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Abstract

The dramatic climate changes during the Quaternary had a major influence on the distribution of biota in mountain ranges with subsequent genetic, demographic and evolutionary consequences. The European Alps provide an excellent model for studying various effects of climate changes on mountain biota. Since large areas of the Alps were covered by a continuous ice shield during the cold periods, the survival for mountain biota was possible only in ice-free areas, such as mountains summits protruding from the ice shield (i.e. nunataks) and peripheral regions. After ice melting in the warm periods, they were able to (re)colonize the Alps.

Two hypotheses have been proposed to describe species' range shifts in response to climate changes: the interglacial contraction hypothesis and the interglacial expansion hypothesis. In the first chapter, we tested these two hypotheses in eight alpine plants that differ in habitat preferences. We take population size as a proxy of range size. In all studied species, irrespective of their habitat preferences, interglacial expansion models are favoured over the interglacial contraction models. During the Last Glacial Maximum (LGM), these species were restricted to mountain ranges, peripheral and/or interior refugia, whereas during the postglacial period they re-colonized large de-glaciated areas. Lacking of suitable habitat types in lowland and competitive exclusion may have prevented them from expanding to lowland areas.

Due to lacking fossil evidence, survival on nunataks remains controversial. The second chapter aims at testing the nunatak hypothesis using two high alpine plants, *Pedicularis aspleniifolia* and *Carex fuliginosa*, which share similar habitats but differ in pollination modes. With the iDDC (integrative distributional, demographic and coalescent) approach, we explicitly tested three glacial survival scenarios: peripheral survival only, nunatak survival

only and nunatak plus peripheral survival. The nunatak plus peripheral survival scenario was supported in *P. aspleniifolia* and the peripheral survival only scenario was supported in *C. fuliginosa*. These results are consistent with current habitat preferences (*P. aspleniifolia* occurs at higher elevations) and the propensity for genetic swamping (expected to be higher in the wind-pollinated *C. fuliginosa*). This is one of the first studies to explicitly test the hypothesis instead of solely using correlative evidence.

Survival in glacial refugia was often invoked to explain the origin and distribution of endemic species in mountain ranges. However, for narrowly endemic species restricted to formerly glaciated areas, other mechanism need to be sought. In the third chapter, we investigated the origin of two narrowly endemic diploid species, *Euphrasia inopinata* and *E. sinuata*, restricted to the formerly heavily glaciated central Eastern Alps. We tested whether they are ancestral diploid remnants of a diploid-polyploid complex or whether they are segregates from a widespread diploid species. To this end, we analysed ITS sequences and AFLP fingerprinting data, identifying that *E. inopinata* and *E. sinuata* are phylogenetically closely related to diploid *E. alpina* instead of *E. minima* and that they did not participate in the formation of allotetraploid *E. minima*. Both lines of evidence indicate that *E. inopinata* and *E. sinuata* are peripheral segregates of *E. alpina*. Shifts to autogamy, genetic drift and geographic isolation may have led to the morphological and ecological differentiation of *E. inopinata* and *E. sinuata*.

Zusammenfassung

Die dramatischen klimatischen Veränderungen während des Quartärs hatten einen großen Einfluss auf die Verbreitung von Gebirgsorganismen mit entsprechenden genetischen, demographischen und evolutionären Folgen. Die europäischen Alpen stellen ein exzellentes Modellsystem dar, um die verschiedenen Auswirkungen von Klimaänderungen auf Gebirgsorganismen zu untersuchen. Nachdem weite Teile der Alpen während der Kaltzeiten von durchgehenden Eismassen bedeckt waren, konnten Gebirgsarten nur in den eisfreien Bereichen, wie auf über das Eisschild hinaus ragenden Gipfeln (Nunataker) oder in Randgebieten der Alpen, überdauern. Nach der Eisschmelze in den wärmeren Perioden konnten diese ehemals vergletscherten Bereiche (wieder-)besiedelt werden.

Zwei Hypothesen wurden aufgestellt, um Arealänderungen von Arten im Zuge vergangener Klimaschwankungen zu beschreiben, die Interglaziale-Kontraktions-Hypothese und die Interglaziale-Expansions-Hypothese. Im ersten Kapitel testen wir diese beiden Hypothesen in acht Gebirgspflanzen mit unterschiedlichen Standortsansprüchen. Dabei nehmen wir Populationsgröße als Surrogat für Arealgröße. Unabhängig von ihren Standortsansprüchen wird in allen untersuchten Arten die Interglaziale-Expansions-Hypothese gegenüber der Interglazialen-Kontraktions-Hypothese unterstützt. Entsprechend waren diese Arten während des letzten eiszeitlichen Maximums auf, interne und/oder periphere, Refugialgebiete innerhalb der Gebirge beschränkt, wovon aus sie im Postglazial die nunmehr eisfreien Bereiche (wieder-)besiedeln konnten. Das Fehlen geeigneter Standorte in den Tieflagen und Ausschluss durch Konkurrenz mögen ein Ausbreiten in Tieflagen während der Kaltzeiten verhindert haben.

Wegen des Fehlens von entsprechenden Fossilfunden ist die Überdauerung auf Nuntakern nicht unumstritten. Im zweiten Kapitel wird die Nuntaker-Hypothese anhand zweier Arten,

Pedicularis aspleniifolia und *Carex fuliginosa*, getestet, die ähnlichen Standortsansprüche, aber unterschiedliche Bestäubungsmodi besitzen. Unter Verwendung der iDDC (integrative distributional, demographic and coalescent) Methode werden drei Szenarien explizit getestet: Überdauerung ausschließlich in peripheren Refugien, Überdauerung ausschließlich auf Nunatakern, Überdauerung sowohl in peripheren Refugien als auch auf Nunatakern. Letzteres ist das bestgestützte Szenario in *P. aspleniifolia*, während für *C. fuliginosa* das Szenario mit Überdauerung ausschließlich in peripheren Refugien die größte Unterstützung findet. Diese Ergebnisse stimmen überein mit den gegenwärtigen Standortsansprüchen der Arten (*P. aspleniifolia* steigt in höhere Lagen) und mit der Anfälligkeit für genetische Verdrängung (höher bei der windbestäubten *C. fuliginosa*). Dies ist eine der ersten Studien, die diese Hypothesen explizit anstatt nur über genetische Korrelationen testet.

Eiszeitliche Überdauerung wurde oft zur Erklärung von Herkunft und Verbreitung endemischer Gebirgsarten herangezogen. Für engräumig verbreitete Arten, die ausschließlich in vormals stark vergletscherten Gebieten vorkommen, müssen allerdings andere Erklärungen gefunden werden. Im dritten Kapitel wird die Herkunft zweier engräumig verbreiteter diploider Arten, *Euphrasia inopinata* und *E. sinuata*, untersucht, die auf vormals stark vergletscherte Bereiche der zentralen Ostalpen beschränkt sind. Wir testeten, ob diese Arten ursprüngliche diploide Reste eines diploid-polyploiden Artenkomplexes oder Abkömmlinge weit verbreiteter diploider Arten sind. Zu diesem Zweck analysierten wir ITS-Sequenzen und AFLP-Fingerprint-Daten. Dabei stellte sich heraus, dass *Euphrasia inopinata* und *E. sinuata* stammesgeschichtlich nächstverwandt mit der diploiden *E. alpina* anstatt der tetraploiden *E. minima* ist und nicht an der Entstehung der allotetraploiden *E. minima* beteiligt waren. Dies deutet darauf hin, dass *Euphrasia inopinata* und *E. sinuata* periphere Abkömmlinge von *E. alpina* sind. Änderungen hin zu Autogamie, genetische Drift und geographische Isolation

haben wahrscheinlich zur morphologischen und ökologischen Differenzierung von *Euphrasia inopinata* und *E. sinuata* beigetragen.

General Introduction

The dramatic climatic fluctuations of the Quaternary had significant influence on phylogeographic patterns and evolutionary history of mountain biota (Comes and Kadereit 1998; Tribsch and Schönswetter 2003; Schönswetter et al. 2005; Lohse et al. 2011). In the European arctic-alpine, large areas were repeatedly covered by ice-sheets resulting in massive range shifts in plants. During the glacial period, with the advance of the ice-cap, plants were forced to migrate to ice-free areas (Schönswetter et al. 2005; Stewart et al. 2010). In the following inter-glacial period, in pace with the ice melting, plants were able to (re)colonize the formerly glaciated areas (Schönswetter et al. 2005; Stewart et al. 2010).

Two hypotheses have been proposed to describe altitudinal and/or latitudinal range shifts of alpine species during the Pleistocene. One is that species had small range sizes during the interglacial period (the interglacial contraction hypothesis, Schmitt 2007; Stewart et al. 2010). These species were widely distributed in peripheral refugia and in lowland refugia during cold periods, and were restricted to mountain regions in the following interglacial period, resulting in decreasing range sizes. Alternatively, species may have had larger range size during the interglacial period (the interglacial expansion hypothesis, Schmitt 2007; Stewart et al. 2010; Theodoridis et al. 2017). These species were restricted to mountain regions across glacial cycles. They survived in nunataks and/or peripheral refugia during glacial periods and (re)colonized deglaciated mountain areas during the interglacial period as ice melted, resulting in increasing range sizes.

Habitat preferences have significant influence on the phylogeographic history of species (e.g. Hodges et al. 2007; Massatti and Knowles 2014). In the context of range dynamics in alpine species, habitat preferences might limit their dispersal to and persistence in lowland habitats during glacial periods. Species that have high competitiveness, wider ecological

tolerances and are dry-adapted might have survived in lowlands, thus experiencing population expansion (Schmitt 2007; Theodoridis et al. 2017). On the contrary, species that have low competitiveness, are explicitly adapted to mountainous habitats and are intolerant to dryness might have been restricted to montane areas (Schmitt 2007; Stewart et al. 2010), thus experiencing population contraction.

Another major research topic, over the last decades, is where plants managed to survive glacial periods (Stehlik 2000; Schönschwetter et al. 2005; Lohse et al. 2011; Schneeweiss and Schönschwetter 2011). Two hypotheses have been proposed: the *tabula rasa* hypothesis and the *nunatak* hypothesis (Stehlik 2000; Stewart et al. 2010). The *tabula rasa* hypothesis refers to that plants have survived in ice-free areas in the periphery of the glacier (Brochmann et al. 1996; Brochmann et al. 2003; Schönschwetter et al. 2005). It has been widely accepted and is supported by both molecular evidence (Gabrielsen et al. 1997; Schönschwetter et al. 2005) and fossil records (Birks and Willis 2008). In contrast, the *nunatak* hypothesis, which refers to that plants have survived on ice-free mountain peaks within the glacier, is more controversial (Gabrielsen et al. 1997; Schneeweiss and Schönschwetter 2011; Westergaard et al. 2011). Because of the absence of fossil evidence, the *nunatak* hypothesis was inferred solely from correlative genetic evidence (e.g. Stehlik et al. 2001; Lohse et al. 2011; Westergaard et al. 2011). However, observed genetic patterns could be swamped by massive immigration from the peripheral refugia, resulting in a failure to detect lineages that survived on nunataks.

Range shifts induced by Quaternary glaciations have been identified as a main driving force fostering contemporary endemism in mountain ranges (Tribsch and Schönschwetter 2003; Tribsch, 2004). The extant distributions of endemic species often coincide with the location of Pleistocene refugia (Tribsch and Schönschwetter 2003; Tribsch 2004). These endemic species have survived in glacial refugia during glacial periods but failed to (re)colonize deglaciated

areas during inter-glacial or the postglacial period. However, for narrowly endemic species currently distributed in formerly heavily glaciated areas, alternative explanations are needed, including (1) *in situ* survival on nunataks and migration to deglaciated areas in close proximity, or (2) survival in peripheral refugia followed by postglacial migration and extinction in source areas due to environmental change, or (3) rapid *in situ* speciation of postglacial (re)colonizers after ice retreating (Kolář et al. 2013). Insights into the origin of narrowly endemics restricted to formerly glaciated areas are essential for the understanding of the effects of Quaternary climate fluctuations on the distribution and evolution of mountain biota.

Research aims

The goal is to address three aspects of the impacts of Quaternary climate fluctuations on alpine plants: (1) testing glacial range dynamic hypotheses, interglacial contraction and interglacial contraction, in the first chapter; (2) testing glacial survival hypotheses, nunatak and tabula rasa, in the second chapter; and (3) investigating the origins of narrowly endemic plants restricted to formerly heavily glaciated areas in the third chapter.

In the first chapter, we employed a model selection approach to test the interglacial contraction hypothesis and the interglacial expansion hypothesis in eight alpine plants found in four habitat types (dry closed alpine swards, moist closed alpine swards, wind-exposed open alpine sward, moist sward and screes and/or subnival cushion formations). We aimed to answer: (1) how do alpine plants respond to climate fluctuations; (2) do the demographic dynamics of alpine plants differ depending on habitat preferences.

In the second chapter, we investigated the glacial survival patterns of two high alpine plants, *Pedicularis aspleniifolia* and *Carex fuliginosa*. By employing the iDDC (integrative distributional, demographic and coalescent) approach, we explicitly tested three glacial

survival scenarios: (1) peripheral survival only (e.g. Schönswetter et al. 2004); (2) nunatak survival only (e.g. Stehlik et al. 2002); (3) nunatak plus peripheral survival (e.g. Escobar García et al. 2012).

In the third chapter, we studied the origin of two narrowly endemic plants, *Euphrasia inopinata* and *E. sinuata*, in a phylogenetic framework employing AFLP and ITS data. These species are exclusively distributed in formerly glaciated areas in the eastern Alps (Ehrendorfer and Vitek 1984). We addressed the following questions: (1) Are they ancestral diploid remnants of a diploid-tetraploid complex? (2) Are they peripheral segregates of a more widespread diploid species?

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Chapter 1

Ecologically divergent high-mountain species from the European Alps responded to Pleistocene climate cooling exclusively by range contraction

Ecologically divergent high-mountain species from the European Alps responded to Pleistocene climate cooling exclusively by range contraction

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Abstract

The Quaternary climate fluctuations profoundly affected range dynamics of high-mountain species. Two alternative hypotheses, interglacial contraction and interglacial expansion, have been proposed to describe range shifts of cold-adapted species in response to climate changes. Whether a species expanded its range during cold periods (interglacial contraction) or during warm periods (interglacial expansion) is expected to depend on the species' habitat preference. Here, we test these hypotheses using eight alpine plant species that differ in their habitat preferences (one insect and one wind-pollinated species each in four main alpine habitat types). Species distribution modelling (SDM) confirmed that changes in range size and in population size are positively correlated, allowing demographic modelling to be used to test these two hypotheses. To this end, genome-wide molecular data (RADseq data) from

populations in the distribution range in the eastern European Alps were employed to compare several demographic scenarios pertaining to the two hypotheses. Although for most species SDMs suggested interglacial population size and range size decrease, genetic data congruently supported the interglacial expansion hypothesis irrespective of the habitat preferences of the studied species. During glacial periods, these species were likely restricted to peripheral and/or interior refugia within the mountain ranges, from where they (re-)colonized in interglacial periods the de-glaciated areas thus acquiring larger distribution ranges. In contrast to Arctic-alpine plants, which, in the temperate regions, mainly experienced interglacial contraction, the interglacial expansion scenario might be common in obligate high-mountain plants. The factors preventing them from expanding to lowland refugia during glacial periods remain, however, unknown. In the context of ongoing global warming, obligate high-mountain plants, although showing interglacial expansion, might be more vulnerable than Arctic-alpine plants.

Keywords: alpine plants, demographic history, range dynamics, Last Glacial Maximum, climate change, European Alps

Introduction

The dramatic climate oscillations during the Quaternary had a major impact on the distribution ranges of cold-adapted species in general and on high-mountain species in particular (Comes & Kadereit, 1998; Hewitt, 1999; Hewitt, 2004; Schönswetter, Stehlik, Holderegger, & Tribsch, 2005; Tribsch & Schönswetter, 2003). During phases of glacial advancing, the distribution of alpine species (i.e., species found mainly above the timberline), was restricted to ice-free areas at the, narrower or broader, periphery of or within the ice-sheets (peripheral and interior refugia, respectively; Schneeweiss & Schönswetter, 2011;

Schönswetter & Schneeweiss, 2019; Schönswetter et al., 2005; Stewart, Lister, Barnes, & Dalén, 2010). During the warm interglacial periods and/or in the postglacial these species could (re)colonize the formerly glaciated areas (Engelhardt, Haase, & Pauls, 2011; Lohse, Nicholls, & Stone, 2011; Schönswetter et al., 2005; Tribsch & Schönswetter, 2003). Understanding these past range dynamics may help us understand how cold-adapted species may respond to the current global climate change (Davis & Shaw, 2001; Sexton, McIntyre, Angert, & Rice, 2009).

Two hypotheses have been proposed to describe altitudinal and/or latitudinal range shifts of alpine species during the Pleistocene. The interglacial contraction hypothesis (Schmitt, 2007; Stewart et al., 2010) suggests that species were able to migrate to and survive in the ice-free periphery of mountain ranges and in lowland regions, resulting in range expansion. In interglacial periods and in the postglacial period, these species retreated to high altitude refugia, resulting in decreasing range sizes. Alternatively, the interglacial expansion hypothesis (Schmitt, 2007; Stewart et al., 2010; Theodoridis et al., 2017) proposes that alpine species were not able to migrate to and/or persist in lowland regions even though they had become climatically suitable. Reasons for this failure are generally unknown, but may include lack of habitats specific to mountainous environments, such as scree habitats, or competitive inferiority compared to lowland species (Birks, 2008). Consequently, during glacial periods alpine species would have been restricted to interior refugia (nunataks) and/or peripheral refugia (i.e. mountainous areas in close proximity to the glacial margin; Holderegger & Thiel-Egenter, 2009; Schönswetter et al., 2005), resulting in range contraction, whereas in interglacial periods and in the postglacial period, these species expanded into formerly glaciated areas, resulting in range expansion.

