1.07	0
<i>P. hirsuta</i>												
ARC	C	11	84	0.14	0.22	61	46.92	1.39	0.29	0.39	0.84	0
BER	C	12	85	0.14	0.21	62	47.69	1.38	0.34	0.39	0.87	0
FLU	?	12	84	0.14	0.22	64	49.23	1.38	0.33	0.42	0.78	0
GAL	C	9	77	0.15	0.22	57	43.85	1.37	0.29	0.32	0.90	0
<u>GAUa</u>	C	11	89	0.15	0.23	66	50.77	1.40	0.39	0.42	0.95	0
<u>GAUb</u>	C	9	80	0.12	0.19	52	40	1.34	0.26	0.35	0.84	0
GRAE	R	9	80	0.17	0.25	64	49.23	1.44	0.24	0.34	1.14	0
GRI	C	11	80	0.15	0.22	59	45.38	1.35	0.34	0.40	1.43	1
GRUE	R	11	80	0.16	0.24	64	49.23	1.39	0.38	0.42	0.89	0
IND	?	8	86	0.15	0.23	62	47.69	1.42	0.20	0.35	1.29	1
LAU	C	12	73	0.15	0.22	57	43.85	1.34	0.29	0.35	0.78	0
MAL	?	9	75	0.16	0.23	56	43.08	1.38	0.22	0.33	1.07	0
OB	?	12	81	0.13	0.20	55	42.31	1.33	0.24	0.29	0.83	0
<u>OLAb</u>	R	8	72	0.15	0.23	55	42.31	1.38	0.18	0.28	0.95	0
SBE	R	10	83	0.15	0.23	61	46.92	1.39	0.24	0.34	0.88	0
SEL	R	10	85	0.14	0.21	60	46.15	1.39	0.23	0.35	1.09	0
<u>SULa</u>	?	9	78	0.14	0.22	58	44.62	1.39	0.26	0.35	1.39	3
<u>SULb</u>	?	10	89	0.17	0.26	71	54.62	1.46	0.27	0.37	1.53	3
TIRa	C	9	81	0.14	0.22	57	43.85	1.38	0.24	0.37	0.89	0
TIRb	C	9	75	0.15	0.22	54	41.54	1.36	0.24	0.32	0.72	0
TIRc	C	7	69	0.11	0.17	40	30.77	1.29	0.12	0.12	1.04	1
VAL	C	10	76	0.13	0.20	50	38.46	1.34	0.20	0.26	1.38	2
ZEI	C	12	81	0.15	0.23	65	50	1.38	0.38	0.43	0.72	0
MURa+ba+bb+c												
<u>MURa</u>	C	24	84	0.18	0.28	66	48.04	1.46	0.52	0.66	1.13	4
<u>MURba</u>	C	5	65	0.11	0.17	32	32.99	1.33*	-	-	1.06	1
<u>MURba</u>	C	4	66	0.13	0.20	36	37.11	1.37*	-	-	1.14	0
<u>MURbb</u>	C	6	71	0.15	0.23	45	46.39	1.46*	-	-	1.04	1
<u>VIVa+b</u>	C	24	81	0.19	0.29	65	67.01	1.42	0.54	0.64	1.13	2
<u>GAUa+b</u>	C	20	96	0.00	0.24	75	57.69	1.39	0.45	0.58	0.80	0
<u>OLAa+b</u>	R	9	72	0.15	0.23	55	42.31	1.37	0.25	0.35	0.97	0
<u>OLAa</u>	R	1	46	-	-	-	-	-	-	-	1.13	0
<u>SULa+b+c</u>	?	22	97	0.19	0.29	84	64.62	1.46	0.52	0.65	1.46	4
<u>SULc</u>	?	3	71	0.11	0.17	39	30.00	1.3*	0.00	0.00	1.49	2

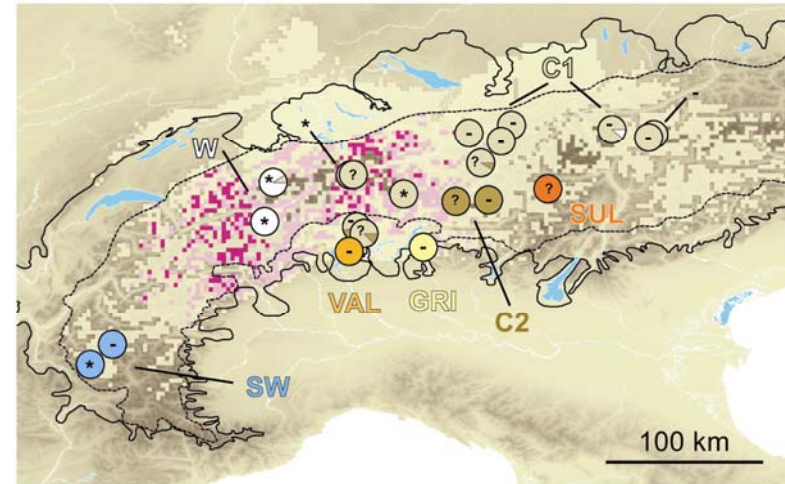
*calculated without rarefaction, instead the mean number of alleles is given

A) BAPS-groups

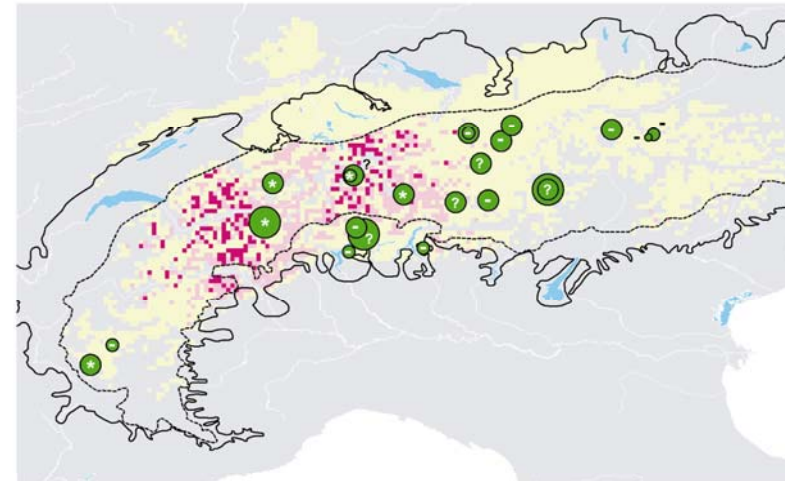
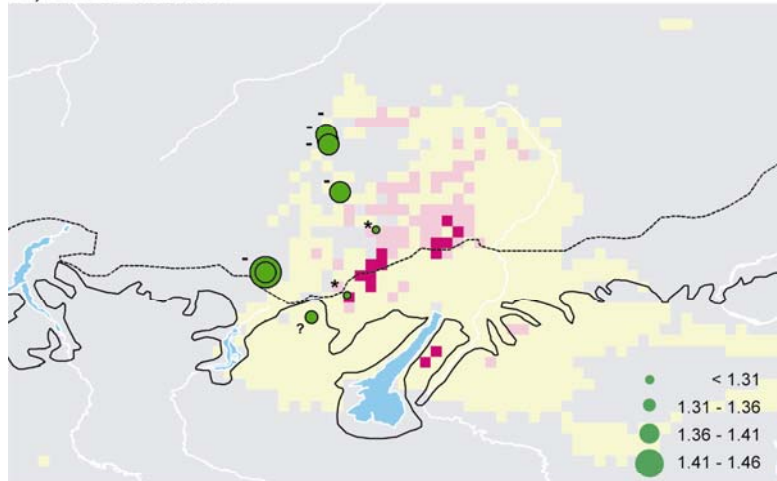
P. daonensis