Species' responses to climate changes are largely dependent on traits of the particular species (Stewart, 2008; Stewart et al., 2010). One key factor for the phylogeographic history of a species is its habitat preference (e.g. Beavis, Sunnucks, & Rowell, 2011; Hodges, Rowell, & Keogh, 2007; Langerhans & DeWitt, 2004; Massatti & Knowles, 2014). Habitat preferences and underlying functional traits of alpine species might have limited the suitability of lowland habitats during glacial periods. Specifically, highly competitive or dry-adapted species and/or those having broader ecological tolerances might have colonized and persisted in lowlands, thus conforming to the interglacial contraction hypothesis (Schmitt, 2007; Theodoridis et al., 2017). In contrast, species that show low competitiveness, are intolerant to dry conditions and/or are exclusively adapted to mountainous habitats might not have colonized lowlands (Schmitt, 2007; Stewart et al., 2010), thus conforming to the interglacial expansion hypothesis.

A good system for evaluating these two hypotheses and the effects of habitat preferences on the demographic history of alpine species are the European Alps (hereinafter referred to as Alps), whose flora has been subject to numerous molecular phylogeographic studies (e.g., Lohse et al., 2011; Scheel & Hausdorf, 2012; Schneeweiss & Schönswetter, 2010; Schönswetter et al., 2005). Major parts of the Alps were covered by a continuous ice sheet during the glacial periods (Ehlers & Gibbard, 2004; Seguinot et al., 2018). Various putative refugia for alpine species, including interior refugia (nunataks, Schneeweiss & Schönswetter, 2010), peripheral refugia (e.g., in the southern or northeastern Alps, Schönswetter et al., 2005), and lowland refugia (Alsos, Alm, Normand, & Brochmann, 2009; Skrede, Eidesen, Portela, & Brochmann, 2006) were identified. During glacial periods, lowlands around the Alps were covered by a variety of habitats, e.g. minerotrophic fen and typical steppe (Janská et al., 2017), supporting the survival of alpine species like *Primula farinosa* (Theodoridis et al., 2017) and *Salix herbacea* (Alsos et al., 2009).

In this study, a model selection approach was employed to test the interglacial contraction and the interglacial expansion hypothesis for eight alpine plants that differ in their habitat preferences and pollination biology. It can be expected that range shifts and, under the assumption that range expansion and contraction correlate with population size increase and decrease, respectively, demographic patterns of these species will differ depending on their habitat preferences. For instance, the glacial history of species that inhabit dry alpine habitats may rather conform to the interglacial contraction hypothesis because these species might have survived in cold and dry habitats (widespread steppe: Janská et al., 2017) in lowland refugia.

Materials and methods

Study species and sampling

From each of two plant genera (louseworts: *Pedicularis*, Orobanchaceae; sedges: *Carex*, Cyperaceae) we selected four study species differing in their habitat preferences. To minimize potential biases due to species-specific characteristics we chose phylogenetically closely related species with similar functional traits. Specifically, all louseworts are insect-pollinated perennials with similar flower, capsule and seed morphology (Fischer, 2008; Günthart, 1926). Three of them belong to the same major clade within the genus (clade 8 of Tkach, Ree, Kuss, Röser, & Hoffmann, 2014); the fourth species (*P. rostratospicata*) has not been investigated yet using molecular data. The sedges are wind-pollinated perennials with similar spike and utricle morphology (Fischer, 2008; Kükenthal, 1909). All four sedge species belong to the same subclade within the core *Carex* clade (Jiménez-Mejías et al., 2016). One representative of each genus can be found in each of the following four habitats (Mucina, Grabherr, & Ellmauer, 1993; Oberdorfer, Schwabe, & Müller, 2001): *P. rostratocapitata* and *C.*

sempervirens inhabit dry closed alpine swards (alliance *Seslerion coeruleae*); *P. rostratospicata* and *C. ferruginea* prefer moist (due to longer lasting snow cover) closed alpine swards (alliance *Caricion ferrugineae*); *P. rosea* and *C. firma* grow in wind-exposed open alpine swards (alliance *Caricion firmae*); *P. aspleniifolia* and *C. fuliginosa* are found in moist (due to longer snow cover) open alpine to subnival swards and screes.

We sampled from the common distribution range of the eight study species, i.e., the eastern Alps, where some of these species are endemic to. 8–24 populations per species were sampled between the years 2013 and 2017, with 1 or 2 individuals per population separated by at least 5 m (Table S1). Sampled leaves were dried in silica gel for DNA extraction. In total, 222 individuals were collected in our study. The number of individuals in each species was: *P. rostratocapitata*, 36; *P. rostratospicata*, 24; *P. aspleniifolia*, 15; *P. rosea*, 24; *C. sempervirens*, 34; *C. ferruginea*, 34; *C. fuliginosa*, 19; *C. firma*, 36. Voucher specimens are deposited in the herbarium of the University of Vienna (WU; Table S1).

Species distribution models

To estimate direction and magnitude of potential range shifts during the last glacial maximum (LGM) species distribution models (SDMs) were constructed. Occurrence data were obtained for all study species from the GBIF database (<https://www.gbif.org/>), the project “Mapping the flora of Austria” (H. Niklfeld and L. Ehrendorfer, University of Vienna, unpubl. data) and the European Vegetation Archive (EVA; Chytrý et al., 2016). Nineteen bioclimate variables for the present and the LGM climate conditions were downloaded from the WorldClim dataset (<http://www.worldclim.org>, Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) at a 2.5 arc-min resolution. Pairwise correlations between variables were checked. After removing highly correlated (> 0.7) variables, eight bio-climate variables were retained (annual mean

temperature, temperature seasonality, mean temperature of wettest quarter, mean temperature of driest quarter, annual precipitation, precipitation seasonality, precipitation of warmest quarter, precipitation of coldest quarter). An ensemble modelling approach implemented in the “Biomod2” package (Thuiller et al., 2016) for R (R Core Team, 2013) was employed to model species distributions. We applied six modelling algorithms: Generalized Linear Model (GLM), Generalized Boosting Model (GBM), Generalized Additive Model (GAM), Classification Tree Analysis (CTA), Artificial Neural Network (ANN) and Random Forest (RF). Pseudo-absences for each species were randomly created with prevalence equal to 0.5 with ten replicates. To evaluate the predictive power of models, datasets were randomly divided into two subsets, 80% for calibrating and 20% for evaluating. To avoid a random effect of data splitting, we repeated the split 10 times. The predictive performance of each model was measured using relative operating characteristic (ROC) values (Swets, 1988). Models with $ROC < 0.75$ were excluded from generating ensemble projections.

The reference area for estimating changes in range and population size was defined as the zone surrounding occurrence points with a radius of 150 km and was calculated as the convex hull around these occurrences using the ‘chull’ function in R. Changes in range size were measured as the number of climatically suitable cells. Changes in population size were measured as the sum of suitability scores, assuming that climatic suitability is positively correlated with carrying capacity and thus population size (Brown & Knowles 2012; He, Prado, & Knowles, 2017; Massatti & Knowles, 2016); cells with suitability scores smaller than 10% of the maximum suitability score were considered as totally unsuitable (following the approach of He et al., 2017; Massatti & Knowles, 2016).

RAD library preparation and SNP calling

Total genomic DNA was extracted using a modified CTAB protocol (Doyle & Doyle, 1987; Jang et al., 2013). The RAD library preparations followed the single-digest protocol from Paun et al. (2016). Each individual was barcoded with P1 barcode (8-bp), an inline barcode (6-bp) and P2 barcode (8-bp); all barcodes differed by at least two nucleotides. After Pst1 restriction enzyme (New England Biolabs, Ipswich, Massachusetts, USA) digestion of 150 ng DNA, P1 adaptors (containing P1 barcode and inline barcode) were ligated in order to distinguish individuals within each sub-library. Then, samples were sheared by sonication using a Bioruptor Pico (Diagenode, Liège, Belgium) using the following settings: 2 cycles of 45 s ‘on’ and 60 s ‘off’ at 4 °C. Subsequently, P2 adaptors (containing P2 barcode) were ligated to differentiate sub-libraries. After 18 cycles of PCR amplification, a Pippin Prep (Sage Science, Beverly, Massachusetts, USA) was used to select a size range from 220 bp to 850 bp. 100 bp single-end Illumina sequencing was performed on altogether 6 RAD libraries at VBCF Vienna (<http://csf.ac.at/facilities/next-generation-sequencing/>).

Libraries were de-multiplexed according to the P1 and P2 adaptor combinations employing BamIndexDecoder.jar in Illumina2Bam (<https://github.com/gq1/illumina2bam>) and process_radtags.pl in STACKS 1.44 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). Reads with poor quality (Phred quality score < 10) and high number of repeats were removed from further analysis. SNPs were called separately for each species using the STACKS package with minimum coverage depth (-m) set to 5, distance between loci within each sample (-M) set to 2, distance between loci across samples (-n) set to 2 and upper bound of the error rate (--bound_high) set to 0.1. Only loci containing no more than 4 SNPs were retained. The first SNP per locus was exported into VCF-format and STRUCTURE-format files.

Inferring population genetic structure

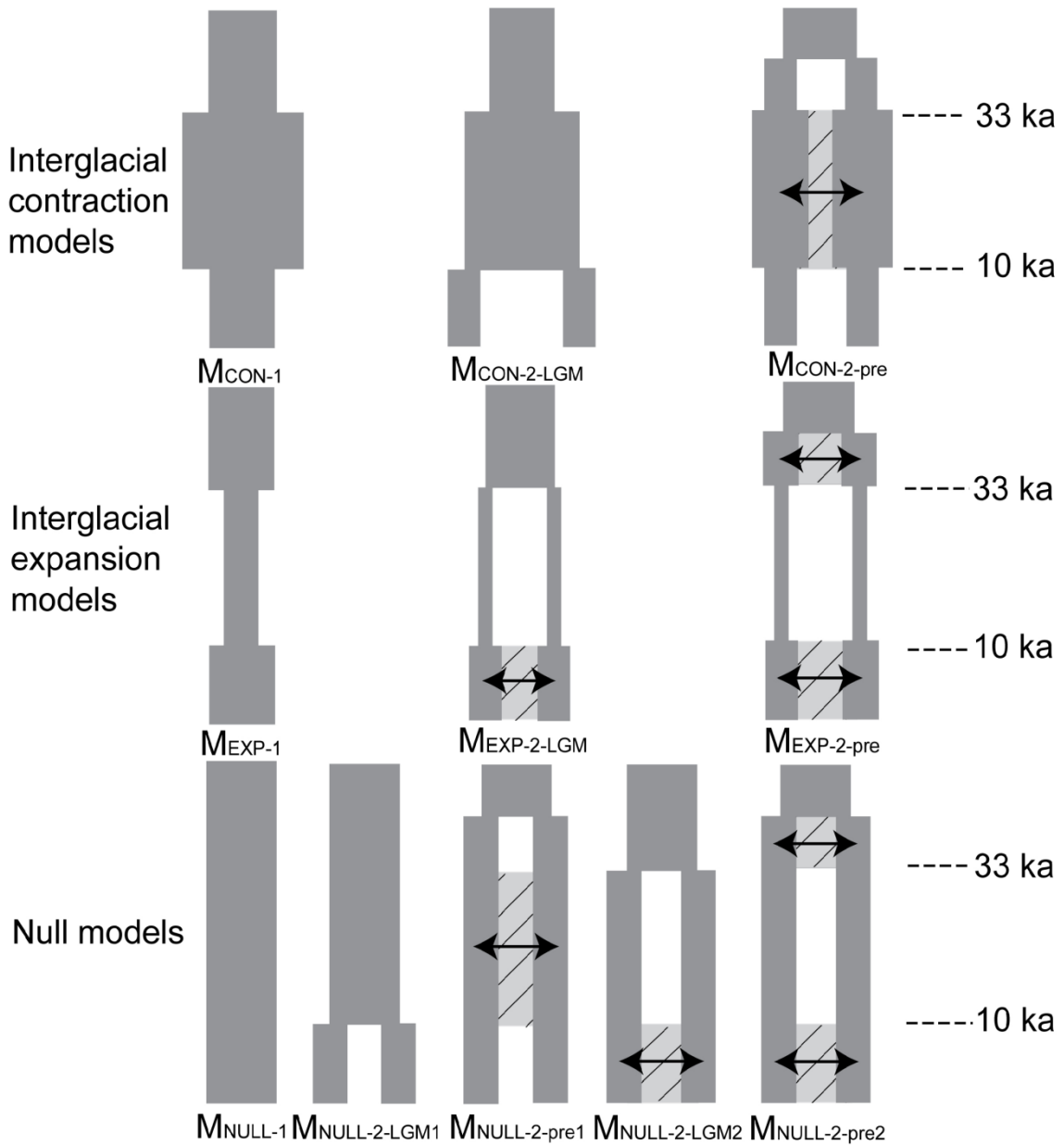
We used a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) to investigate the population structure within each species. The program was run using the admixture model with 10^6 Markov Chain Monte Carlo iterations after a burn-in of 10^5 iterations. All parameters were left at their default values. Number of clusters (K) was explored within the range from 1 to 8 with 10 replicates each. The best K was chosen based on ΔK (Evanno, Regnaut, & Goudet, 2005) as implemented in STRUCTURE HARVESTER (<http://taylor0.biology.ucla.edu/structureHarvester/>, Earl & vonHoldt, 2012). Results were plotted in R (R Core Team, 2013).

Demographic model comparisons

We tested 11 models representing different demographic histories during the Last Glacial Maximum (LGM; Fig. 1). For species without genetic structure (see Results), three one-deme models were tested. These models correspond to the interglacial population contraction hypothesis (M_{CON-1}) with increased population size during the LGM, the interglacial population expansion hypothesis (M_{EXP-1}) with decreased population size during the LGM, and to a constant population size model (M_{NULL-1}) with no change in population size. Points of population size change were set at the beginning of the Last Glacial Maximum (33,000 years ago, Clark et al., 2009) and at the end of the LGM (10,000 years ago). For those species that show genetic structure (see Results), eight two-deme models were evaluated. Four models were derived from M_{CON-1} and M_{EXP-1} by adding a deme split either at the beginning of the phase with reduced population size (i.e., at the end of the LGM in $M_{CON-2-LGM}$, at the beginning of the LGM in $M_{EXP-2-LGM}$), assuming that lineage differentiation is more likely in case populations are small and isolated, or before the LGM ($M_{CON-2-pre}$ and $M_{EXP-2-pre}$), reflecting that lineage differentiation may precede the LGM. In all four models, gene-flow between populations was permitted only during phases of increased population sizes, when, as

suggested by current distributions of intraspecific gene pools, lineages became geographically close. These models ($M_{\text{CON-2-LGM}}$, $M_{\text{EXP-2-LGM}}$, $M_{\text{CON-2-pre}}$, $M_{\text{EXP-2-pre}}$) were compared to four constant population size models ($M_{\text{NULL-2-LGM1}}$, $M_{\text{NULL-2-LGM2}}$, $M_{\text{NULL-2-pre1}}$, $M_{\text{NULL-2-pre2}}$) that had the same temporal patterns of deme split and gene flow as their counterparts with populations size changes, but did not show any population size change (Fig. 1).

Fig. 1 Six demographic models representing the hypothesized demographic histories and five null models used for model evaluation. Black arrows indicate gene flow between populations during the time period indicated by hatched areas. The duration of the cold period (from 33 to 10 ka) is indicated.



The composite likelihoods of these models were calculated based on the joint site frequency spectra (SFS) using FASTSIMCOAL2 2.6 (Excoffier, Dupanloup, Huerta-Sánchez, Sousa, & Foll, 2013; Excoffier & Foll, 2011). VCF-format files were converted to ARLEQUIN-format using PGDSPIDER 2.1.1.2 (Lischer & Excoffier, 2011). Observed 1-D and 2-D minor allele SFS (MAF and jointMAF) were generated using ARLEQUIN 3.5.2.2 (Excoffier & Lischer, 2010). Priors of estimated parameters were drawn from unbounded uniform distributions. We performed 50 independent estimations per model per species with random seeds. For all runs, we performed 10^5 simulations and 10–40 cycles of the Expectation Conditional Maximization (ECM) algorithm, with a stopping threshold of 0.001. Model fit was evaluated based on the Akaike Information Criterion (AIC, Akaike, 1973) values and Δ AIC (i.e., the difference between the AIC score from the current model and the overall lowest AIC score) calculated from the best maximum composite likelihood across 50 replicate runs. Following Burnham and Anderson (2004), Δ AIC values of at least 10 are interpreted that this model has essentially no support.

Results

Species distribution models

Habitat suitability scores derived from SDMs based on current climate conditions match well the actual distributions of the study species in the Alps (Fig. 2). Under the LGM climate condition, range expansion (i.e., a higher number of suitable cells, Table 1) was predicted for all species except *P. rostratospicata* and *P. rosea* (Fig. 2), which were inferred to have experienced range contraction (i.e., a lower number of suitable cells, Table 1) being essentially restricted to the Alps. Changes in range size (number of climatically suitable cells) positively correlated with changes in population size (sum of suitability scores of all cells) in

all species but *P. rostratocapitata* (Table 1). Here, areas northeast of the Alps were reconstructed to be climatically suitable, but had generally low suitability scores (< 200), resulting in an inferred range expansion, but decrease in population size (Table 1).

Fig. 2 Predicted habitat suitability of the study species in the Alps at present and during the Last Glacial Maximum (LGM). The dashed lines indicate the current distribution areas of each species in the Alps.

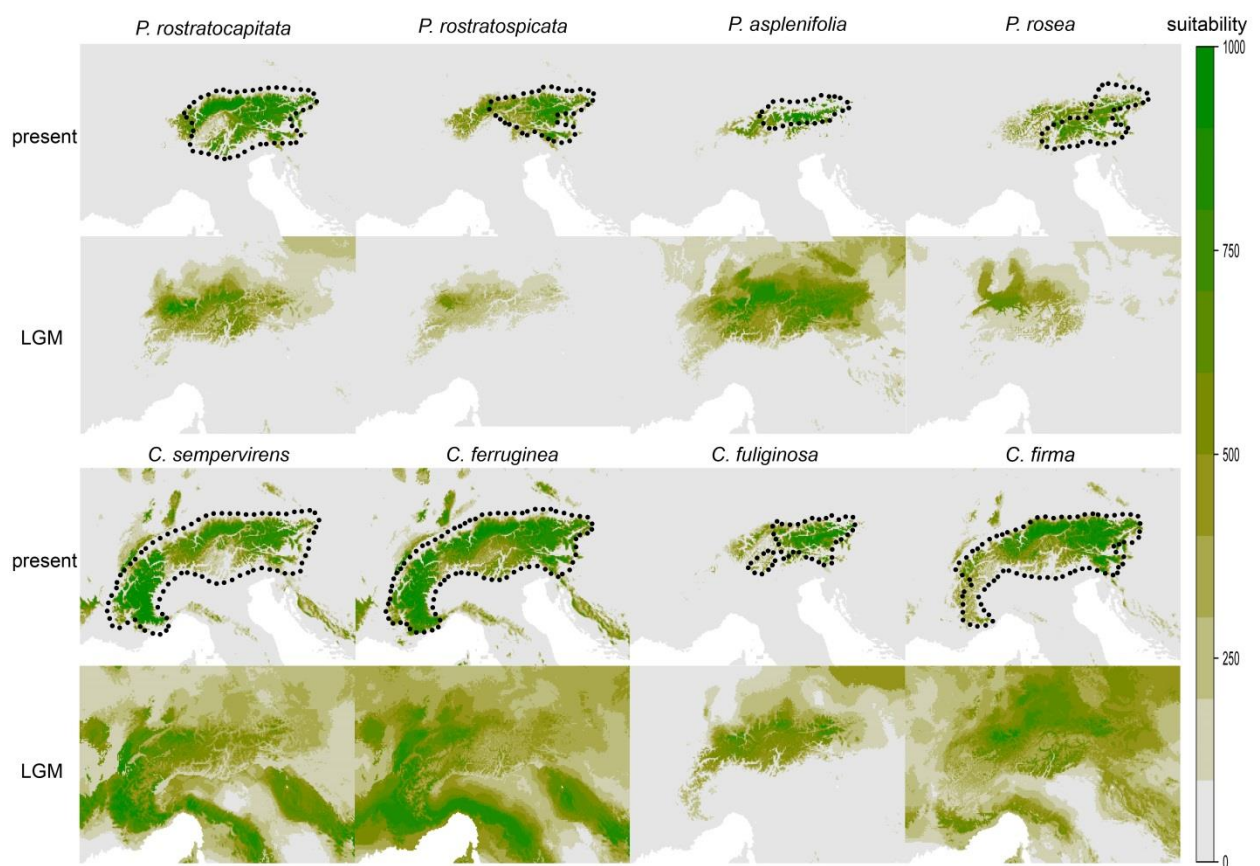


Table 1 Range size (measured via the number of climatically suitable cells) and population size (measured as the sum of suitability scores) of the eight studied species at present and during the Last Glacial Maximum (LGM).

		<i>P. rostratocapitata</i>	<i>C. sempervirens</i>	<i>P. rostratospicata</i>	<i>C. ferruginea</i>	<i>P. rosea</i>	<i>C. firma</i>	<i>P. aspleniifolia</i>	<i>C. fuliginosa</i>
Number of climatically suitable cells	present	6,124	13,717	4,438	15,342	4,364	10,153	1,873	3,864
	LGM	9,314	33,116	1,731	33,628	2,412	28,993	7,357	7,990
Sum of suitability scores	present	3,331,205	6,442,116	2,044,242	7,985,013	1,781,221	4,878,377	985,941	1,729,355
	LGM	2,104,494	11,911,246	293,799	15,586,762	448,736	11,061,101	3,378,872	2,078,515

RAD datasets

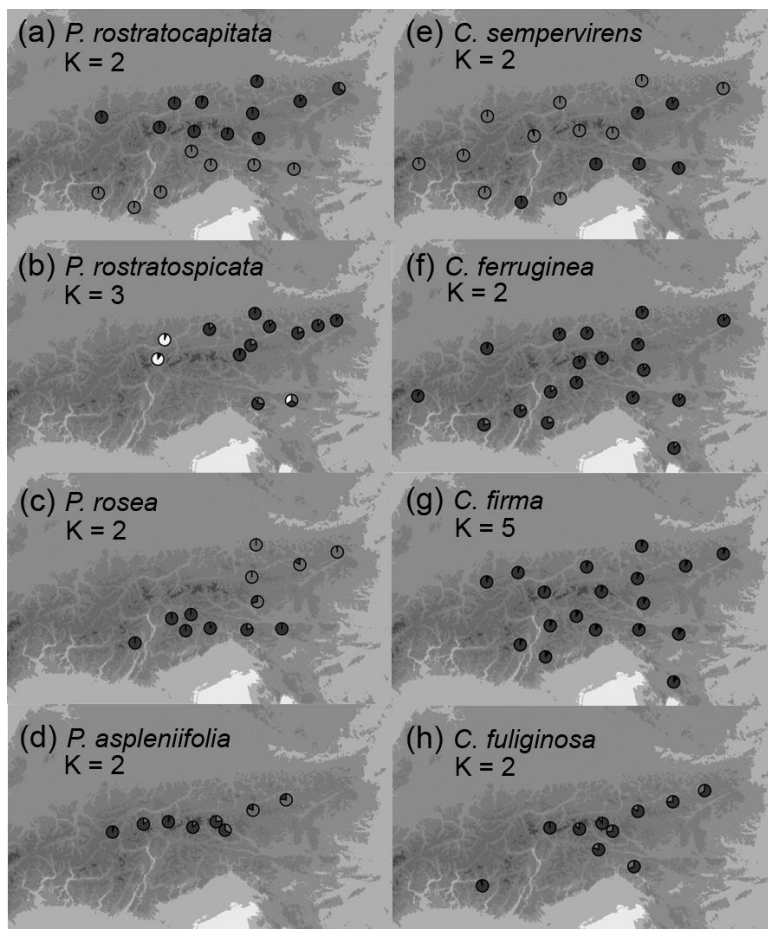
In total, we obtained more than 300 million reads from six Illumina lanes after quality filtering, with more than 900,000 reads per individual. To remove possible paralogues, loci with more than 4 SNPs were discarded. All loci containing missing data were removed to allow estimation of SFS. For analyses, only one SNP per locus was retained to avoid linkage. The final datasets contained 6531 SNPs for *P. rostratocapitata*, 6110 SNPs for *P. rostratospicata*, 5458 SNPs for *P. aspleniifolia*, 5531 SNPs for *P. rosea*, 2462 SNPs for *C. sempervirens*, 6484 SNPs for *C. ferruginea*, 5035 SNPs for *C. fuliginosa* and 4234 SNPs for *C. firma*.

Population structure

In *P. rostratocapitata*, *P. aspleniifolia*, *P. rosea*, *C. sempervirens*, *C. ferruginea*, and *C. fuliginosa* $K=2$ was identified as the optimal number of clusters, whereas in *P. rostratospicata* and in *C. firma*, the best supported number of K were $K=3$ and $K=5$, respectively (Fig. 3). However, in several species the suggested number of clusters lacked a meaningful interpretation, i.e., the proportion of membership to a particular cluster did not exceed 0.5 in any individual. This was the case for the third cluster (i.e., the least frequent cluster) in *P. rostratospicata*, and for all clusters but the first one (i.e., the most frequent cluster) in *C. ferruginea*, *C. fuliginosa*, and *C. firma*. These low-frequency clusters may indicate the presence of gene pools more widespread in unsampled areas. This might be especially the case for *C. sempervirens* and *C. firma*, which are continuously distributed in the entire Alps and in neighbouring mountain ranges (Meusel, Jager, Rauschert, & Weinert, 1978); it is, however, less likely for *C. fuliginosa*, which also occurs in the Carpathians, but whose Alpine range is fully covered (the close relative *C. misandra* is in Europe restricted to

high latitudes, Meusel et al., 1978) and *P. rostratospicata* subsp. *rostratospicata*, whose closest relative, *P. rostratospicata* subsp. *helvetica*, is geographically and ecologically well-separated. Alternatively, in cases of $K=2$, it might be the result of the Delta K method, which by design cannot evaluate $K=1$ (Evanno et al., 2005). Ignoring such low-frequency clusters, we eventually used $K=1$ for *Carex ferruginea*, *C. firma* and *C. fuliginosa* and $K=2$ for *C. sempervirens* and the four *Pedicularis* species.

Fig. 3 Genetic structures of eight study species revealed by STRUCTURE analysis. (a) *P. rostratocapitata*; (b) *P. rostratospicata*; (c) *P. rosea*; (d) *P. aspleniifolia*; (e) *C. sempervirens*; (f) *C. ferruginea*; (g) *C. firma*; (h) *C. fuliginosa*. The number of optimal clusters, K , is given and these are indicated by different colours (clusters present only in minute proportions are not discernible).



Demographic scenario comparisons

For all investigated species scenarios representing an increase of population size during the interglacial period (models M_{EXP}) had the smallest AIC scores and alternative models (models M_{CON} and M_{NULL}) had essentially no support (ΔAIC values well above 100; Table 2). In species tested using the two-deme model (all *Pedicularis* species and *C. sempervirens*), where either $M_{\text{EXP-2-LGM}}$ (*P. aspleniifolia*, *P. rosea*, *P. rostratospicata*, *C. sempervirens*) or $M_{\text{EXP-2-pre}}$ (*P. rostratocapitata*) had the lowest AIC value, models differing in the timing of the population split (at or before the onset of the LGM; i.e., model $M_{\text{EXP-2-LGM}}$ versus $M_{\text{EXP-2-pre}}$) had similar support in *P. rosea*, *P. rostratospicata* and *C. sempervirens* (ΔAIC values 2–5).

Table 2 Comparison of demographic models based on ΔAIC values.

	M_{CON-1}	M_{EXP-1}	M_{NULL-1}	$M_{CON-2-LGM}$	$M_{NULL-2-LGM}$	$M_{EXP-2-LGM}$	$M_{NULL-2-LGM2}$	$M_{CON-2-pre}$	$M_{NULL-2-pre1}$	$M_{EXP-2-pre}$	$M_{NULL-2-pre2}$
<i>P. rostratocapitata</i>	-	-	-	1136	1082	335	3507	1459	1331	0	3176
<i>C. sempervirens</i>	-	-	-	213	453	0	560	135	425	2	499
<i>P. rostratospicata</i>	-	-	-	883	916	0	903	872	781	3	773
<i>C. ferruginea</i>	1196	0	2251	-	-	-	-	-	-	-	-
<i>P. rosea</i>	-	-	-	2068	2439	0	2463	1770	2355	5	2367
<i>C. firma</i>	2357	0	3442	-	-	-	-	-	-	-	-
<i>P. asplenifolia</i>	-	-	-	915	875	0	857	820	758	15	797
<i>C. fuliginosa</i>	360	0	799	-	-	-	-	-	-	-	-

Discussion

In this study, we used a model evaluation approach to test alternative hypotheses with respect to the demographic history in response to the climatic fluctuations during the last 33,000 years in eight alpine plants species differing in their habitat preferences. The interglacial contraction and the interglacial expansion hypothesis have, however, been formulated in terms of range size, not population size. Range size, in the absence of an LGM fossil record approximated via the number of suitable cells (Liu, Berry, Dawson, & Pearson, 2005; Theodoridis et al., 2017), and population size, approximated via suitability scores (Brown & Knowles, 2012; He, Edwards, & Knowles, 2013; He et al., 2017), positively correlate in all but one of the investigated species (*P. rostratocapitata*; Table 1). Therefore, we are confident that (at least for the herein investigated species) demographic models are suitable for testing the hypotheses of interglacial contraction and expansion, respectively.

For cold-adapted species evidence for the interglacial contraction hypothesis prevails (Espíndola et al., 2012; Kropf, Kadereit, & Comes, 2003; Westergaard et al. 2011; but see Theodoridis et al. 2017), and this is actually also expected for the investigated species (increased LGM range sizes: Table 1) with the exception of *P. rosea* and *P. rostratospicata*. However, in all studied species and thus irrespective of their habitat preferences and the pollination syndrome scenarios with decreased population sizes are strongly favoured over alternative demographic scenarios, which, given the largely positive correlation of range size and population size, suggests that LGM ranges were reduced for most or all species. This may indicate that during glacial periods these species retreated essentially only to peripheral and/or interior refugia (Schönswetter et al., 2005), which are comparatively small and thus expected

to harbour smaller populations only, whereas during interglacial periods, they (partially) re-colonized larger mountain areas which were formerly glaciated.

It has been suggested that the interglacial expansion hypothesis should apply to species intolerant to dry conditions and/or of low competitiveness, as dry glacial steppes and their constituent vegetation types (e.g., boreal dwarf shrubs: Frenzel, 1992; Lang, 1994) have prevented those species from expanding into lowland areas (Birks, 2008; Schmitt, 2007). Indeed, these traits are found in *Primula farinosa*, a weak competitor (Lindborg & Ehrlén, 2002) of mostly wet and open habitats (Theodoridis, Randin, Broennimann, Patsiou, & Conti, 2013), one of the few cold-adapted species for which the interglacial expansion hypothesis has been supported (Theodoridis et al., 2017). Similarly, such traits can explain the inferred demographic history of investigated species from moister habitats (e.g., *P. rostratospicata* and *C. ferruginea*) and from species of open vegetation types (e.g., *C. fuliginosa* and *P. aspleniifolia*), where competition is presumably low (quantitative data on competitive abilities of the studied species are not available). Although the investigated species cover a broad range of alpine habitat types, including closed alpine swards, where competition is expected to be relatively high at least in the context of alpine habitats, competitiveness of alpine species may be generally too low to keep up against lowland species of that time (Callaway et al., 2002; Choler, Michalet, & Callaway, 2001).

One common feature of the studied species is that they are obligate high-mountain plants with distribution ranges essentially constrained to the Alps and adjacent mountain systems. This contrasts with species of Arctic-alpine distribution, for which (at least in the temperate zone) the interglacial contraction hypothesis has been supported also by fossil data (Alsos et al., 2009; Birks, 2008; Skrede et al., 2006), even though these species might regularly co-occur with our study species (e.g., the Arctic-alpine *Dryas octopetala* with *C. firma* and *P. rosea* or

the Arctic-alpine *Saxifraga oppositifolia* with *P. aspleniifolia*: Mucina et al., 1993). It seems plausible that the absence in Arctic regions of the investigated species and of other species restricted to central European mountain ranges is a consequence of the same factors that prevented alpine plants to survive in climatically suitable lowland refugia during glacial periods (Fig. 2). The ultimate nature of these factors remains, however, unknown and may be both, more general (e.g., adaptation to high light-saturation in photosynthesis, Billings & Mooney, 1968) or rather idiosyncratic (e.g., seed versus site limitation: Dullinger & Hülber, 2011).

One potential caveat of our study is that the sampling is restricted to the eastern Alps, although several of the investigated taxa show a broader distribution. This geographic restriction allows the number of demographic models to be tested low (maximum K was 2), but the inclusion of members of other gene pools, as may be suggested by the Structure results, may cause inflated estimates of population sizes (Beerli, 2004), thus potentially biasing against the interglacial contraction hypothesis. Inclusion of undersampled gene pools can be excluded for *P. rostratospicata* and *P. rosea*, where the entire ranges of the studied nominate subspecies were covered (*P. rostratospicata* ssp. *helvetica* and *P. rosea* ssp. *allionii* are allopatric and differ morphologically and partly ecologically from the nominate subspecies, indicating a deep split), and is unlikely for *C. fuliginosa*, where the entire Alpine range was covered; still, also these three species followed the interglacial expansion scenario.

Conclusion

In many phylogeographic studies, SDMs were used to infer past distribution ranges of species (e.g. Beatty & Provan, 2010; Peterson, Martínez-Meyer, & González-Salazar, 2004; Waltari & Guralnick, 2009). However, a caveat of the SDM approach is that it only takes climate

variables into account, whereas other variables, such as biotic interaction (e.g., competition, facilitation), topography or (micro-)habitats, may at least be co-responsible for the distribution range of a species (Peterson, 2003). Therefore, SDM predictions are reliable only if the distribution range of a species is mainly determined by climatic factors, otherwise its distribution range will be over-predicted. This appears to be the case at least in some of the herein investigated species, for which SDMs suggested increased range and population sizes (Table 1). Thus, even though large lowland areas were inferred to be climatically suitable during the LGM period, other factors might have constrained distribution ranges of studied species to mountain ranges. Consequently, caution is warranted when interpreting past distribution ranges of high alpine plants inferred using SDM approaches.