P. hirsuta



B) Allelic richness



Climate suitability from consensus models at LGM (21 kyr):



Population characterisation:

- * Refugial population
- Colonized population
- ? Unclear population history

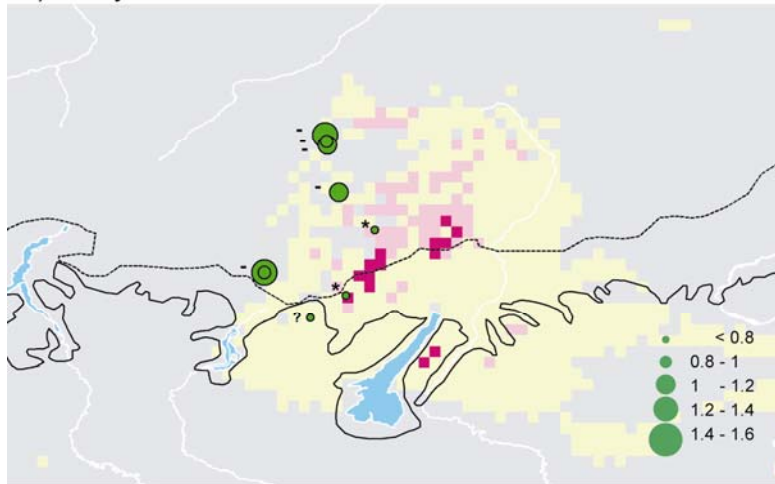
Lakes and rivers

Maximum extend of LGM glacier

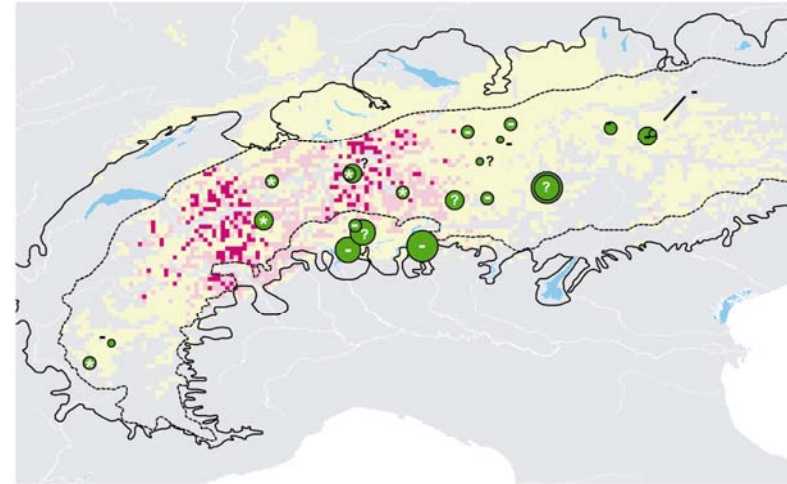
LGM snowline

C) Rarity index

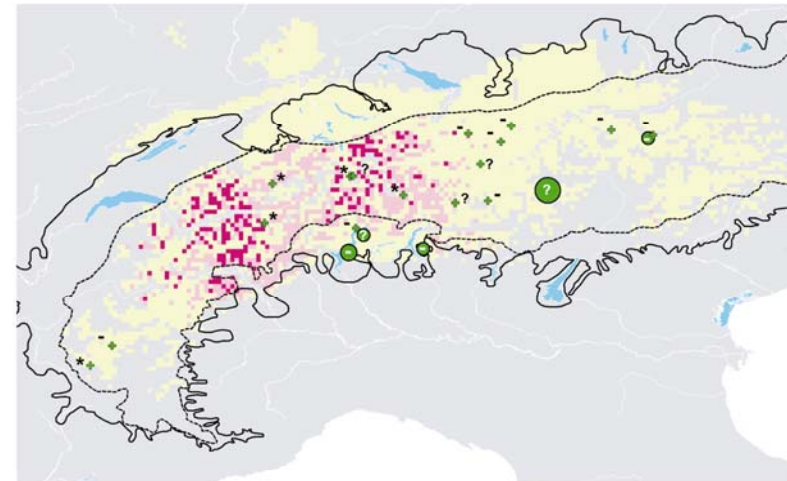
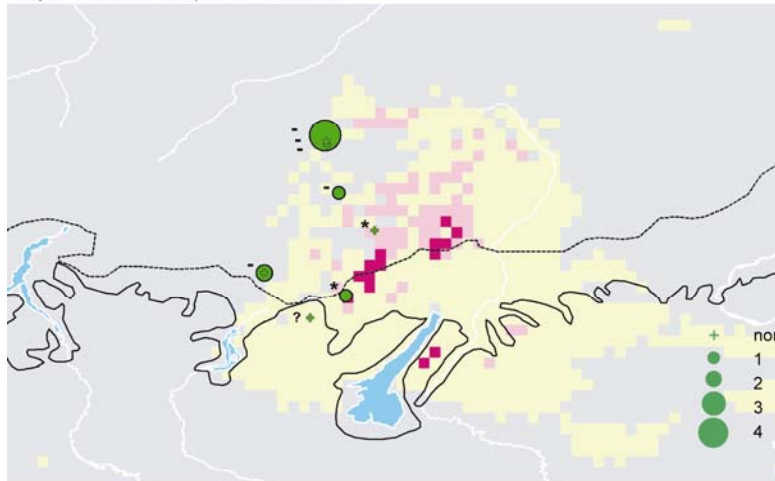
P. daonensis



P. hirsuta



D) Number of private alleles



Climate suitability from consensus models at LGM (21 kyr):

- 3-4 models
- 5 models
- 6-7 models

Population characterisation:

- * Refugial population
- Colonized population
- ? Unclear population history

Lakes and rivers

Maximum extent of LGM glacier

LGM snowline

Figure 3 Population structure (A) and population genetic indices, namely B) allelic richness (Pb), C) rarity and D) the number of private alleles for *P. daonensis* (left) and *P. hirsuta* (right) overlaid onto consensus maps of glacial refugial areas predicted with paleodistribution models for LGM, 21,000 years ago. The ‘population characterization’ as refugial population or colonized population is based on the present and past consensus models.