Recent global warming has already changed the distribution of alpine plants and it is predicted to continue doing so (Chen, Hill, Ohlemüller, Roy, & Thomas, 2011; Dullinger et al., 2012; Lenoir, Gégout, Marquet, de Ruffray, & Brisse, 2008), particularly for high-altitude species with restricted ranges (Hülber et al., 2016). Upward migrations, with the recent increase in temperature as the most likely cause, were shown for several taxa (Bahn & Körner, 2003; Parolo & Rossi, 2008; Walther, Beißner, & Burga, 2005). Recent studies indicate that the warm limit of the distribution ranges of species is particularly sensitive to alterations of the competitive environment due to climate change (Rumpf et al., 2018), i.e. the retreat at the rear edge might be more pronounced than the expansion at the leading (=cold) edge. Thus, although interglacial expansion indicates that alpine species actually profited from climate warming by allowing them to extend their ranges during the Holocene, the limited nature of available altitudinal space and the evident incapability to reach geographically distant yet ecologically suitable regions (such as the Arctic) renders species of the European temperate mountain systems highly threatened under current global warming.

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Conflict of interest statement

No conflict.

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Table S1 Collection information of the eight study species.

Species	Region ¹	Latitude	Longitude	Voucher number ²
<i>Pedicularis rostratocapitata</i>	A, Müritzsteger Alpen	47°41'26"	15°36'12"	Schneeweiss 288
	A, Ennstaler Alpen	47°29'21"	14°42'37"	Schneeweiss 292
	A, Steiner Alpen	46°22'28"	14°33'54"	Schneeweiss 303
	A, Gurktaler Alpen	46°52'41"	13°44'49"	Schneeweiss 290
	A, Salzkammergut-Berge	47°49'02"	13°42'16"	Schneeweiss 302
	SL, Julische Alpen	46°26'37"	13°38'27"	Schneeweiss 297
	A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 291
	A, Goldberggruppe	46°57'12"	13°01'20"	Schneeweiss 289
	I, Südliche Karnische Alpen	46°26'30"	12°38'04"	Schneeweiss 298
	A, Kitzbüheler Alpen	47°28'30"	12°25'40"	Schneeweiss 378
	A, Venedigergruppe	46°59'38"	12°14'59"	Schneeweiss 299
	I, Dolomiten	46°39'58"	12°11'16"	Schneeweiss 296
	A, Rofangebirge und Brandenberger Alpen	47°27'27"	11°47'35"	Schneeweiss 301
	I, Vizentiner Alpen	45°59'52"	11°29'24"	Schneeweiss 295
	A, Stubai Alpen	47°03'40"	11°26'39"	Schneeweiss 304
	I, Gardaseeberge	45°44'43"	10°51'31"	Schneeweiss 293
	A, Lechquellengebirge	47°13'25"	10°06'23"	Schneeweiss 300
	I, Bergamasker Alpen	45°58'48"	10°01'28"	Schneeweiss 294
<i>Pedicularis rostratospicata</i>	A, Müritzsteger Alpen	47°41'26"	15°36'12"	Schneeweiss 305
	A, Hochschwabgruppe	47°36'44"	15°10'40"	Schneeweiss 308
	A, Ennstaler Alpen	47°29'21"	14°42'37"	Schneeweiss 309
	A, Steiner Alpen	46°22'28"	14°33'54"	Schneeweiss 311
	A, Totes Gebirge	47°35'57"	14°03'00"	Schneeweiss 306
	SL, Julische Alpen	46°19'44"	13°46'47"	Schneeweiss 316
	A, Salzkammergut-Berge	47°49'2"	13°42'16"	Schneeweiss 310
	A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 307
	A, Ankogelgruppe	47°07'57"	13°21'02"	Schneeweiss 315
	A, Loferer und Leoganger Steinberge	47°33'11"	12°38'52"	Schneeweiss 312
	A, Karwendel	47°22'51"	11°36'11"	Schneeweiss 313
	A, Stubai Alpen	47°03'40"	11°26'39"	Schneeweiss 314
<i>Pedicularis</i>	A, Rottenmanner und Wölzer	47°26'26"	14°24'52"	Schneeweiss 83

<i>aspleniifolia</i>	Tauern			
	A, Schladminger Tauern	47°16'29"	13°38'33"	Schneeweiss 107
	A, Goldberggruppe	46°57'27"	13°01'11"	Schneeweiss 66
	A, Glocknergruppe	47°04'57"	12°46'42"	Schneeweiss 113
	A, Venedigergruppe	46°59'38"	12°14'59"	Schneeweiss 317
	A, Zillertaler Alpen	47°04'49"	11°40'12"	Schneeweiss 286
	A, Stubai Alpen	47°02'24"	11°05'51"	Schneeweiss 285
	CH, Samnaungruppe	46°54'38"	10°22'40"	Schneeweiss 287
<i>Pedicularis rosea</i>	A, Müzzsteger Alpen	47°51'26"	15°36'12"	Schneeweiss 318
	A, Ennstaler Alpen	47°29'30"	14°43'35"	Schneeweiss 381
	A, Karawanken	46°25'53"	14°18'22"	Schneeweiss 328
	A, Gurktaler Alpen	46°52'41"	13°44'49"	Schneeweiss 319
	A, Salzkammergut-Berge	47°49'02"	13°42'16"	Schneeweiss 327
	A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 321
	I, Julische Alpen	46°24'57"	13°29'37"	Schneeweiss 325
	I, Südliche Karnische Alpen	46°26'30"	12°38'04"	Schneeweiss 326
	I, Dolomiten	46°39'58"	12°11'16"	Schneeweiss 324
	I, Dolomiten	46°23'58"	12°03'49"	Schneeweiss 322
	I, Dolomiten	46°36'02"	11°43'54"	Schneeweiss 323
	I, BrentaGruppe	46°11'50"	10°52'56"	Schneeweiss 320
<i>Carex sempervirens</i>	A, Müzzsteger Alpen	47°40'56"	15°36'22"	Schneeweiss 329
	A, Steiner Alpen	46°22'28"	14°33'54"	Schneeweiss 345
	A, Rottenmanner und Wölzer Tauern	47°26'24"	14°25'14"	Schneeweiss 331
	A, Salzkammergut-Berge	47°49'02"	13°42'16"	Schneeweiss 344
	SL, Julische Alpen	46°26'37"	13°38'27"	Schneeweiss 337
	A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 332
	A, Goldberggruppe	46°57'12"	13°01'20"	Schneeweiss 330
	I, Südliche Karnische Alpen	46°26'30"	12°38'04"	Schneeweiss 338
	A, Venedigergruppe	46°59'38"	12°14'59"	Schneeweiss 339
	I, Dolomiten	45°52'19"	11°48'06"	Schneeweiss 334
	A, Rofangebirge und Brandenberger Alpen	47°27'27"	11°47'35"	Schneeweiss 343
	I, Ötztaler Alpen	46°54'06"	11°11'11"	Schneeweiss 333
	I, Gardaseeberge	45°48'42"	10°53'37"	Schneeweiss 335
	A, Lechquellengebirge	47°13'25"	10°06'23"	Schneeweiss 342

<i>Carex ferruginea</i>	I, Bergamasker Alpen	45°58'14"	10°02'27"	Schneeweiss 336
	CH, Plattagruppe	46°35'02"	9°32'33"	Schneeweiss 341
	CH, Tessiner Alpen	46°26'41"	8°30'15"	Schneeweiss 340
	A, Mürzsteger Alpen	47°40'56"	15°36'22"	Schneeweiss 346
	A, Steiner Alpen	46°22'28"	14°33'54"	Schneeweiss 361
	SL, Dinarische Alpen	45°35'12"	14°26'53"	Schneeweiss 383
	A, Gurktaler Alpen	46°52'09"	13°44'56"	Schneeweiss 347
	A, Salzkammergut-Berge	47°49'2"	13°42'16"	Schneeweiss 360
	A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 348
	I, Julische Alpen	46°24'57"	13°29'37"	Schneeweiss 354
	A, Glocknergruppe	47°03'53"	12°46'32"	Schneeweiss 349
	A, Kitzbüheler Alpen	47°28'30"	12°25'40"	Schneeweiss 379
	A, Venedigergruppe	46°59'38"	12°14'59"	Schneeweiss 356
	I, Dolomiten	46°39'58"	12°11'16"	Schneeweiss 353
	A, Rofangebirge und Brandenberger Alpen	47°27'27"	11°47'35"	Schneeweiss 359
	I, Dolomiten	46°30'43"	11°35'06"	Schneeweiss 355
	I, Vizentiner Alpen	45°59'52"	11°29'24"	Schneeweiss 352
	I, Brentagruppe	46°11'50"	10°52'56"	Schneeweiss 351
	A, Lechquellengebirge	47°13'25"	10°06'23"	Schneeweiss 358
<i>Carex fuliginosa</i>	I, Bergamasker Alpen	45°58'14"	10°02'27"	Schneeweiss 350
	CH, Tessiner Alpen	46°26'41"	8°30'15"	Schneeweiss 357
	A, Hochschwabgruppe	47°37'24"	15°09'05"	Schneeweiss 279
	A, Rottenmanner und Wölzer Tauern	47°26'26"	14°24'52"	Schneeweiss 82
	A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 362
	I, Julische Alpen	46°22'36"	13°30'56"	Schneeweiss 284
	A, Goldberggruppe	46°57'27"	13°01'11"	Schneeweiss 71
	A, Glocknergruppe	47°04'57"	12°46'42"	Schneeweiss 114
	A, Karnischer Hauptkamm	46°39'16"	12°41'42"	Schneeweiss 283
	A, Venedigergruppe	46°59'38"	12°14'59"	Schneeweiss 282
	A, Zillertaler Alpen	47°00'47"	11°33'55"	Schneeweiss 280
	I, Bergamasker Alpen	46°03'46"	10°00'06"	Schneeweiss 281
<i>Carex firma</i>	A, Mürzsteger Alpen	47°40'56"	15°36'22"	Schneeweiss 363
	A, Ennstaler Alpen	47°29'30"	14°43'35"	Schneeweiss 382
	A, Steiner Alpen	46°22'28"	14°33'54"	Schneeweiss 376

SL, Snežnik	45°35'12"	14°26'53"	Schneeweiss 384
A, Gurktaler Alpen	46°52'41"	13°44'49"	Schneeweiss 364
A, Salzkammergut-Berge	47°49'02"	13°42'16"	Schneeweiss 375
SL, Julische Alpen	46°26'37"	13°38'27"	Schneeweiss 370
A, Schladminger Tauern	47°17'21"	13°36'42"	Schneeweiss 365
A, Glocknergruppe	47°04'11"	12°46'14"	Schneeweiss 366
I, Südliche Karnische Alpen	46°26'30"	12°38'04"	Schneeweiss 371
A, Kitzbüheler Alpen	47°28'30"	12°25'40"	Schneeweiss 380
A, Lechquellengebirge	47°13'25"	10°6'23"	Schneeweiss 373
I, Dolomiten	46°39'58"	12°11'16"	Schneeweiss 369
I, Dolomiten	46°30'43"	11°35'06"	Schneeweiss 372
I, Vizentiner Alpen	45°59'52"	11°29'24"	Schneeweiss 368
A, Stubai Alpen	47°03'40"	11°26'39"	Schneeweiss 377
I, BrentaGruppe	46°11'50"	10°52'56"	Schneeweiss 367
A, Lechtaler Alpen	47°22'56"	10°49'49"	Schneeweiss 374

¹ A = Austria; CH = Switzerland; I = Italy; SL = Slovenia

² vouchers are deposited at the herbarium of the University of Vienna (WU)

Chapter 2

**An explicit test of Pleistocene survival in peripheral versus
nunatak refugia in two high mountain plant species with
contrasting pollination syndromes**

An explicit test of Pleistocene survival in peripheral versus nunatak refugia in two high mountain plant species with contrasting pollination syndromes

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Abstract

Pleistocene climate fluctuations had profound influence on the biogeographic history of many biota. As large areas in higher latitudes and high mountain ranges were covered by glaciers, biota were forced either to peripheral refugia or to interior refugia (nunataks), but nunatak survival remains controversial as it solely relies on correlative genetic evidence. Here, we test the nunatak hypothesis using two high alpine plant species of contrasting pollination modes (insect-pollinated *Pedicularis aspleniifolia* and wind-pollinated *Carex fuliginosa*) in the European Alps, a geographic model system to study Pleistocene biogeography. Employing the iDDC (integrative distributional, demographic and coalescent) approach, which couples species distribution modelling, spatial and temporal demographic simulation and Approximate Bayesian Computation, we explicitly test three hypotheses of glacial survival: (1) peripheral survival only, (2) nunatak survival only, and (3) nunatak plus peripheral survival. In *P. aspleniifolia* the nunatak plus peripheral survival hypothesis was supported by Bayes Factors ($BF > 14$), whereas in *C. fuliginosa* the peripheral survival only hypothesis could not be unambiguously distinguished from the nunatak plus peripheral survival

hypothesis ($BF < 2$). These results are consistent with current habitat preferences (*P. aspleniifolia* extends to higher elevations) and the potential for genetic swamping (expected to be higher in the wind-pollinated *C. fuliginosa*). Although the persistence of plants on nunataks during glacial periods has been debated and studied over decades, this is one of the first studies to explicitly test the hypothesis instead of solely using correlative evidence.

Keywords: Alps, Pleistocene glaciation, nunataks, peripheral refugia, coalescent simulations

Introduction

Pleistocene climate fluctuations had profound influence on the biogeographic history of many biota (Hewitt, 1996, 2004). During the glacial periods, large areas in higher latitudes and in high mountain ranges were covered by ice sheets. It is of particular interest to identify where plants and animals occurring in formerly glaciated areas managed to survive these periods (Gabrielsen, Bachmann, Jakobsen, & Brochmann, 1997; Schönswetter, Stehlik, Holderegger, & Tribsch, 2005; Wachter et al., 2012). Species might have retreated to unglaciated areas in the, narrower or broader, periphery of the glaciers (peripheral refugia) as supported by fossil data (Birks & Willis, 2008) and by molecular data (Comes & Kadereit, 1998; Schönswetter et al., 2005; Stehlik, 2000; Tollefsrud, Bachmann, Jakobsen, & Brochmann, 1998) for many plant species. Alternatively, species may have survived within the ice shield (interior refugia) on ice-free mountain peaks, so-called nunataks (nunatak survival hypothesis, Schneeweiss & Schönswetter, 2011; Schönswetter et al., 2005; Stehlik, 2000). Due to usually lacking fossil evidence, nunatak survival is essentially inferred from molecular data only (e.g., Lohse, Nicholls, & Stone, 2011; Schönswetter & Schneeweiss, in press; Stehlik, Blattner, Holderegger, & Bachmann, 2002; Stehlik, Schneller, & Bachmann, 2001; Westergaard et al., 2011). The incidence of nunatak survival may, however, be underestimated, especially in species with high gene flow, as during (re-)colonization signals of nunatak survival can be

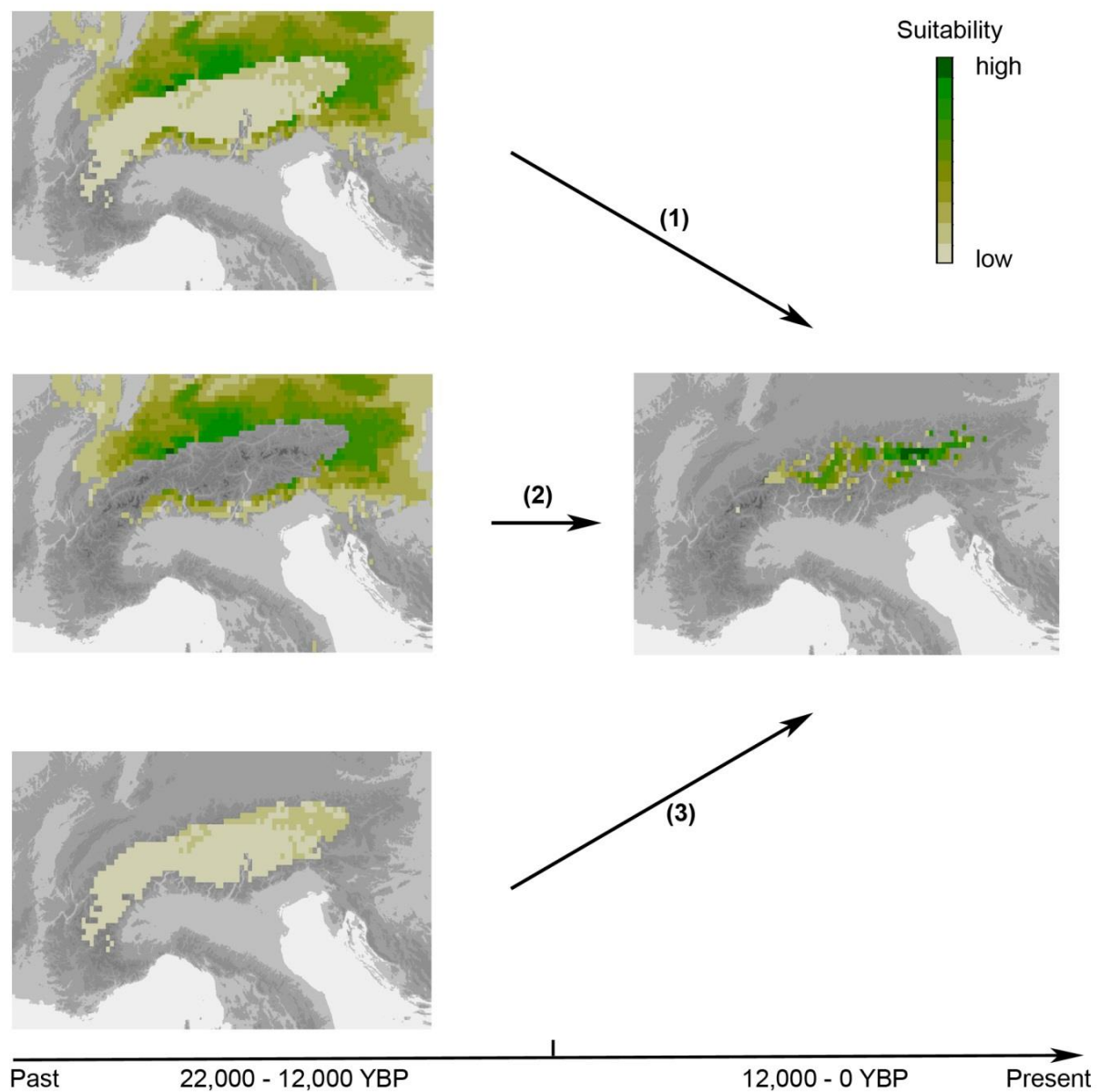
genetically swamped by migrants from peripheral refugia (Gabrielsen et al., 1997; Schneeweiss & Schönswetter, 2011; Tollefsrud et al., 1998). The hypotheses of survival in peripheral versus interior refugia are, however, not mutually exclusive, and for some species both types of refugia were inferred (Escobar García et al., 2012).