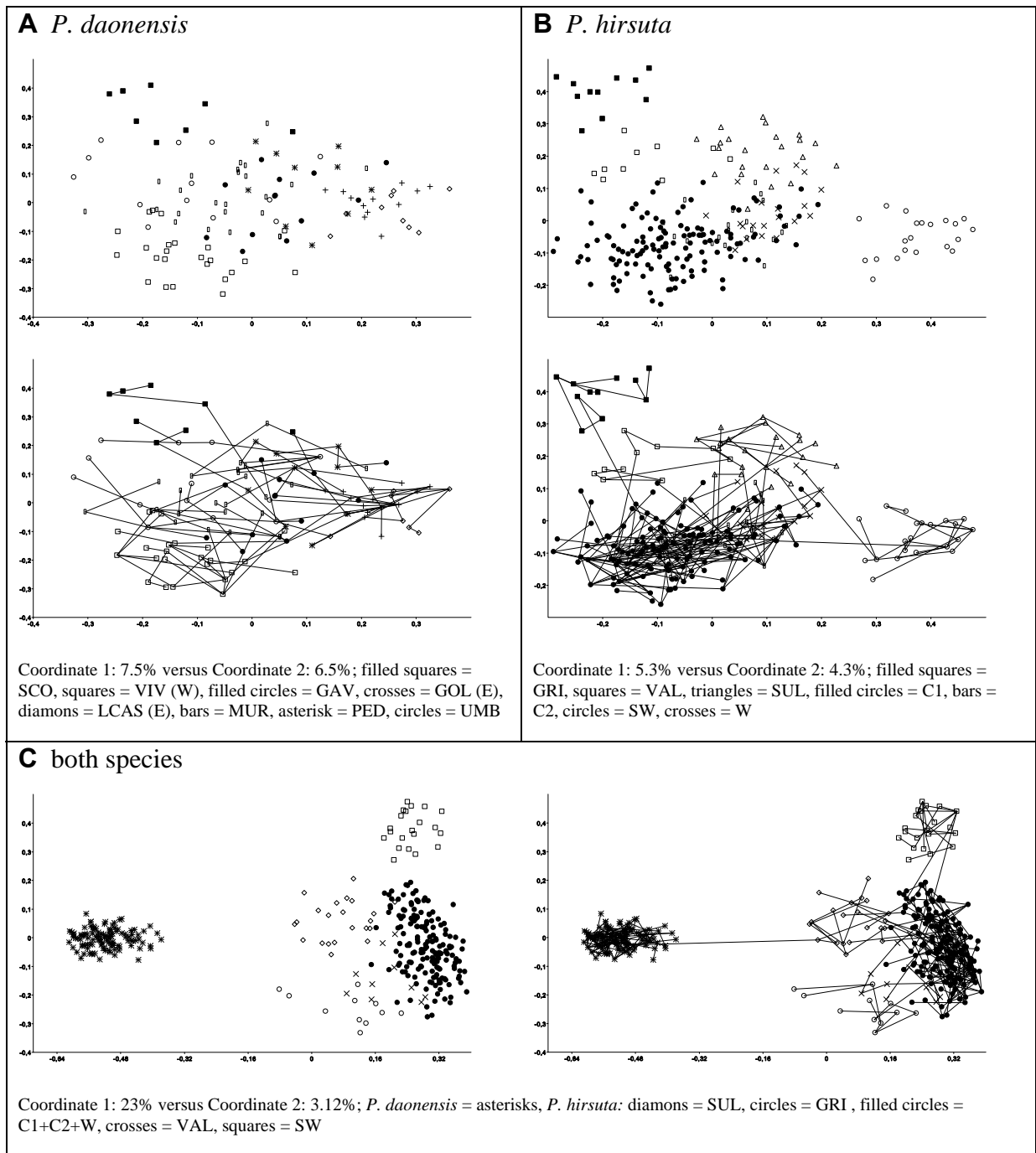


Figure 4 Principle coordinate analyses with minimum spanning tree of *Primula daonensis* (A) and *P. hirsuta* (B) and both species (C) for 113 AFLP multilocus phenotypes from 8 populations and 234 AFLP multilocus phenotypes from 21 populations respectively. Symbols indicate the populations in A), the genetic groups derived with BAPS in B) and C) (except *P. daonensis*), whereby all individuals from a population are assigned to the BAPS group, where most of the populations' individuals belong to. Axes are scaled using the square root of the eigenvalues.

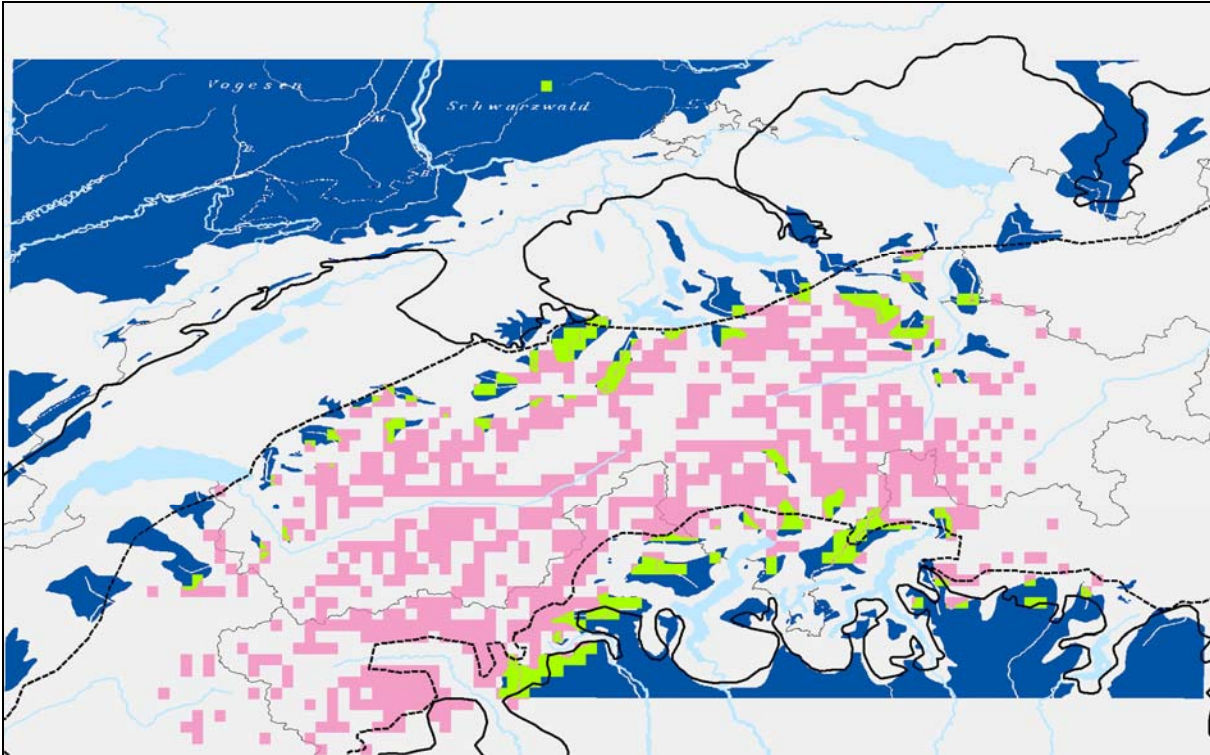
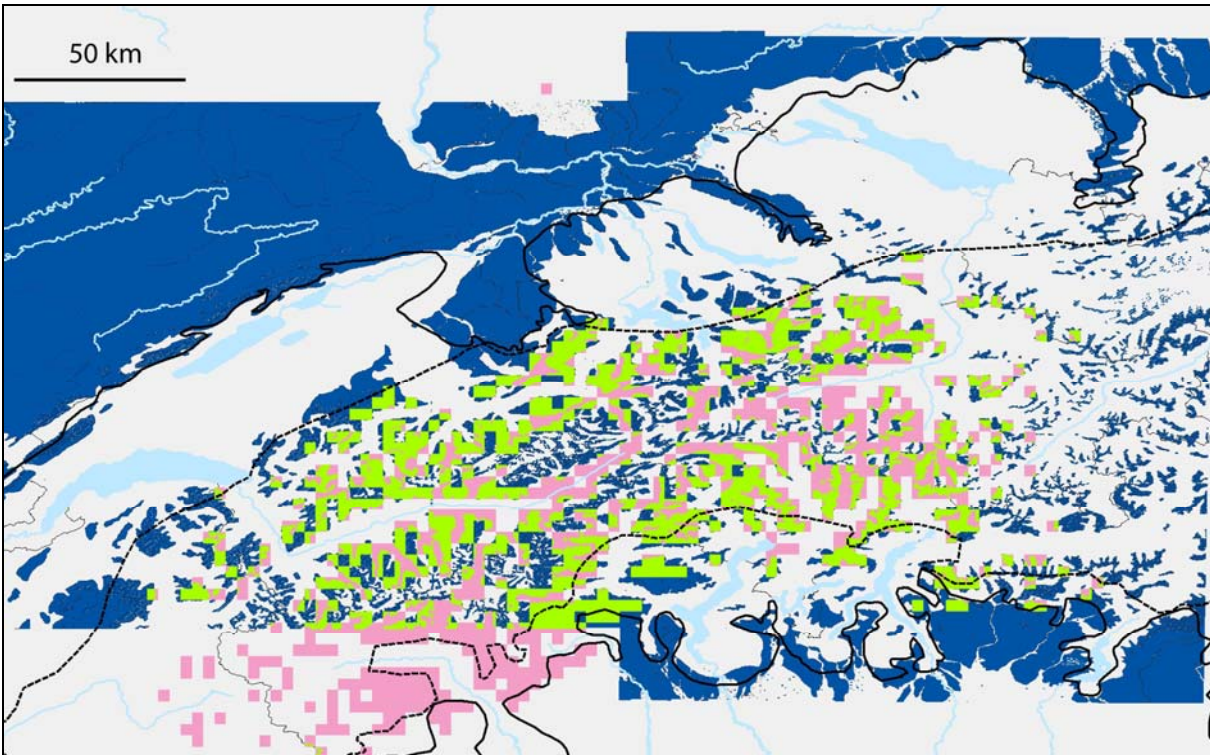
A**B**

Figure 5 Potential habitat of *Primula hirsuta* during LGM (21,000 years ago) in the central Alps as inferred from the overlap of paleodistribution of *P. hirsuta* with A) always ice-free areas redrawn from Hess et al. (1967) and B) ice-free areas and nunataks redrawn from Jäckli (1962). Rose= LGM distribution of *P. hirsuta* as predicted by the majority of models (5-7 models), dark blue= ice-free areas and nunataks, green= overlap of both areas. For further descriptions see Figure 1 or 3.

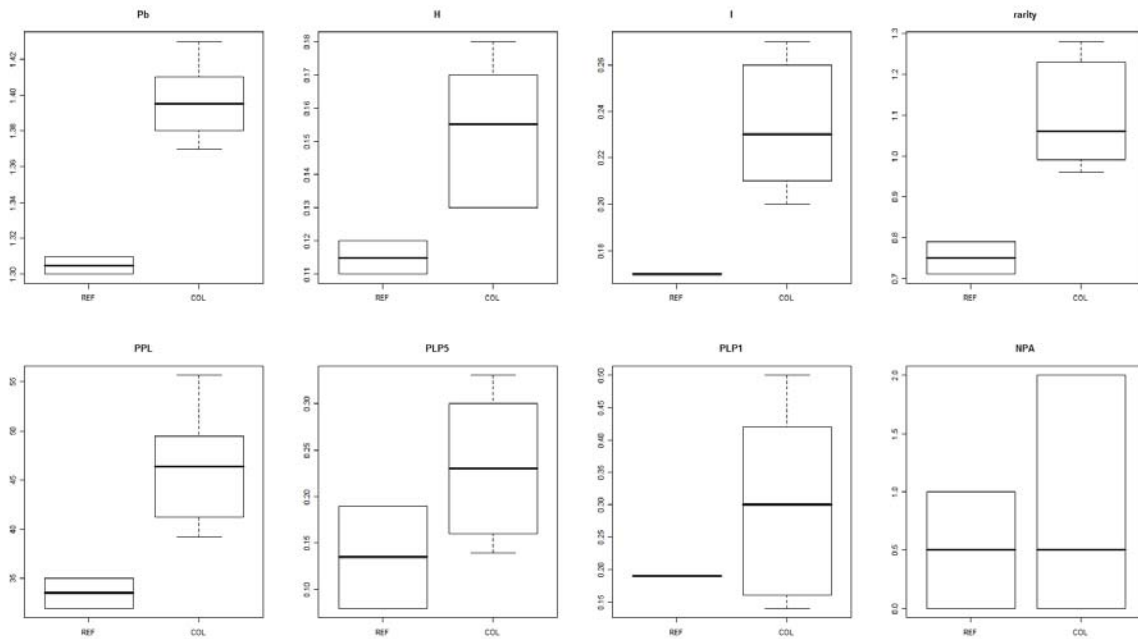
Evaluation of modeled refugia with population genetic patterns

Often high within-species diversity (Hewitt 1996, Stehlik et al., 2002a, Stehlik et al., 2002b, Tribsch et al., 2002) or rarity (Comps et al., 2001, Widmer & Lexer, 2001, Paun et al., 2008) are used as indicators for glacial refugial areas. We tested whether the sampled populations which are situated within modeled refugial areas exhibit higher diversity indices and/or rarity than the sampled populations which are not situated within modeled refugial areas (Table 4; Figure 3 B-D). In *P. hirsuta* significantly higher diversities (expected heterozygosity, Shannon's information index and allelic richness) are detected in refugial areas compared to colonized areas (Figure 6 B; Figure 3 B), but rarity is not significantly different (Figure 6 B; Figure 3 C). Only four populations harbor private fragments and these are not related to modeled refugial areas (Figure 6 B; Figure 3 D), though for two of them (SUL and IND) the consensus models do not allow to discriminate between refugial and colonized populations.

In *P. daonensis* we find the opposite diversity pattern. Populations in model-predicted colonized areas exhibit significantly higher diversities (expected heterozygosity, Shannon's information index, allelic richness and the percentage of polymorphic markers) than populations in model-predicted refugial areas (Figure 6 A; Figure 3 B). Also rarity is significantly higher in colonized populations and they tend to have more private fragments (Figure 6 A; Figure 3 C+D).

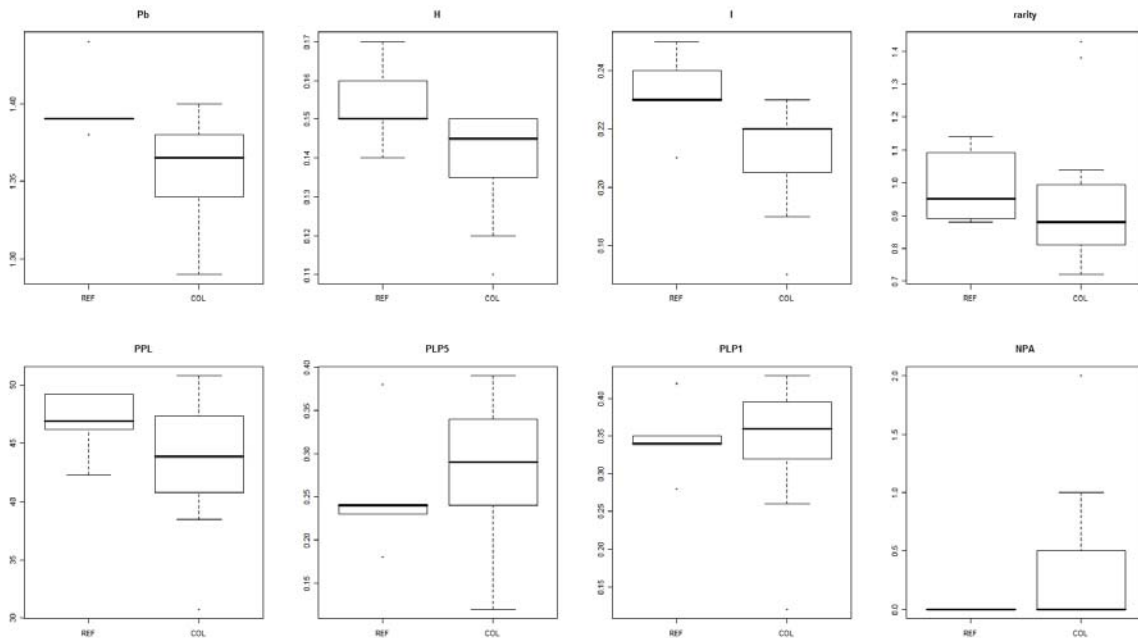
In both species modeled refugial areas harbor fewer private alleles than colonized areas, which is contrary to our hypothesis. No fragments are shared between all refugial populations (49 individuals) of *P. hirsuta*, which do not occur in colonized populations, whereas 13 fragments were exclusively found in colonized areas (122 individuals). In *P. daonensis* only two fragments are private to populations in glacial refugial areas (19 individuals), while 29 are exclusively found in colonized areas (82 individuals).