The iDDC (integrative Distributional, Demographic and Coalescent modelling) approach provides a powerful framework allowing different glacial survival scenarios to be explicitly tested (Brown & Knowles, 2012; He, Edwards, & Knowles, 2013; He, Prado, & Knowles, 2017; Papadopoulou & Knowles, 2016). Briefly, using demographic models corresponding to the hypotheses to be tested, genetic patterns are simulated under the coalescent. These models, which often are informed by species distribution modelling (SDM), are then evaluated by comparing them to the empirical genetic pattern using an Approximate Bayesian Computation (ABC) framework. In the context of glacial survival, demographic models differ with respect to whether species are allowed to persist in central glaciated and/or peripheral unglaciated areas during the glacial period (Fig. 1).

Here, glacial survival patterns of two plant species, *Pedicularis aspleniifolia* and *Carex fuliginosa*, were investigated in the European Alps, a geographic model system to study Pleistocene range shifts (Escobar García et al., 2012; Lohse et al., 2011; Schönswetter et al., 2005). Both species are perennial herbs found exclusively in the alpine and, particularly *P. aspleniifolia*, in the subnival zone. As species that can cope with cold harsh environments are likely to be able to survive in extreme habitats like nunataks (Lohse et al., 2011; Stehlik et al., 2002), they are excellent candidates to test glacial survival hypotheses. In addition, their current distribution ranges encompass both areas situated in formerly glaciated regions, where they may have survived on nunataks, and areas outside the former ice-sheet, where they may have survived in peripheral refugia (Fig. 2). The study species do, however, differ in their pollination systems. Specifically, *P. aspleniifolia* is insect-pollinated and *C.*

fuliginosa is wind-pollinated. As gene flow is expected to be stronger in wind-pollinated species (Govindaraju, 1988), *C. fuliginosa* may be more prone to genetic swamping than *P. aspleniifolia*. Using RAD sequencing data analyzed with the iDDC approach, we test three glacial survival scenarios identified previously, i.e., peripheral survival only, nunatak survival only, and nunatak plus peripheral survival (e.g., Escobar García et al., 2012; Schönswetter, Tribsch, Stehlik, & Niklfeld, 2004; Stehlik et al., 2002).

Fig. 1 Schematic of the three glacial survival scenarios used in the simulations. Suitability of cells derived from species distribution modelling (SDM) for the Last Glacial Maximum (22,000–12,000 years before present [YBP]) were modified to comply with the different glacial survival scenarios (1) nunatak plus peripheral survival, (2) peripheral survival only and (3) nunatak survival only (see text for details); suitabilities for the postglacial (12,000–0 YBP) were taken from the SDM for the present. Grey cells represent unsuitable areas of different altitude.



Materials and methods

Molecular data generation

Leaf material of 18 *Carex fuliginosa* and 14 *Pedicularis aspleniifolia* individuals was collected from nine and seven populations, respectively, across the species' entire distributional ranges in the eastern Alps (Table 1, Fig. 2). Leaf material was stored in silica gel. DNA extractions were performed following Jang et al. (2013). Single enzyme (Pst1) digested RAD libraries (Baird et al., 2008) were constructed using the protocol described in Paun et al. (2015), and sequenced on the Illumina HiSeq2000 platform in Vienna Biocenter Core Facilities (<https://www.vbcf.ac.at>).

Table 1 Collection information of the study species.

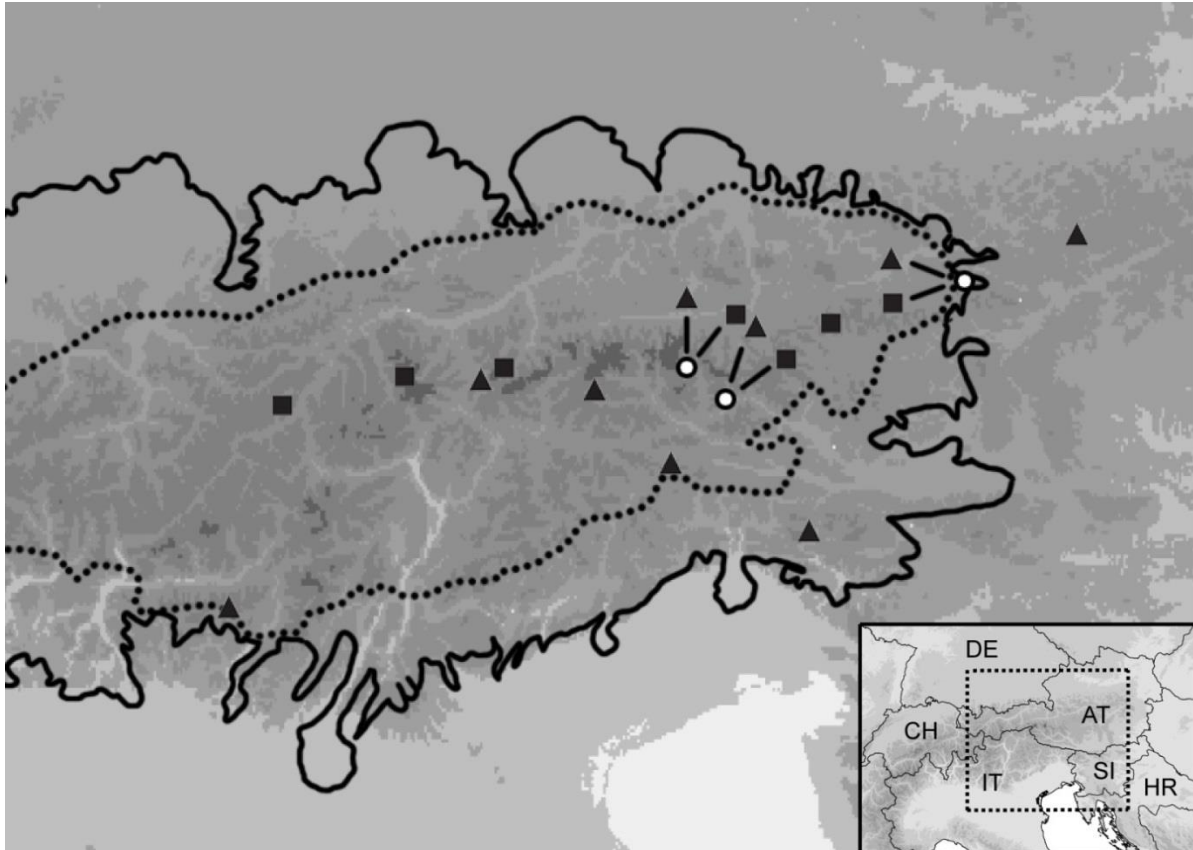
Species	Region ¹	Coordinates ²	Voucher number ³
<i>P. aspleniifolia</i>	A, Rottenmanner und Wölzer Tauern	47°26'/14°25'	Schneeweiss 83
	A, Schladminger Tauern	47°16'/13°38'	Schneeweiss 107
	A, Goldberggruppe	46°57'/13°01'	Schneeweiss 66
	A, Glocknergruppe	47°04'/12°46'	Schneeweiss 113
	A, Zillertaler Alpen	47°05'/11°39'	Schneeweiss 286
	A, Stubai Alpen	47°02'/11°05'	Schneeweiss 285
	CH, Samnaungruppe	46°54'/10°22'	Schneeweiss 287
<i>C. fuliginosa</i>	A, Hochschwabgruppe	47°36'/15°10'	Schneeweiss 279
	A, Rottenmanner und Wölzer Tauern	47°26'/14°25'	Schneeweiss 82
	I, Julische Alpen	46°22'/13°30'	Schneeweiss 284
	A, Goldberggruppe	46°57'/13°01'	Schneeweiss 71
	A, Glocknergruppe	47°04'/12°46'	Schneeweiss 114
	A, Karnischer Hauptkamm	46°38'/12°42'	Schneeweiss 283
	A, Venedigergruppe	46°59'/12°14'	Schneeweiss 282
	A, Zillertaler Alpen	47°00'/11°33'	Schneeweiss 280
	I, Bergamasker Alpen	46°03'/10°00'	Schneeweiss 281

¹ A = Austria; CH = Switzerland; I = Italy

² latitude/longitude

³ vouchers deposited in the herbarium of the University of Vienna (WU)

Fig. 2 Sampled populations of *Pedicularis aspleniifolia* (squares) and *Carex fuliginosa* (triangles). The dashed line indicates the snowline (i.e., altitude above which snow does not melt in climatically average years) during the Last Glacial Maximum (LGM), the solid line indicates the maximum extent of the ice-sheet during the LGM. The right insert shows the position of the study area.



Raw reads were de-multiplexed by allowing for a single mismatch at the barcodes using Illumina2Bam (<https://github.com/gq1/illumina2bam>) and STACKS 1.44 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). Reads with low quality scores (< 10) were discarded. SNP calling was conducted employing the `denovo_map.pl` pipeline in STACKS (Catchen et al., 2013) with default settings except that the minimum number of identical reads required to build stacks (-m) was set to 5, the number of mismatches allowed to merge loci to catalogs (-n) was set to 2, and the upper bound of error rate (--bound_high) was set to 0.01.

iDDC approach

Species occurrence data were obtained from the GBIF database (<https://www.gbif.org/>), the project “Mapping the flora of Austria” (H. Niklfeld & L. Ehrendorfer, University of Vienna, unpubl. data) and the European Vegetation Archive (EVA; Chytrý et al., 2016). Distributions of the two species were modelled for both, the present and the last glacial maximum (LGM) period. Nineteen bio-climate variables representing current and past (LGM) climatic conditions were downloaded from the Worldclim database (<http://www.worldclim.org/>, Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) at a 2.5 arc minutes resolution. Eight bio-climate variables were retained for further analyses (annual mean temperature, temperature seasonality, mean temperature of wettest quarter, mean temperature of driest quarter, annual precipitation, precipitation seasonality, precipitation of warmest quarter, precipitation of coldest quarter) after removing highly correlated (> 0.7) variables. SDMs were calibrated by linking these climatic data to the species occurrence data using the ensemble modelling approach implemented in the package “Biomod2” (Thuiller, Georges, Engler, & Breiner, 2016) of R (R Core Team, 2013). Thereby, we selected six models, including Generalized Linear Model (GLM), Generalized Boosting Model (GBM), Generalized Additive Model (GAM), Classification Tree Analysis (CTA), Artificial Neural Network (ANN), Random Forest (RF). To evaluate model quality for each species and modelling technique, the available occurrence data was randomly split into one part for calibrating the models (80%) and the remaining data for evaluating them (20%). To avoid random effects of splitting, we repeated this procedure ten times. Only models with relative operating characteristic (ROC) values (Swets, 1988) > 0.75 were used to subsequently generate ensemble projections of potential species distribution under current climate and under climatic conditions corresponding to the LGM. Ensemble predictions were defined as the means of projected

occurrence probabilities of single models. Pseudo-absence data were randomly generated with prevalence equal to 0.5 with ten replicates.

In order to render the subsequent demographic modelling computationally feasible, we up-scaled the cell sizes of the SDMs from the original 2.5×2.5 arc minutes to 7.5×7.5 arc minutes (i.e., merging nine cells resulting in a cell covering ca. 192 km²) using ArcGIS 9 (ESRI, Redlands, California, USA). Values for these larger cells were calculated as the mean value from the nine smaller cells.

Habitat suitabilities at the LGM were modified according to the three main scenarios (Fig. 1). Specifically, in the peripheral survival only scenario (Peri), areas within the glacial snow-line were considered totally uninhabitable (i.e. suitability was set to zero). The location of the ancestral population was either in the ice-free eastern part (Peri_{East} scenario) or in the ice-free southern part (Peri_{South} scenario) of the Alps. In the nunatak survival only scenario (Nun), suitabilities in glaciated areas were reduced by 85% (as in Massatti & Knowles, 2016) and those in ice-free areas were set to zero, respectively; an ancestral population in the central glaciated area of the Alps was used. In the nunatak plus peripheral survival scenario (Nun+Peri), the suitability of cells within the glacial snow-line (Schönswetter et al., 2005) was decreased by 85%. The single ancestral population (i.e., the geographic starting point for the demographic modelling) was located either in the ice-free eastern part (Nun+Peri_{East} scenario), the ice-free southern part (Nun+Peri_{South} scenario), or in the central glaciated part of the Alps (Nun_{Central}+Peri scenario); using two ancestral populations was computationally not feasible with the available resources.

For each scenario, 10⁶ demographic simulations were performed in SPLATCHE 2.01 (Ray, Currat, Foll, & Excoffier, 2010). Demographic parameters, including migration rate m , maximum carrying capacity k , and population size of ancestral population N_{anc} , were drawn

from uniform priors through ABCtoolbox 2.0 Beta (<https://bitbucket.org/phaentu/abctoolbox-public/>, Wegmann, Leuenberger, Neuenschwander, & Excoffier, 2010); specifically, the priors were $m \sim U(0.01, 0.5)$, $k \sim U(1 \times 10^3, 2 \times 10^4)$, $N_{anc} \sim U(2 \times 10^3, 5 \times 10^6)$ for ancestral population located in a peripheral area and $N_{anc} \sim U(5 \times 10^2, 5 \times 10^4)$ for ancestral population located in the glaciated central Alps, where populations are expected to have been smaller. The generation times of both species were set to 40 years, following a re-scaling approach (the actual generation times very probably are considerably shorter) similar to the one used by Massatti and Knowles (2016). Cells with suitability less than 10% of the maximum suitability were treated as totally unsuitable (i.e., their suitabilities were set to zero) to remove non-zero, though minuscule, suitabilities mostly outside the mountain ranges (Fig. 3). Subsequently, habitat suitabilities derived from the SDMs larger than zero were classified into 10 categories in increments of 10% of the maximum suitability found in the particular species using a modified python script from X-ORIGIN (He et al., 2017). The carrying capacity of each cell was scaled according to its suitability. From generation 1 (22 kya) to 250 (12 kya), corresponding to the glacial period, demographic modelling used the modified SDM predictions for the LGM, as described above, whereas for generations 251 to 550 (present time) the modelling used the SDM predictions based on the current climate.

Genetic data sets matching the dimensions of the empirical data set were simulated on each of the 10^6 demographic simulations. For both simulated and empirical data sets, summary statistics were calculated in ARLEQUIN 3.5 (Excoffier & Lischer, 2010), including mean number of alleles over loci for each population, mean number of alleles over loci and population, mean heterozygosity over loci for each population, mean heterozygosity over loci and population, mean total heterozygosity, global F_{is} , global F_{st} and pairwise population F_{st} . A total of 40 and 59 summary statistics were computed for *P. aspleniifolia* and *C. fuliginosa*,

respectively. These numbers differ because of the different number of populations analysed for the two species.

To identify the best supported scenario we employed Approximate Bayesian Computation with ABCtoolbox 2.0 Beta (Wegmann et al., 2010). Instead of using all computed summary statistics directly, we converted summary statistics to Partial Least Squares (PLS) components using the R package “pls” (Mevik & Wehrens, 2007) with Box-Cox treatment (Box & Cox, 1964). The number of PLS components to be used was determined based on the Root Mean Squared Error (RMSE) plots. For each scenario, 5,000 (0.5 %) simulated genetic data sets that are closest to the empirical data set were retained for parameter estimation and model selection. A post-sampling regression adjustment was applied using General Linear Models (Leuenberger & Wegmann, 2010). Marginal densities were used to evaluate models. For validation, *P*-values were calculated to check if the models are capable to generate the empirical data (Wegmann et al., 2010). Additionally, we checked whether parameter estimations are unbiased using 1,000 pseudo-observations; a uniform distribution of posterior quantiles is expected if estimation of the parameter is unbiased (Wegmann et al., 2010).