A *Primula daonensis*



h	I	PB7	PPL	PLP5	PLP1	RARITY	PRIVAL
0.037*	0.035*	0.038*	0.034*	0.11	0.215	0.037*	0.503

B *Primula hirsuta*



h	I	PB6	PPL	PLP5	PLP1	RARITY1	PRIVAL
0.037*	0.022*	0.011*	0.116	0.258	0.546	0.349	0.316

Figure 6 Boxplots of various genetic indices of *Primula daonensis* (A) and *P. hirsuta* (B) comparing populations situated in glacial refugia (“REF”: *P. daonensis*: LCAS, PED; *P. hirsuta*: GRAE, GRUE, OLAB, SBE, SEL) versus colonized areas (“COL”: *P. daonensis*: GAVa, MURc, SCO, UMB, VIVa, VIVb; *P. hirsuta*: ARC, BER, GAL, GAUa, GAUb, GRI, LAU, TIRa, TIRb, TIRc, ZEI, VAL) as indicated by SDMs and paleodistribution models. Whiskers extend to the most extreme data point within the interquartile range from the box. P-values from one-tailed randomization tests using 10,000 Monte Carlo iterations comparing the two groups are given below the boxplots, significant differences are marked with an asterisk.

Discussion

Glacial refugia and postglacial re-colonisation of *Primula daonensis*

Paleodistribution models indicate that refugial areas of *P. daonensis* were located east of the extant main distribution range (west of the Adige). The three refugial areas predicted by most (6-7) of the models (Figure 1 B) – northern Alpi Giudicarie to southeastern Adamello, Brenta and Monte Baldo – overlap with hypothetical refugial areas for silicicolous subnival, calcicolous subnival and calcicolous upper alpine species, respectively, based on geological and paleoenvironmental data (Tribsch & Schönswetter, 2003). The latter two mountain ranges seem less likely to have served as refugia since *P. daonensis* is a predominantly silicicolous species. On the other hand it also grows in the predominantly calcareous Alpi Giudicarie. However Hegi (1966) stated that in Val Daone (Alpi Giudicarie) granitic and calcareous/dolomite rock alternate at a small scale, and *P. daonensis* always grows on granite. The area on Monte Lessini, predicted by fewer models (5), predominantly consists of calcareous rock, similarly to Brenta and Monte Baldo, and accordingly the same interpretations apply here. Areas predicted by fewer (5) models which are located farther northwest of the three refugial areas predicted by most of the models (6-7), may also be less likely to have served as refugia, because those are situated higher than 300 m above the LGM snowline (according to Tribsch & Schönswetter, 2003). This altitude today represents the approximate upper limit of prospering populations of mountain plants in the centralmost part of the Eastern Alps (Reisigl & Pitschmann 1958, Körner 1999, Tribsch & Schönswetter, 2003). To summarize, based on predictions by most (6-7) of the paleodistribution models as well as paleoenvironmental and geological evidence, the area from the northern Alpi Giudicarie to the southeastern Adamello seems most likely to have served as refugium for *P. daonensis* during the last glacial maximum.

The distributional shift (Figure 2 A) – measured from the southernmost/easternmost limit of the glacial distribution to the northernmost/westernmost limit of the extant range – is comparably small, at most c. 60 km. The postulated refugial area is located within the modern range of *P. daonensis*, although southeast of its main distributional range (west of the Adige). Following the species distribution models, the most likely refugial area, the area from the northern Alpi Giudicarie to the southeastern Adamello, was ecoclimatologically stable and consequently populations presumably did not move, while the western and northern parts of the species extant distribution range were not suitable during the LGM and presumably colonized during the Holocene. Surprisingly, populations in the presumably colonized areas

have high diversities and rarities and harbor private alleles, while populations in the potential refugial area are genetically impoverished, have low numbers of rare alleles and only one of the populations has a private allele (Figure 3 B-D; Figure 6 A; Table 4). Based on theoretical and empirical studies, a decrease in genetic diversity from refugia towards newly colonized areas is expected, and such pattern indicates the direction of migration (Hewitt 1996, Widmer & Lexer, 2001, Stehlik et al., 2002a, Tribsch et al., 2002).

Several explanations for the discrepancy of population genetic patterns and paleodistribution modeled refugial areas found here can be offered. A first explanation would be that the paleodistribution models failed to correctly predict refugial areas, and that these indeed were located in the northern and western parts of the extant distribution. This seems unlikely because i) predicted areas overlap with hypothetical refugial areas proposed by (Tribsch & Schönswetter, 2003) and ii) predicted refugial areas for three other *Primula* species (*P. latifolia*, *P. marginta*, *P. hirsuta*) were congruent with population genetic patterns indicating that predictions by the paleodistribution models are correct.

A second explanation for high diversity and rarity indices found in colonized areas is hybridization. *P. daonensis* has been observed to hybridize with five other *Primula* species, namely *P. hirsuta* (Val di Sole south of the Ortler (Prosser & Scortegagna, 1998, Prosser, 2000), Val Seriana in the Alpi Orobie and Alp Muraunza northwest of the Ortler (Kadereit et al., in prep.), *P. latifolia* (Alpi Orobie (Hegi 1966)), *P. minima* (Alpi Giudicarie (Hegi 1966), hybrids are probably sterile (Richards 1993)), *P. auricula* L. (Alpi Giudicarie (Hegi 1966)) and *P. spectabilis* (Alpi Giudicarie (Hegi 1966)). According to these geographical locations of observed hybrids in the literature, the sampled northwestern populations could only be affected by hybridization with *P. hirsuta* in Alp Muraunza. We can exclude this possibility with our molecular data which provide evidence for hybridization or introgression of *P. daonensis* in two populations of *P. hirsuta* but not vice versa. Although the possible existence of undetected hybrid populations in areas beyond the locations mentioned above should be considered, this seems unlikely since none of the sampled populations showed morphologically intermediate characters between different *Primula* species. Additionally, hybridisation with *P. spectabilis* and *P. auricula* is less likely to occur since these are limited to basic soils which should result in less geographic overlap. Furthermore, no evidence for hybridization/introgression was found in the two *P. daonensis* populations STE (nine individuals) and VIV (eight individuals) with admixture analyses using a different AFLP dataset comprising six other *Primula* species (results not shown; five admixture analyses were run with predefined groups for each species comparison separately and included i) three

populations of *P. latifolia* (27 individuals) from the southwestern Alps (Col Sommeiller and Vallon de Fontanalba; F) and the Engadin (Albulapass; CH), ii) two populations of *P. lutea* Vill. (= *P. auricula* L., 11 individuals) from the Central Alps (Geißspitze, Vorarlberg and Schmirntal; Austria) together with three populations of *P. auricula* L. (= *P. balbisii* Lehm., 27 individuals) from the southern Central Alps (M. Fumante, Carega Massif; I), the Apennini (Balzi dell'Ora, Corno alle Scale; I) and the southeastern Alps in Slovenia, iii) one population of *P. spectabilis* (five individuals) from the southern Central Alps (M. Fumante, Carega Massif; I), iv) one population of *P. albenensis* (eight individuals) from the southern Central Alps (Val d'Ancogno; I), and v) one population of *P. recubariensis* (eight individuals) from the southern Central Alps (M. Fumante, Carrega Massif; I).