Results

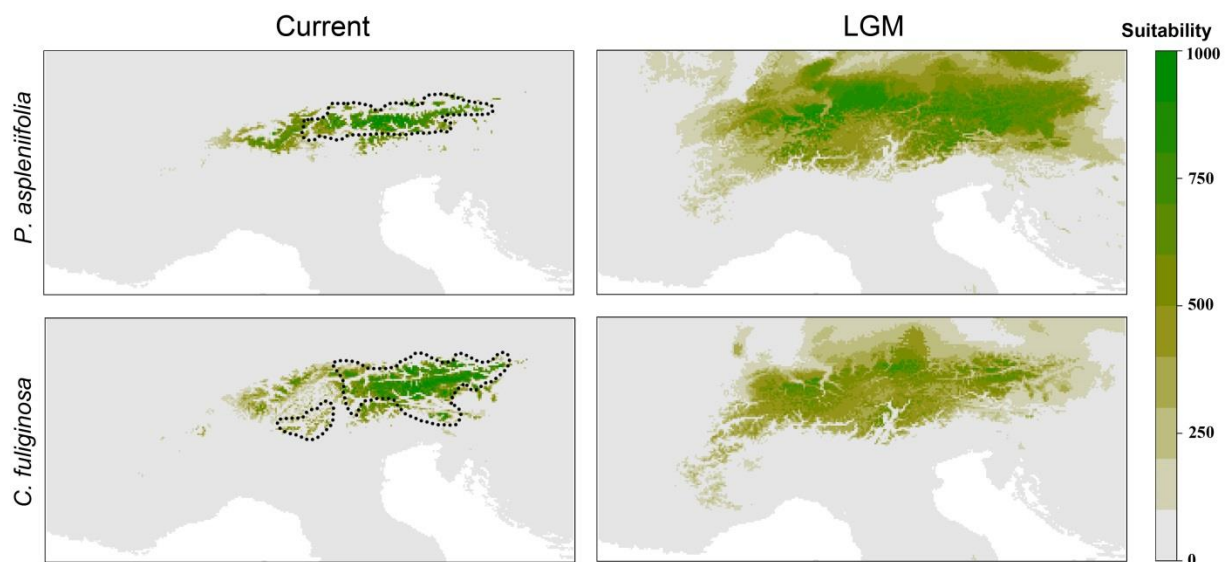
RAD sequencing

After de-multiplexing and quality filtering, more than 900,000 reads were obtained per individual. We further filtered out loci containing missing data or more than 4 SNPs. Only one SNP per locus was used for further analysis to avoid any linkage disequilibrium. The final data sets contained 5,504 SNPs for *P. aspleniifolia* and 4,976 SNPs for *C. fuliginosa* (available on Dryad at <https://datadryad.org/review?doi=doi:10.5061/dryad.p7p0j5m>).

Model evaluation

Based on current climate data, SDM predicted suitable areas for *P. aspleniifolia* and *C. fuliginosa* that were mostly congruent with their current distribution ranges (Fig. 3). According to the projections at LGM conditions, major parts of the Alps as well as peripheral areas (mostly adjacent mid-elevation mountain ranges) were suitable for *P. aspleniifolia* and, to a lesser extent, also for *C. fuliginosa* (Fig. 3).

Fig. 3 Projected suitabilities of *Pedicularis aspleniifolia* and of *Carex fuliginosa* under current climate conditions and under climate conditions of the Last Glacial Maximum. The dashed lines indicate the current distribution ranges of the two species.



Based on the RMSE plots (Fig. S1), 4–7 PLS components were retained for calculating the distance between simulated and empirical data sets. In *P. aspleniifolia*, the nunatak plus peripheral survival scenario with the ancestral population located in the eastern Alps (Nun+Peri_{East}) best explained the empirical genetic pattern, followed by the peripheral survival only scenario with the ancestral population located in the southern Alps (Peri_{South}; BF = 14.30; Table 2). All remaining scenarios were clearly rejected (BF > 90). In accordance, the Nun+Peri_{East} scenario better reproduced the empirical data (P -value = 0.987) compared to all alternative scenarios (P -value ≤ 0.065). In *C. fuliginosa*, the best supported model was the

peripheral survival only scenario with the ancestral population located in the eastern Alps (Peri_{East}; Table 2), followed by the nunatak plus peripheral survival scenario with the ancestral population located in the eastern Alps (Nun+Peri_{East}; BF = 1.97). For these models, *P*-values were 0.486 and 0.112, respectively (Table 2). The remaining scenarios were clearly rejected (BF > 60) and had *P*-values ≤ 0.034. In both species, prior distributions of parameter estimates in the best supported models were distinct from the posterior distribution (Fig. S2), indicating that the data have power to estimate parameters. Parameter estimates were not unbiased, as posterior quantiles of all parameters departed from a uniform distribution (Kolmogorov–Smirnov test, Fig. S3).

Table 2 Comparison of Pleistocene survival scenarios of the study species.

Species	Model ¹	Marginal density	Bayes factor	<i>P</i> -value
<i>P. aspleniifolia</i>	Nun	9.73×10 ⁻²⁰	>100	<0.001
	Nun+Peri _{East}	1.07×10 ⁻⁴	0	0.987
	Nun+Peri _{South}	3.08×10 ⁻⁸	>100	0.002
	Nun _{Central} +Peri	2.63×10 ⁻¹³	>100	<0.001
	Peri _{East}	1.11×10 ⁻⁶	96.39	0.031
	Peri _{South}	7.48×10 ⁻⁶	14.30	0.065
<i>C. fuliginosa</i>	Nun	4.28×10 ⁻⁹⁸	>100	<0.001
	Nun+ Peri _{East}	2.78×10 ⁻⁵	1.97	0.112
	Nun+ Peri _{South}	5.79×10 ⁻⁷	94.65	0.034
	Nun _{Central} +Peri	6.85×10 ⁻¹¹⁵	>100	<0.001
	Peri _{East}	5.48×10 ⁻⁵	0	0.486
	Peri _{South}	7.84×10 ⁻⁷	69.89	0.023

¹ Nun, nunatak survival, the index indicating (where necessary) the location of the ancestral population (Central, central Alps); Peri, peripheral survival, the index indicating (where necessary) the location of the ancestral population (East, eastern Alps; South, southern Alps).

Discussion

Concerning the debate whether nunatak survival does matter, the answer may be species-specific rather than universal (Gabrielsen et al., 1997; Tollefsrud et al., 1998; Wachter et al., 2016; Westergaard et al., 2011). Species traits affecting, for instance, dispersal capabilities shape current genetic patterns both through glacial survival *per se* (via, for instance, genetic bottlenecks; Schönswetter, Paun, Tribsch, & Niklfeld, 2003; Wachter et al., 2012) and through post-glacial recolonization (via, for instance, gene flow or long distance dispersal; Paun, Schönswetter, Winkler, IntraBioDiv Consortium, & Tribsch, 2008; Schönswetter, Tribsch, Barfuss, & Niklfeld, 2002). As shown in this study, although *P. aspleniifolia* and *C. fuliginosa* have similar habitat preferences and current distribution ranges, unambiguous evidence for nunatak survival was only found in *P. aspleniifolia*.

In *P. aspleniifolia*, both nunatak and peripheral areas appear to have acted as refugia during the LGM. This is evident from the support for the nunatak plus peripheral survival scenario with the ancestral population located in the eastern Alps (Nun+Peri_{East}; Table 2), an area that acted as glacial refugium also for other alpine plants (Schönswetter et al., 2005). Nunatak and peripheral survival were shown to jointly contribute to current genetic patterns in some high alpine plants (Escobar García et al., 2012; Schneeweiss & Schönswetter, 2011; Schönswetter & Schneeweiss, in press), and this appears also to be the case in *P. aspleniifolia*.

In contrast, in *C. fuliginosa*, only peripheral areas in the easternmost Alps could be unambiguously confirmed as refugia, although based on Bayes Factors a nunatak survival cannot be ruled out (Table 2). A lack of nunatak survival in *Carex fuliginosa* would agree with a, compared to *P. aspleniifolia*, lower tolerance against harsh climate conditions expected to have occurred at Pleistocene nunataks. Such a lower tolerance is suggested by the current altitudinal distributions, as *C. fuliginosa* is restricted to the alpine zone (i.e., the zone

with closed swards) whereas *P. aspleniifolia* frequently extends into the subnival zone (i.e., the zone with open vegetation). Alternatively, however, traces of nunatak survival may have been genetically swamped (Gabrielsen et al., 1997; Tollefsrud et al., 1998) after (re-)colonization following deglaciation and subsequent gene flow between immigrants from peripheral refugia and *in situ* inhabitants in *C. fuliginosa*. The potential for genetic swamping is expected to be higher in wind-pollinated species such as *C. fuliginosa* than in insect-pollinated species such as *P. aspleniifolia*. Wind-pollination has been shown to mediate postglacial gene flow among refugia (Liepelt, Bialozyt, & Ziegenhagen, 2002). For taxa prone to genetic swamping, neither a correlative genetic approach nor a modelling approach as used here may allow to detect nunatak survival, even if based on genomic data such as RAD-seq data. In those cases, valuable information may be obtained by using mostly uniparentally inherited markers not prone to homogenization, as is the case for plastid or mitochondrial sequences (Schönswetter & Schneeweiss, in press).

As with any ABC approach, model validation is essential, because ABC will always produce posterior distributions independent of model quality (Bertorelle, Benazzo, & Mona, 2010; Wegmann et al., 2010). In our case, compared to alternative models, the most supported model had higher probability of generating data similar to the empirical one (high *P*-values) than the alternative models, indicating that the model evaluation results are robust. The posterior quantiles from pseudo-observations of all estimated parameters showed departure from a uniform distribution (Fig. S3), suggesting that they are estimated inaccurately and that their biological interpretation should be avoided (Wegmann et al., 2010). However, in this study, we were not interested in the specific parameter values, as our primary objective was to distinguish alternative glacial survival scenarios. We acknowledge that testing more refined scenarios would be desirable, but there will be data-imposed limits. In any case, testing simple models does not compromise the biological insights from our study, which is

whether populations surviving on nunataks within the ice-sheet during glaciation period contributed to the history of alpine plant species.

Conclusion

In phylogeographic studies, multiple demographic histories may lead to similar genetic patterns. For example, high genetic diversity may be the outcome of either secondary contact or of temporally stable populations (Nettel, Dodd, Afzal-Rafii, & Tovilla-Hernández, 2008; Ursenbacher et al., 2008), or both geographic isolation and founder events might generate high genetic differentiation between populations (Gugerli et al., 2001; Schönswetter, Popp, & Brochmann, 2006). Using model-based approaches as applied here allows the genetic pattern to be explicitly linked to phylogeographic history of species (He et al., 2013; Massatti & Knowles, 2016). Thus, we could unambiguously demonstrate nunatak survival within the heavily glaciated central Alps in *P. aspleniifolia*. Although the persistence of plants on nunataks during glacial periods has been debated and studied over decades (Gabrielsen et al., 1997; Schneeweiss & Schönswetter, 2011; Tollefsrud et al., 1998; Westergaard et al., 2011, in press), this is one of the first studies to explicitly test the hypothesis instead of solely using correlative evidence.

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Data accessibility

Genetic data (SNP data) for *Pedicularis aspleniifolia* and *Carex fuliginosa* are available at Dryad (<https://datadryad.org/review?doi=doi:10.5061/dryad.p7p0j5m>).

Author contributions

D.P. and G.S. conceived the study; D.P. and G.S. collected specimens; W.W. provided distribution data; K.H. advised on SDM methods; D.P. conducted laboratory work and data analyses; D.P., K.H., W.W. and G.S. wrote the manuscript.

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Fig. S1 Change in Root Mean Square Error with increasing number of Partial Least Squares (PLS) components for the most supported model in (A) *Pedicularis aspleniifolia* (nunatak plus peripheral survival model with the ancestral population located in the eastern Alps) and (B) *Carex fuliginosa* (peripheral survival only model with the ancestral population located in the eastern Alps). Nanc = population size of the ancestral population; k = maximum carrying capacity; m = migration rate.

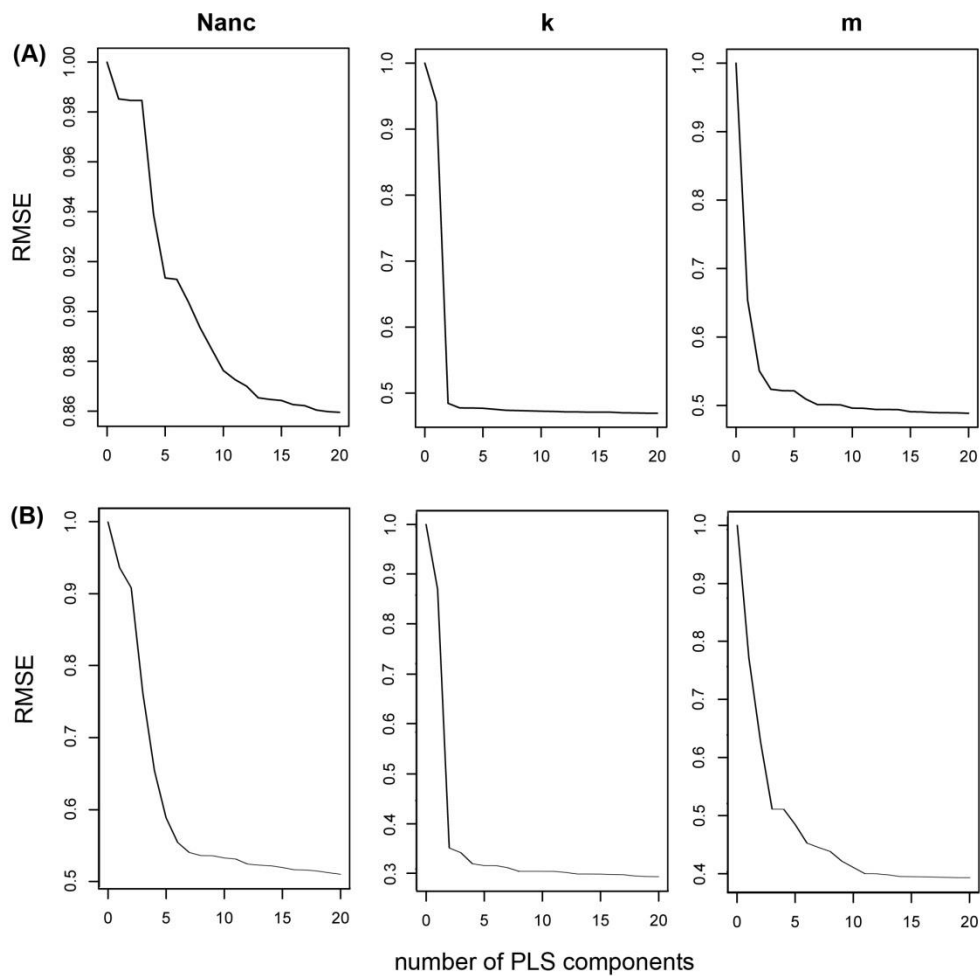


Fig. S2 Prior distributions (black line) and posterior distributions (grey line) of parameter estimates in (A) *Pedicularis aspleniifolia* (nunatak plus peripheral survival model with the ancestral population located in the eastern Alps) and (B) *Carex fuliginosa* (peripheral survival only model with the ancestral population located in the eastern Alps). Nanc = population size of the ancestral population; k = maximum carrying capacity; m = migration rate.

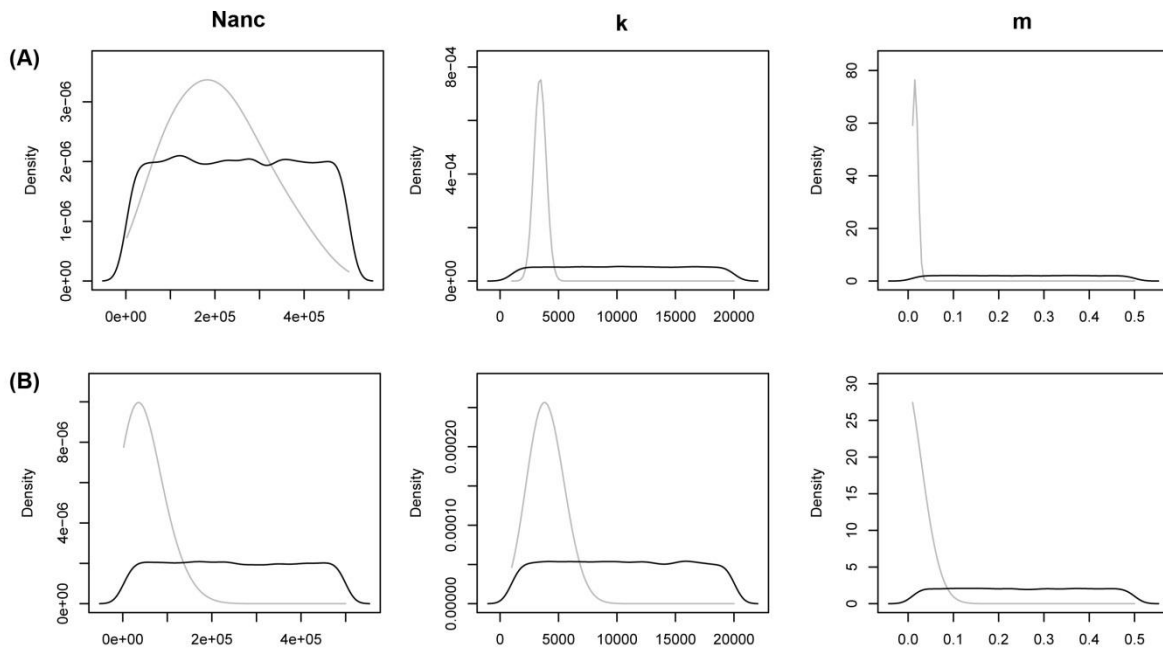
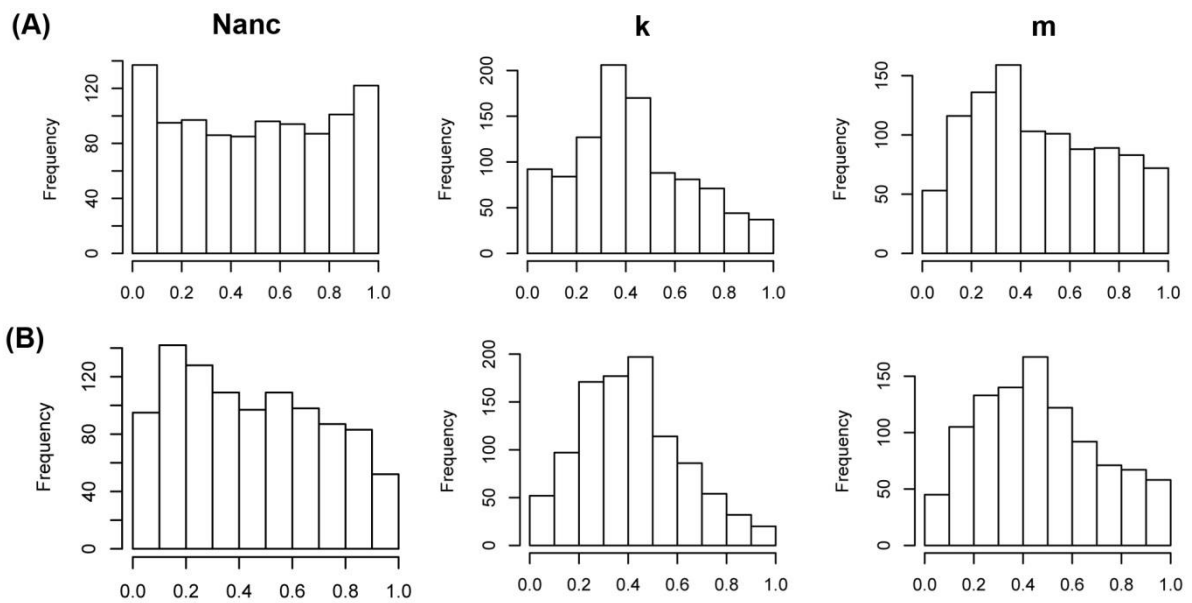


Fig. S3 Distribution of posterior quantiles of parameters for (A) *Pedicularis aspleniifolia* (nunatak plus peripheral survival model with the ancestral population located in the eastern Alps) and (B) *Carex fuliginosa* (peripheral survival only model with the ancestral population located in the eastern Alps). Departures from a uniform distribution were tested with a Kolmogorov-Smirnov test (p -values <0.001 in all six tests). Nanc = population size of the ancestral population; k = maximum carrying capacity; m = migration rate.