Third, secondary contact of divergent lineages colonizing previously uninhabitable areas from separate refugia has been shown to cause high diversities (Petit et al., 2003, Heuertz et al., 2004, Schönswetter et al., 2004) and this explanation might apply to the genetic pattern found in *P. daonensis*, too. However, the very low level of genetic structuring of *P. daonensis* suggests that the species survived the LGM in only one refugium.

Forth, following Hewitt (1996), slow expansions retaining a broad advancing front ('phalanx') would allow more alleles to survive with less genotypic divergence among populations and areas, compared to extensive rapid continued expansions ('pioneer'), which would produce considerable homozygosity with derived genotypes spread over large areas of the colonized range. *Salix herbacea* is an example for the first mode of expansion. It had a continuous range in Central Europe during the last glaciation and northward colonization from there probably occurred on a broad front maintaining diversity as the climate warmed (Alsos et al., 2009). Phalanx expansion is conceivable to be the underlying mechanism of postglacial expansion of *P. daonensis* and would provide a plausible explanation for the lack of genetic depletion associated with the colonization of western and northern parts of the extant distribution range. Additionally, the rather small range shifts in *P. daonensis* likely resulted in constant gene flow between populations that colonized newly available habitats and refugial populations and prevented the expected loss of diversity and rarity with migration. However, it seems unreasonable to assume that this mode of expansion only took place in *P. daonensis* and not in other *Primula* species (*P. hirsuta*, *P. marginata*, *P. latifolia*) (see first article for *P. marginata* and *P. latifolia*), even though those species presumably survived in several glacial refugial areas and consequently had different re-colonization patterns. Finally this scenario (phalanx expansion) still would not explain the genetic poverty of refugial populations and, maybe even more surprising, the low levels of rarity. Rare

markers are often found to correlate with refugia (Comps et al., 2001, Widmer & Lexer, 2001, Paun et al., 2008).

It seems that the peculiar genetic structure in *P. daonensis* can not be explained by the rather simple processes assumed to be associated with Quaternary range shifts alone. Two possible scenarios, which were not investigated here, or a combination of them may account for these patterns. i) Extant factors such as population size, frequency of populations and differences in gene flow strongly influence patterns of genetic variation and confound the genetic imprints of glacial history. ii) Complex patterns of consecutive range shifts during several glacial cycles and perhaps also during climatically unstable glacials (Ganopolski & Rahmstorf, 2001, Paillard, 2001) result in ambiguous genetic signals. Sampling more populations across the whole distribution range of *P. daonensis* and assessing population sizes and density as well as quantifying gene flow could lead to new insights.

Populations east of the Adige which are located c. 50 km east of the main distribution range were not sampled (Figure 1 A), and consequently only paleodistribution models can be interpreted. Although these populations are located in a hypothetical glacial refugia for silicolous upper alpine to subnival plants (southern Dolomiti, Alpi di Val di Fiemme (Tribusch & Schönswetter, 2003)), paleodistribution models indicate that they did not survive *in situ*, but rather have colonized this region either from southern peripheral refugia on Monte Baldo and/or Monte Lessini or from western refugia. The latter scenario seems more plausible because the former two predominantly calcareous mountain chains would probably allow survival of small populations on siliceous intrusions and so render these areas less likely refugia.

Glacial refugia and postglacial recolonization of *Primula hirsuta*

Refugial areas predicted by most models (6-7) are located on different nunataks in the inner parts of the Alps (central Alpine nunataks), and additional areas south of the last glacial maximum snowline but principally within the iceshield (peripheral nunataks) are predicted by fewer models (5) from the Valle d'Aoste to an area west of Lago di Como (Figure 1 D, Figure 2 B). Both, central Alpine and peripheral nunataks overlap with glacial-morphologically reconstructed 'never glaciated areas' and nunatak areas (Figure 5). Predicted peripheral nunataks are identical with 'potential peripheral refugia' on siliceous bedrock defined by Schönswetter et al., (2005) on the basis of geological and paleoenvironmental data. These authors demonstrated that phylogeographic patterns of several alpine plant species confirmed the locations of the 'potential peripheral refugia' they had defined and thus concluded that the

area between the Valle d'Aoste and the Lago di Como presumably served as refugium for *Androsace alpina*, partly *A. brevis*, partly *Phyteuma globulariifolium* and *Ranunculus glacialis*. The area between Lago di Como and the Dolomiti was hypothesized as refugium for *Androsace alpina*, partly *A. brevis*, *A. wulfeniana*, *Eritrichium nanum*, *Phyteuma globulariifolium*, *Ranunculus glacialis*, and *Saponaria pumila*.

The central Alpine nunataks identified for *P. hirsuta* (Figure 1 D; Figure 2 B) partly overlap with five floristically rich nunatak areas proposed by Brockmann-Jerosch & Brockmann-Jerosch (1926), reviewed and mapped in (Stehlik, 2000): species distribution models (SDMs) predicted glacial distribution of *P. hirsuta* i) around the high mountains of Monte Rosa and in the valleys of Visp – but rather in the outer and lower parts of this area and not at the highest altitudes – ii) in Avers and iii) the Rothorn-mountains near Arosa, while they did not predict glacial distribution of *P. hirsuta* in iv) Simplon and in v) very small and restricted areas of the high mountains of Engadin and Bernina even though the species is frequent today in the latter two areas. The central Alpine refugium in the Monte Rosa mountains and the valleys of Simplon was also shown to be a refugium for *Eritrichium nanum* based on genetically distinct genotypes detected with AFLPs and cpDNA (Stehlik et al., 2001, Stehlik et al., 2002a, Stehlik, 2003). To summarize, based on predictions by the majority of SDMs (5-7), geological and paleoenvironmental as well as floristic evidence and supported by phylogeographic studies of other alpine plants, *P. hirsuta* survived the last glacial maximum on central Alpine as well as peripheral nunataks.

Restricted gene flow among refugial populations is shown to lead to inter-population genetic differentiation resulting in well defined refugial gene pools and high levels of regional diversity in the refugial areas (Comes & Kadereit, 1998, Petit et al., 2003, Hampe & Petit, 2005). In the case of nunatak survival these different gene pools should be distributed throughout the formerly glaciated inner Alps. In *P. hirsuta* four genetic groups are situated within the formerly glaciated inner parts of the Alps (Figure 3 A). Variation among these groups is higher than variation among the populations within the groups and F_{st} values are clearly higher than in *P. daonensis* (Table 3), which presumably survived in a single refugium. Additionally various refugial populations in the inner Alps have high diversities (Figure 3 B; Table 4). These findings are congruent with the scenario of nunatak survival, which is indicated by the paleodistribution maps. Moreover, significantly higher diversities in the refugial areas predicted by the paleodistribution maps are found and confirm the predictions of the SDMs.

While large parts of the Central Alps represent climatically stable habitats (Figure 2 B, green), the eastern part of the extant distribution was re-colonized, based on the paleodistribution models. However, many populations in the Eastern Alps have high diversities. Extant factors, not investigated here, like large population sizes and/or high population densities, which presumably increase recent gene flow might explain the high diversities found in these eastern populations, similarly to patterns found in the *Ranunculus alpestris* group (Paun et al., 2008). Admixture of divergent lineages coming from separated refugia, as often shown (Schönswetter et al., 2004, Petit et al., 2003, Heuertz et al., 2004), can be excluded as a reason to cause the high diversities, because most of the eastern populations are not mixed, but belong to the same genetic group.

High diversity and rarity detected in the distinct population in Suldental (SUL) first seems to indicate nunatak survival (Figure 3 B+C; Table 4), but the paleodistribution models do not support this. Indeed, admixture analyses have revealed hybridization and/or introgression with *P. daonensis*, also reported from this valley (herbarium specimen: “W. Gutermann & L. Schratt-Ehrendorfer, 29/07/1982” and “Arnold, 23/06/1913 Staatssammlung München”) which explains the high divergence, diversity and rarity found.

Peripheral survival in the southern Central Alps

Two distinct geographic populations exist at the southern Alpine periphery (GRI in the Grigna Meridionale, east of Lago di Como and VAL in the Valcuvia, east of Lago Maggiore), which harbor private alleles and exhibit high rarity but low diversities (Figure 3). High rarity together with private alleles and the association of these distinct geographical populations with well-known southern Alpine peripheral refugial areas (Schönswetter et al., 2005), as well as with one of the most important areas of endemism in the Eastern Alps (Tribsch, 2004), argue that these areas have been glacial refugia for those populations, once or repeatedly. This is additionally supported by morphological divergence and geographic distance to the main distributional range of *P. hirsuta* (Moser 1998, Jeßen & Lehmann, 2005). From these populations obviously no recolonization of inner Alpine areas started, since they represent distinct genetic groups, which are restricted to small discrete areas at the southern Alpine periphery. Furthermore, no admixture with other genetic groups is detected. Therefore, these populations are regarded as ‘relict populations’, in contrast to refugial populations from which recolonization of inner Alpine areas started. Interestingly, in both regions the populations grow on calcareous rock, in contrast to the remaining populations of *P. hirsuta*. Regarding their high level of divergence, these populations were probably separated for several glacial

cycles. Diversification presumably took place in these glacial peripheral refugial areas and was associated with an edaphic shift.

It seems likely that an ecoclimatological shift was associated with the edaphic shift. This could account for the fact that the species distribution models (SDMs) did not predict these areas (VAL and GRI) during the last glacial maximum. Another explanation for this discrepancy between SDMs and biological as well as paleoenvironmental evidence could be that the spatial resolution of the paleodistribution maps is too low. This might lead at a small scale to the situation that some refugial areas are not predicted whereas close-by areas are predicted, which is the case in GRI. Downscaling of the paleoclimate predictors would refine the paleodistribution maps and probably make them more realistic.

Besides VAL and GRI, two other populations, ARC and IND, are located at the southern-alpine periphery within the former ice-shield but south of the last glacial maximum (LGM) snowline (Figure 1 D; Figure 3 A). Similarly to VAL and GRI, population genetic patterns in IND support refugial survival – high genetic diversity, rarity and one private allele (Table 4; Figure 3 B-D). However, from the paleodistribution models this area cannot be clearly identified as a suitable area during the LGM, but areas very close to the sampled population are predicted, similar to the case of GRI. The population in ARC has an average genetic diversity, but low rarity and no private fragments, probably indicating gene flow with other near-by populations. All three populations around the Lago Maggiore (ARC, IND, VAL) grow at low altitudes under 1100 m (Table 1), which is untypically low in *P. hirsuta* and therefore an indicator for peripheral refugial populations. To summarize, the northern parts around the Lago Maggiore are indicated as refugial areas by the majority of models (5-7) and population genetic findings also support this. Thus, even though SDMs predictions do not include the areas of the sampled populations (ARC & IND), presumably due to the low spatial resolution, we hypothesize that this area at the northern Lago Maggiore has been a refugium for *P. hirsuta*. ARC and IND belong to genetic groups (C1 & C2) which are mainly distributed in the inner Alps (Figure 3 A). This indicates that from these populations, in contrast to VAL and GRI, recolonization of inner Alpine regions started.

Based on predictions by the majority of models (5-7), population genetic patterns as well as paleoenvironmental and biological evidence from other species, the area around the Lago Maggiore and the Lago di Como in the southern Central Alps has served as a peripheral refugium i) from which recolonization of central Alpine parts started and ii) for relict populations, in which divergence during glacial isolation took place.

Schlußbetrachtungen

Teil 1 Eiszeitliche und nacheiszeitliche Geschichte der vier untersuchten *Primula* Arten und Artbildung von *P. latifolia* und *P. marginata*

Durch die Kombination von Artenverbreitungs-Modellen (engl. species distribution models, SDMs) mit der geographischen Verbreitung innerartlicher genetischer Variation konnten folgende Szenarien eiszeitlichen Überdauerns und postglazialer Rekolonisierung vorher vereister Gebiete entworfen werden:

Primula marginata hatte in der Würmeiszeit zwei voneinander getrennte Refugialgebiete, westlich (südwestliches Refugium) und östlich (südöstliches Refugium) der Alpi Marittime am Rande der Vergletscherung. Die zur Eiszeit vergletscherten inneren Meeralpen wurden postglazial vom südöstlichen Refugium aus besiedelt, während die nördlichen Teile des heutigen Verbreitungsgebiets in den Alpi Cozie wahrscheinlich vom südöstlichen Refugium aus besiedelt wurden.

Primula latifolia hatte ebenfalls mehrere Refugialgebiete, die innerhalb der Vergletscherung lagen, in peripheren Nunatakern am südöstlichen Rand der Südwest-Alpen von den Alpi Marittime bis zu den Alpi Cozie und in zentralen Nunatakern in den Alpi Marittime und möglicherweise auch in den Alpi Cozie und/oder den Alpi Graie. Periphere Nunataker sind eisfreie Gebiete oberhalb des Gletschers, die außerhalb der eiszeitlichen Schneegrenze der Alpen liegen, und somit in klimatisch durchschnittlichen Sommern schneefrei waren. Zentrale Nunataker sind eisfreie Gebiete in den inneren Alpenregionen oberhalb der Schneegrenze, in denen Habitate für Pflanzen nur in windexponierten und wahrscheinlich meist südexponierten Berggipfeln verfügbar waren. Im Gegensatz zu den Nord- und den Zentralalpen waren die klimatischen Bedingungen in den Südalpen günstiger für ein eiszeitliches Überdauern auf Nunatakern (Landolt 1992).

Artbildung Die Eiszeitrefugien von *P. marginata* und *P. latifolia* waren großteils getrennt, überlappten aber kleinräumig am südlichen Eisrand in den Alpi Marittime. Dieses Überlappungsgebiet ist aber zu klein, um auf sympatrische Artbildung hinzudeuten. Vielmehr belegen die überwiegend getrennten Refugialgebiete beider Arten unsere Hypothese allopatrischer Artbildung in isolierten eiszeitlichen Refugien

Primula daonensis hatte nur ein Refugialgebiet, das sich von den Alpi Giudicarie bis zum südöstlichen Adamello erstreckte und periphere und zentrale Nunataker umfasst. Von dort aus wurden die heutigen Verbreitungsgebiete westlich und nördlich davon, und vermutlich auch

das Teilareal östlich der Adige besiedelt. Bei dieser Art steht die geographische Verteilung genetischer Variation nicht im Einklang mit den Refugialgebieten der Artenverbreitungs-Modelle (engl. species distribution models, SDMs). Rezente Faktoren wie Populationsgröße und/oder Populationsdichte und unterschiedliche Muster im Genfluß haben eventuell die eiszeitlichen Einflüsse auf molekularer Ebene verwischt, oder aufeinanderfolgende Arealverschiebungen im Zuge mehrerer Eiszeit-Warmzeit-Wechsel haben widersprüchliche genetische Muster generiert.