Chapter 3

Ancestral remnants or peripheral segregates? Phylogenetic relationships of two narrowly endemic *Euphrasia* species (Orobanchaceae) from the eastern European Alps



Ancestral remnants or peripheral segregates? Phylogenetic relationships of two narrowly endemic *Euphrasia* species (Orobanchaceae) from the eastern European Alps

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Abstract. Endemism in mountain ranges is considered to be the result of a number of factors, including restriction to refugia during Pleistocene climate fluctuations. However, isolation in glacial refugia cannot explain the origin of narrowly endemic taxa restricted to formerly heavily glaciated areas. Here, we investigate the phylogeny of two narrowly endemic species, *Euphrasia inopinata* and *E. sinuata* (Orobanchaceae), found exclusively in formerly heavily glaciated areas of the eastern European Alps. As both species are diploid and very similar to the widespread (allo)polyploid *E. minima*, we test whether the restricted distributions of *E. inopinata* and *E. sinuata* are relictual, i.e. the two species are ancestral diploid remnants of a polyploid complex, or whether they are derived, i.e. the two species are peripheral segregates of a more widespread diploid. Based on internal transcribed spacer (ITS) sequence and amplified fragment length polymorphism (AFLP) fingerprint data it is shown that *E. inopinata* and *E. sinuata*, whose diploid ploidy level is confirmed for all analysed individuals via flow cytometry, are phylogenetically closely related to diploid *E. alpina* s. l. (series *Alpinae*) instead of *E. minima* (series *Parviflorae*). In addition, there is no evidence that these two diploid species participated in the formation of allotetraploid *E. minima*. Thus, *E. inopinata* and *E. sinuata* are interpreted as peripheral segregates of the widespread *E. alpina* s. l. Shifts in pollination system from allogamy in *E. alpina* s. l. to autogamy in *E. inopinata* and *E. sinuata*, genetic drift in small populations and geographic isolation at the periphery of the range of *E. alpina* s. str. probably contributed to the morphological and ecological differentiation of *E. inopinata* and *E. sinuata*.

Keywords: Alps; endemics; *Euphrasia*; peripheral segregate; polyploidy.

Introduction

Mountain ranges host a considerable number of endemic species (Barthlott et al. 1996; Körner 2003), which is

considered to be the result of the complex origin and histories of mountain ranges, being shaped by geographic isolation, climate changes and strong microhabitat

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differentiation (Körner 1995; Agakhanjanz and Breckle 1995). Distribution areas of narrowly endemic species, for instance in the European Alps (hereinafter simply referred to as Alps), often coincide with Pleistocene refugia (Tribsch and Schönswetter 2003; Tribsch 2004), suggesting that Pleistocene climate fluctuations had major impacts on the biogeography of endemic taxa. For narrowly endemic taxa restricted to formerly heavily glaciated areas, however, alternative explanations have to be sought. These include in situ survival on nunataks, survival in periglacial areas and extirpation in those source areas due to postglacial environmental changes, or rapid in situ speciation of postglacial (re)colonizers (Kolář *et al.* 2013).

Members of the genus *Euphrasia* (Orobanchaceae) from the eastern Alps form a group well suited to study hypotheses on the origin of narrowly distributed species in formerly strongly glaciated areas. Apart from several widely distributed species, *Euphrasia* in this region includes two locally endemic species, *E. inopinata* restricted to a few populations in the central Alps (Ötztal Alps, Tyrol) and *E. sinuata* found in two disjoint areas in the northern calcareous Alps (Rofan Mountains, Tyrol) and in the central Alps (Kitzbühler Alps, Tyrol; Fig. 1; Ehrendorfer and Vitek 1984). Both species are

found in the subalpine and lower alpine zone, but differ edaphically as *E. inopinata* occurs on siliceous substrate, whereas *E. sinuata* grows on limestone and dolomites. As the current distribution areas of these species are not associated with any peripheral glacial refugium (Schönswetter *et al.* 2005), Ehrendorfer and Vitek (1984) suggested nunatak survival during the last glacial period for both species.

Both *E. inopinata* and *E. sinuata* are taxonomically assigned to series *Parviflorae* (Ehrendorfer and Vitek 1984), which otherwise in the study region only includes tetraploids (Yeo 1978). Thus, the two diploid species might actually be relics of formerly more widely distributed diploids that have been largely replaced by tetraploids, i.e. in the Alps by the morphologically and ecologically extremely plastic *E. minima* (Vitek 1998). Based on morphological data, it has been suggested that the diploids *E. inopinata* and *E. sinuata* were involved in the formation of *E. minima*, a presumable allopolyploid (Yeo 1978), either directly as one of the parents (Ehrendorfer and Vitek 1984) or indirectly via introgression into an allotetraploid derivative of *E. alpina* subsp. *christii* (from series *Alpinae*) and *E. hirtella* (from series *Euphrasia*, formerly, nomenclaturally incorrectly, named series *Grandiflorae*), transferring the character of small flowers to *E. minima* (Vitek 1986).

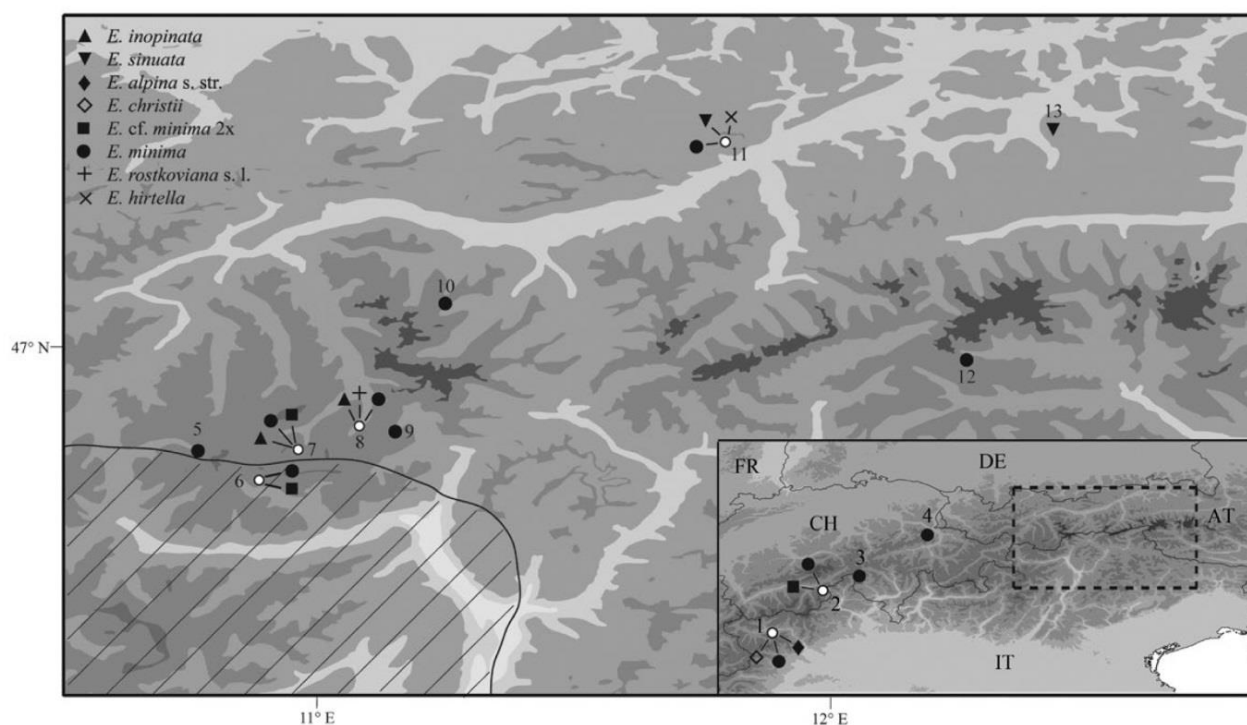


Figure 1. Locations of sampled populations of the investigated *Euphrasia* species (see Table 1 and Supporting Information—Table S1 for detailed information). The hatched area indicates the north-eastern edge of the distribution range of *Euphrasia alpina* s. str. (Vitek 1985, modified using data from <http://florafauna.it/>). The insert shows sampled locations in the western Alps as well as the position of the sampling area shown in the main map. Country abbreviations: AT, Austria; CH, Switzerland; DE, Germany; FR, France; IT, Italy.

An alternative hypothesis is that *E. inopinata* and *E. sinuata* are the result of the evolution of high-elevation dwarf forms from other large-flowered diploids, such as *E. alpina* subsp. *alpina* (series *Alpinae*; Fig. 7 in Vitek 1986). As morphological traits in *Euphrasia* are, however, variable and ecologically convergent (Yeo 1978; Vitek 1998; Twyford et al. 2018), hypotheses derived on the basis of phenotypic comparison can be potentially misleading. Hence, for elucidating the taxonomic position and evolution of *E. inopinata* and *E. sinuata* molecular data are needed.

Here we study phylogenetic relationships of the two narrow endemics *E. inopinata* and *E. sinuata* in the eastern Alps. To this end, we use nuclear DNA sequences from the internal transcribed spacer (ITS) of the rRNA operon as well as the amplified fragment length polymorphism (AFLP) fingerprinting method (Vos et al. 1995). Nuclear ITS has been recently used for DNA barcoding of British *Euphrasia* taxa (Wang et al. 2018), and although not resolving at the species level ITS sequences allow series *Euphrasia* and *Alpinae* to be unambiguously distinguished. Amplified fragment length polymorphisms have several advantages, including whole genome coverage, bi-parental inheritance and independence from prior sequence information, and have also been widely used for polyploid plants (e.g. Hedrén et al. 2001; Guo et al. 2005; Dixon et al. 2009; Pachschröck et al. 2015; Winkler et al. 2017). Specifically, we want to test the hypotheses (i) that *E. inopinata* and/or *E. sinuata* are parental taxa of the allotetraploid *E. minima* (i.e. an origin of *E. minima* from *E. inopinata*/*E. sinuata* × species from series *Euphrasia*, specifically *E. officinalis* or *E. hirtella*) and (ii) that they are segregates from more widespread diploid species not involved in the origin of *E. minima*, which instead might have originated from a crossing between *E. alpina* subsp. *christii* and *E. hirtella*.

Methods

Studied species and sampling

None of the investigated taxa is protected and none of the sampled populations is located in protected areas. Whole individuals from *E. inopinata*, *E. sinuata*, *E. alpina* subsp. *alpina*, *E. alpina* subsp. *christii* (the latter two jointly referred to as *E. alpina* s. l.), *E. officinalis* subsp. *rostkoviana*, *E. officinalis* subsp. *picta* (the latter two jointly referred to as *E. officinalis* s. l.) and *E. hirtella* were collected and stored in silica gel during 2015 and 2016 (Fig. 1; Table 1; see Supporting Information—Table S1). Per sampling site, whose size did not exceed 1 m², 1–18 individuals were collected. As *E. inopinata* and *E. sinuata* are consistently very small in all parts (Ehrendorfer and Vitek 1984) and may be confused with small-flowered

forms of *E. minima*, we explicitly targeted individuals with relatively small flower size to increase the chance to recover diploids. Herbarium vouchers are deposited in the Natural History Museum Vienna (W) and the University of Vienna (WU); however, due to the dwarfish size of many of the sampled individuals, after genome size measurement and AFLP fingerprinting no material was left to be used as herbarium voucher; for those photographic images are provided as vouchers [see Supporting Information—Table S1].

Ploidy level determination

Flow cytometry (FCM) of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei was used for estimation of relative DNA content (Suda and Trávníček 2006) from silica gel-dried samples. Similar amounts of desiccated sample and fresh internal reference standard were combined in a Petri dish containing 0.5 mL of cold (5–10 °C) Otto I buffer (0.1 M citric acid, 0.5 % Tween 20) and chopped with a razor blade. *Bellis perennis* (2C = 3.38 pg; Schönschetter et al. 2007) was selected as primary reference standard (Doležel et al. 1998). After filtration through a 42 µm nylon mesh, samples were stained for 10 min at room temperature in a solution containing 1 mL of Otto II buffer (0.4 M Na₂HPO₄·12H₂O, 2-mercaptoethanol and DAPI at final concentrations of 4 µg mL⁻¹). The relative fluorescence intensity of 3000 particles was recorded using a Partec PA II flow cytometer (Partec, Münster, Germany) equipped with an HBO mercury arc lamp after incubation for 5 min at room temperature. If the coefficient of variation (CV) of the G0/G1 peak of a sample exceeded the 5 % threshold, the analysis was discarded and the sample re-measured.

DNA sequencing and AFLP fingerprinting

Total genomic DNA was extracted from similar amounts of silica-dried tissue (ca. 5 mg) applying a CTAB protocol (Doyle and Doyle 1987) with modifications (Jang et al. 2013). Internal transcribed spacer sequences were amplified using the primers ITS4 and ITS5 from White et al. (1990). The PCR reaction mix (10 µL in total) contained 5 µL ReddyMix PCR Master Mix (Thermo Fisher Scientific, Vienna, Austria), 1.6 µL Trehalose Dihydrate (1 M; Sigma, Vienna, Austria), 0.1 µL bovine serum albumin (BSA) (20 mg mL⁻¹, Thermo Fisher Scientific), 0.3 µL dimethyl sulfoxide (DMSO) (100 %, Thermo Fisher Scientific), 1 µL of each primer (5 µM µL⁻¹) and 1 µL of genomic DNA with unknown concentration. The PCR condition was: denaturation for 2 min at 94 °C; 35 cycles each of 30 s at 95 °C, 30 s at 94 °C, 1 min at 72 °C; 2 min at 72 °C. PCR products were checked on a 1.5 % agarose gel. PCR products were purified using the mixture EXO/FastAP (Exonuclease I and Thermosensitive

Table 1. Sampling locations of investigated *Euphrasia* species (see [Supporting Information—Table S1](#) for detailed information).