Primula hirsuta hat die Würm-Eiszeit in mehreren Refugialgebieten überdauert, welche vorwiegend oberhalb des Gletscherstroms auf zentralen Nunatakern lagen, aber ebenso am Gletscherrand in peripheren Nunatakern. In zwei Refugialgebieten am südlichen Gletscherrand, im Valcuvia östlich des Lago Maggiore und im Grigna-Gebirge östlich des Lago di Como, blieben möglicherweise über mehrere Eiszeit-Warmzeit-Wechsel hinweg Reliktpopulationen erhalten, welche sich morphologisch, edaphisch und genetisch von den anderen Populationen absetzen und nicht zur Wiederbesiedlung der inneren Alpen beitragen. Diese Reliktpopulationen können als Beispiele für Populationen betrachtet werden, bei denen Differenzierung in eiszeitlichen Refugien stattgefunden hat.

Teil 2 Artenverbreitungs-Modelle (engl. species distribution models, SDMs) zur Rekonstruktion von eiszeitlicher und nacheiszeitlicher Geschichte alpiner Pflanzen

Potentielle Refugialgebiete, auf die alpine Gebirgspflanzen während der Eiszeiten beschränkt waren, können auf der Grundlage verschiedener Daten lokalisiert werden, wie z.B. durch Vergleiche rezenter Verbreitungsmuster, der geographischen Verbreitung von floristischem Arten- und Endemitenreichtum (Brockmann-Jerosch & Brockmann-Jerosch, 1926, Stehlik, 2000, Tribsch & Schönswetter, 2003, Tribsch, 2004). Schönswetter et al., (2005) schlugen potentielle Refugialgebiete am Gletscherrand vor, die sie auf der Grundlage von Geomorphologie, dem geologischen Untergrund, der maximalen Ausdehnung des Gletschers zur Würmeiszeit (vor 21.000 Jahren) und der Position der Schneegrenze (Höhenlage oberhalb derer Schnee in klimatisch durchschnittlichen Jahren nicht abschmilzt, sondern liegen bleibt) ermittelten.

Die Lage dieser potentiellen Refugialgebiete wurde in zahlreichen Studien mit phylogeographischen Mustern einzelner Pflanzenarten verglichen und so die eiszeitliche und nacheiszeitliche Geschichte von einzelnen Alpenpflanzen rekonstruiert (Stehlik, 2003, Diadema et al., 2005, Schönswetter et al., 2005, Naciri & Gaudeul, 2007, Parisod & Besnard,

2007, Szövényi et al., 2009). Die genaue Lage sogenannter Nunataker, eisfreier Gebiete innerhalb des Gletschers, die über diesen hinausragen (Jäckli 1962, Hess et al., 1967, Florineth 1998, Florineth & Schlüchter, 1998, Kelly et al., 2004), wurde bisher hingegen selten in solche vergleichenden Untersuchungen eingeschlossen (vergleiche aber Schönswetter et al., (2003b) and Diadema et al., (2005)). Betrachtet man alle oben genannten Gebiete, ergibt sich eine Fülle von potentiellen Refugialgebieten für Alpenpflanzen während der Eiszeiten, hauptsächlich am Gletscherrand, aber auch innerhalb der Vergletscherung. SDMs ermöglichen eine Einschränkung auf jene potentiellen Refugialgebiete, die für die untersuchten Alpenpflanzen auf der Grundlage der klimatischen Nische, die diese Arten heute einnehmen, tatsächlich klimatisch geeignet waren. Der Einbezug der rezenten klimatischen Nische von Arten stellt eine neue Datenquelle dar, die bei einem vergleichenden Ansatz phylogeographischer Muster mit potentiellen Refugialgebieten, beschrieben auf der Grundlage der oben genannten Daten, nicht berücksichtigt wird.

Anhand der vier in den beiden Aufsätzen untersuchten *Primula* Arten wird deutlich, dass durch die SDMs Erkenntnisse über Refugialgebiete gewonnen werden, die durch einen Vergleich potentieller Refugialgebiete mit phylogeographischen Mustern der einzelnen Arten nicht hätten erlangt werden können. Überraschenderweise liegen die modellierten Refugialgebiete bei drei von vier Arten fast vollständig innerhalb der Vergletscherung, mit Ausnahme von *P. marginata*. Bei *P. hirsuta*, *P. daonensis* und *P. latifolia* spielten also periphere und zentrale Nunataker eine entscheidende Rolle, während potentielle Refugialgebiete außerhalb der Vergletscherung nur bei *P. marginata* entscheidend waren. Bei *P. latifolia* und *P. daonensis* konnten durch die SDMs manche potentiellen Refugialgebiete ausgeschlossen werden, die man sonst in Betracht gezogen hätte, weil sie an die heutigen Verbreitungsgebiete angrenzen; bei *P. daonensis* die Gebiete außerhalb der Schneegrenze zwischen dem Lago di Como und dem Lago di Garda sowie die südlichen Dolomiten und bei *P. latifolia* die Alpi Pennine. Bei *P. hirsuta* und *P. latifolia* lieferten die SDMs klare Hinweise darauf, dass es beiden Arten möglich war, oberhalb der Gletscherströme (Nunataker) in verschiedenen Bergketten an günstigen Stellen zu überdauern, wahrscheinlich auf südexponierten Hängen und Felsen oder an windgefegten Graten. Bei *P. marginata* wurden potentielle Refugialgebiete am Gletscherrand, die mit der heutigen Verbreitung überlappen, von den SDMs bestätigt. Die Ergebnisse aus den SDMs werden von den populationsgenetischen Ergebnissen in drei von vier Arten unterstützt, *P. daonensis* stellt eine Ausnahme dar, bei der die populationsgenetischen Daten den SDMs widersprechen.

Daraus ergeben sich auch neue Rekolonisierungs-Szenarien. Während wir am Beginn dieser Arbeit davon ausgegangen sind, dass *P. daonensis* und *P. hirsuta* von südlichen Refugialgebieten am Gletscherrand aus die inneren Alpen besiedelt haben, gehen wir nun davon aus, dass *P. hirsuta* sowohl aus südlichen Refugialgebieten am Gletscherrand, als auch von zentralen Nunatakern ihr heutiges Verbreitungsgebiet besiedelt hat. In *P. daonensis* sind die Verhältnisse komplex und bleiben nach wie vor ungeklärt.

Zusammenfassend kann gesagt werden, dass es SDMs ermöglichen, realistischere Szenarien über die eiszeitliche Verbreitung und die postglazialen Rekolonisierungswege zu entwerfen, die dann mit Hilfe von populationsgenetischen Mustern validiert werden können. Dieser Ansatz ist vielversprechend und trägt zu einem besseren Verständnis der komplexen Prozesse bei, die zu den genetischen und floristischen Mustern von Biodiversität, die wir heute in den Alpen finden, geführt haben.

Weitere Verbesserungen erfordern i) eine höhere räumliche Auflösung der Paläo-Klimamodelle, um die SDMs zu verfeinern und detailliertere, realistischere und besser interpretierbare Paläo-Verbreitungskarten zu erstellen. ii) Den Alpen benachbarten Gebirgszüge sollten wenn möglich mit in die Analysen eingeschlossen werden, um die Rolle dieser Gebiete für die eiszeitliche Verbreitung und die nacheiszeitliche Rekolonisierung sowie für Artbildungsprozesse mit zu erfassen. iii) Wichtig wäre, Paläo-Verbreitungsgebiete auch für Zeiträume zwischen dem Würm-Maximum und heute zu modellieren, um Rekolonisierungsbewegungen aus den Refugialgebieten in die eisfrei werdenden Alpenregionen nachzuvollziehen. Daraus könnte man Erkenntnisse über den Zusammenhang zwischen Arealverschiebungen alpiner Pflanzen und klimatischen Veränderungen gewinnen, die helfen könnten, die zukünftige Entwicklung der alpinen Flora besser vorherzusagen.

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