Species	Location no.	Region ¹	Latitude/longitude	Herbarium
<i>E. minima</i>	1	I, Alpi Graie: Vallone di Laures	45°41'13"/7°24'25"	WU:GMS-266
	2	CH, Alpi Lepontine: Gruppo del Monte Leone	46°15'27"/8°03'57"	WU:GMS-267
	3	CH, Alpi Lepontine: Alpi Ticinesi	46°26'41"/8°30'15"	WU:GMS-269
	4	CH, Glarner Alpen	46°58'18"/9°23'50"	WU:GMS-268
	5	I, Alpi Venoste/Ötztaler Alpen	46°49'12"/10°41'53"	WU:GMS-270
	6	I, Alpi Venoste/Ötztaler Alpen	46°45'20"/10°49'09"	WU:GMS-264
	7	A, Ötztaler Alpen	46°49'07"/10°54'02"	WU:GMS-256
	8	A, Ötztaler Alpen	47°51'57"/11°01'24"	W:NHM2014-0014158
	9	I, Alpi Venoste/Ötztaler Alpen	46°51'23"/11°05'37"	WU:GMS-261
	10	A, Stubai Alpen	47°06'57"/11°11'49"	WU:GMS-273
	11	A, Rofengebirge und Brandenberger Alpen	47°26'37"/11°45'54"	WU:GMS-274
	12	A, Hohe Tauern: Venedigergruppe and Lasörlinggruppe	47°00'04"/12°15'10"	WU:GMS-272
<i>E. sinuata</i>	11	A, Rofengebirge und Brandenberger Alpen	47°26'37"/11°45'54"	WU:GMS-251
	13	A, Kitzbüheler Alpen	47°28'22"/12°25'49"	W:NHM2014-0014161
<i>E. inopinata</i>	7	A, Ötztaler Alpen	46°49'07"/10°54'02"	WU:GMS-249
	8	A, Ötztaler Alpen	47°51'57"/11°01'24"	W:NHM2014-0014157
<i>E. cf. minima</i> 2x	2	CH, Alpi Lepontine: Gruppo del Monte Leone	46°15'27"/8°03'57"	WU:GMS-255
	6	I, Alpi Venoste/Ötztaler Alpen	46°45'20"/10°49'09"	WU:GMS-254
	7	A, Ötztaler Alpen	46°49'07"/10°54'02"	WU:GMS-253
<i>E. alpina</i> s. str.	1	I, Alpi Graie: Vallone di Laures	45°41'13"/7°24'25"	WU:GMS-276
<i>E. christii</i>	1	I, Alpi Graie: Vallone di Laures	45°41'13"/7°24'25"	WU:GMS-277
<i>E. rostkoviana</i> s. l.	8	A, Ötztaler Alpen	46°51'57"/11°01'24"	W:NHM2014-0014158
<i>E. hirtella</i>	11	A, Rofengebirge und Brandenberger Alpen	47°26'37"/11°45'54"	W:NHM2014-0014155

¹I = Italy; CH = Switzerland; A = Austria.

Alkaline Phosphatase, Thermo Fisher Scientific) following the manufacturer's instructions. For each sequencing reaction, a mixture was made with 5 µL of purified PCR product, 0.4 µL of BigDye terminator V3.1 (Applied Biosystems, Foster City, CA, USA), 1.8 µL of BigDye buffer (5×, Applied Biosystems), 1 µL of primer (5 µM µL⁻¹), 2 µL of Trehalose Dihydrate (1 M, Sigma) and 2.8 µL of water (Alfa Aesar, Karlsruhe, Germany). Cycle-sequencing products were sequenced on an ABI 3730 DNA Analyzer capillary sequencer (Applied Biosystems).

Amplified fragment length polymorphism fingerprinting was performed with the following selective primer combinations (fluorescent dye in brackets): EcoRI

(6-FAM)-ATG/MseICTT, EcoRI (VIC)-ACG/MseI-CAA and EcoRI (NED)-AGC/MseI-CTG. In two samples, DNA was replaced by water to test for systematic contamination. In addition, 10 samples were replicated in order to calculate the error rate using AFLPTOOLS (<https://github.com/geneva/AFLPTools>). The AFLP laboratory procedure followed that of Rešetnik et al. (2014).

Data analyses

Internal transcribed spacer sequences were assembled and edited using SeqMAN II 5.05 (DNASTAR Inc., Madison, WI, USA). Sequences downloaded from GenBank (Gussarova et al. 2008; Wang et al. 2018) were

combined with our newly obtained sequences and aligned using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>; Edgar 2004). The best-fit substitution model was selected using jMODELTEST 2.1.10 (Darriba *et al.* 2012) based on the Akaike information criterion (AIC). The general time reversible (GTR) model with gamma-distributed substitution rates was selected for our data set. Maximum likelihood (ML) analysis was done using RAxML 8.2.3 (Stamatakis 2014) employing the fast bootstrap approach (Stamatakis *et al.* 2008) with 1000 replicates. Bayesian inference (BI) was conducted using MrBAYES 3.2.7 (Ronquist *et al.* 2012), employing four independent Markov chain Monte Carlo (MCMC) of 2×10^7 generations and sampling trees every 5000 generations. Chain convergence was assessed by visual inspection of the traces and via effective sample size (ESS) values calculated with TRACER 1.6 (Rambaut *et al.* 2018). As in all cases ESS values were safely above 200, the four runs were combined after discarding the first 10 % of each MCMC as burn-in.

Raw AFLP electropherograms were analysed in PEAKSCANNER 1.0 (ABI, Foster City, CA, USA) with default parameters. Automatic scoring was performed in R (RCore Team 2011) using the package RawGeno 2.0 (Arrigo *et al.* 2009). Based on visual inspection of the electropherograms with GENOGRAPHER 1.6 (formerly available at <http://hordeum.oscs.montana.edu/genographer>), the scored range was set to 140–500 bp. Nineteen individuals were excluded because at least one of the primer combinations did not work well. Minimum reproducibility of each marker was set to 85 %. Minimum bin width and maximum bin width were left at the default of 1 and 1.5 bp, respectively.

To infer the genetic relationships among individuals from AFLP data, a neighbour-net analysis (Bryant and Moulton 2004) using Jaccard distances was performed in SPLITSTREE 4.14.2 (Huson and Bryant 2006). Additionally, the genetic structure among samples was analysed via a principal coordinate analysis (PCoA) on the basis of Jaccard distances using the R package vegan (Oksanen *et al.* 2013) and via the Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000). For the Structure analysis, the admixture model with correlated allele frequencies was used. The maximal number of groups (K) was set to 1–10. The run length of the MCMC was 10^6 iterations with additional 10^5 iterations as burn-in; each run was replicated 10 times. The best K was chosen based on Delta K (Evanno *et al.* 2005) using STRUCTURE HARVESTER 0.6.94 (Earl and vonHoldt 2012). The result was plotted with DISTRICT 1.1 (Rosenberg 2004). As the program STRUCTURE assumes Hardy–Weinberg equilibrium, which may not hold in potentially selfing *Euphrasia* species, we analysed the data also with INSTRUCT (Gao *et al.* 2007). As the best supported grouping was $K = 1$, which in light of

results from other methods (see Results) seems overly conservative, and groupings with $K > 1$ failed to yield biologically meaningful results (all but one gene pool were essentially empty, i.e. only minimal proportions of some individuals were assigned to those; data not shown), we did not pursue INSTRUCT analyses any further.

Results

Flow cytometry

The FCM analysis yielded two distinct groups of relative DNA amount [see Supporting Information—Fig. S1]. Taking into account available information on ploidy level in the investigated *Euphrasia* species (Ehrendorfer and Vitek 1984 ; Vitek 1985), we consider these two size classes to correspond to diploids and tetraploids, respectively. Consequently, 92 individuals were identified as diploids, and 235 individuals were identified as tetraploids. Among the diploids, 28 individuals are *E. inopinata* (12 individuals from the locus classicus and 16 individuals from the neighbouring valley south of the village Vent, both Ötztal) and 59 individuals are *E. sinuata* (17 individuals from the locus classicus in Rofan Mountains, and 42 individuals from Kitzbühler Horn). In addition, five individuals initially determined as *E. minima* turned out to be diploids (hereinafter referred to as *E. cf. minima* 2x). Relative genome sizes of the three diploid entities overlapped strongly and showed no interpretable pattern. Tetraploid *E. minima* was found to co-occur with *E. cf. minima* 2x, both populations of *E. inopinata*, and the western population of *E. sinuata* (Rofan Mountains), but was absent from the eastern population of *E. sinuata* (Kitzbühler Horn).

Molecular data

The newly obtained ITS sequences are available from GenBank [see Supporting Information—Table S1]. In both ML and BI analyses *E. inopinata* and *E. sinuata* were placed within *Euphrasia* series *Alpinae* (bootstrap support [BS] 89, posterior probability [PP] 1.00; Fig. 2). Samples of *E. minima* did not form a monophyletic group, but were found in four clades. Most individuals were grouped in a distinct clade (BS 100, PP 1.00) of unclear relationship. Two individuals (EM91-1 and EM91-4; BS 96, PP 1.00) were inferred as sister to *E. oakesii* and *E. randii*, yet this group received only limited support (BS 59, PP 0.88); together these were sister (BS 98, PP 1.00) to a hardly supported clade (BS < 50, PP 0.57) comprising species of series *Euphrasia*, such as *E. hirtella* and *E. officinalis*. One individual of *E. minima* (EM88-1) was clustered with a group of Palaearctic species (including,

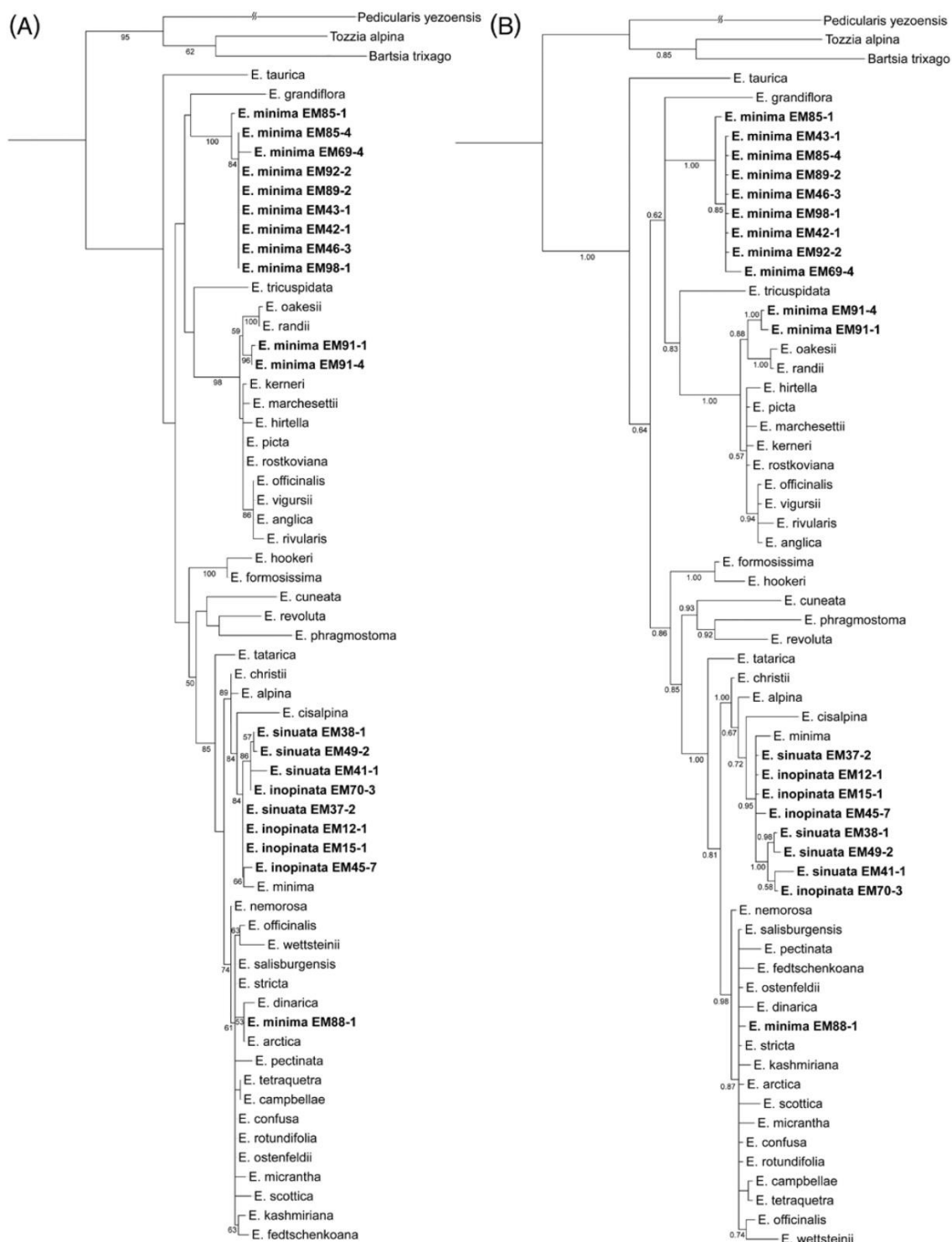


Figure 2. Phylogenetic placement of *Euphrasia minima*, *E. sinuata* and *E. inopinata* based on nuclear ITS sequences analysed using (A) maximum likelihood (numbers at nodes are bootstrap support values of at least 50) and (B) Bayesian inference (majority rule consensus tree, numbers at nodes are posterior probabilities of at least 0.5). Newly obtained sequences are marked in bold.

among others, *E. nemorosa* or *E. arctica*; BS 74, PP 0.98). Finally, one individual (the one used in Gussarova *et al.* 2008) grouped with *E. inopinata* and *E. sinuata* (BS 84, PP 0.95).

With the three AFLP primer combinations 343 fragments sized from 141 to 477 bp were successfully scored in 119 individuals, resulting in on average 63 bands per individual. The error rate estimated among replicated individuals was

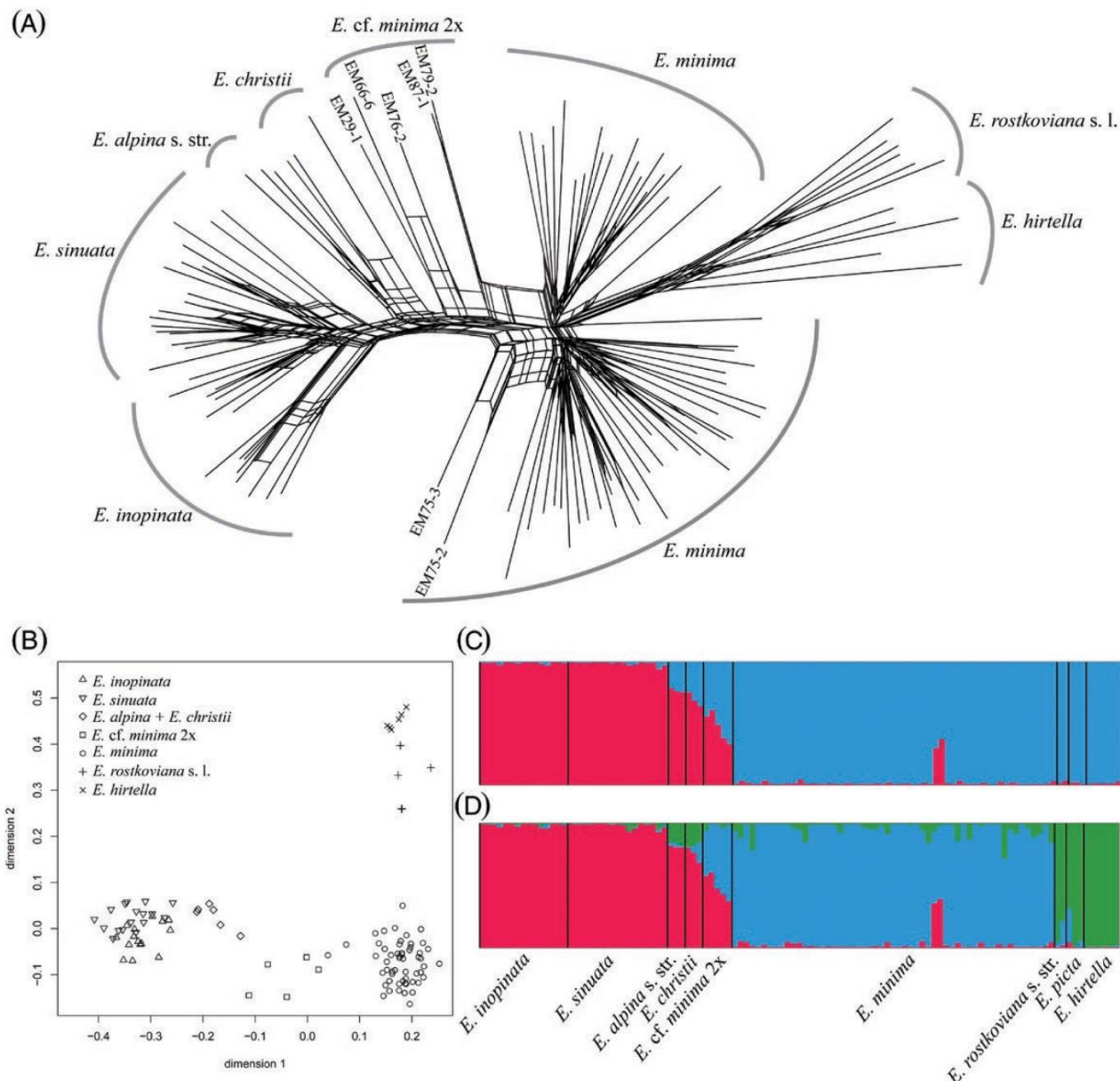


Figure 3. Genetic structure of *Euphrasia inopinata*, *E. sinuata* and related species estimated from AFLP fingerprint data. (A) Neighbour-net based on Jaccard distances (intermediate individuals discussed in the text are indicated); (B) scatter-plot of the first two coordinates of a PCoA using Jaccard distances; (C, D) population structure estimated using the Bayesian clustering approach implemented in Structure at (C) $K = 2$ and (D) $K = 3$.

4.58 %. The neighbour-net revealed three main groups (Fig. 3A). The first group (referred to as Parviflora Group) contained all tetraploid *E. minima*, the second group (referred to as Grandiflora Group) contained the diploids *E. hirtella* and *E. officinalis* s. l. Two individuals of tetraploid *E. minima* (EM75-3 and EM75-2) were clearly separated from the Parviflora Group. The third group (referred to as Alpina Group) contained the diploids *E. alpina*, *E. christii*, *E. inopinata* and *E. sinuata*. Whereas each population of *E. inopinata* and *E. sinuata* was found to be distinct, there

was no split supporting the separation of the two species. The individuals of *E. cf. minima* 2x (EM76-2, EM66-6, EM29-1, EM87-1 and EM79-2) were situated between the Alpina Group on one side and the Parviflora Group and the Grandiflora Group on the other side. The same three groups were identified by PCoA (Fig. 3B), where the Alpina Group was separated from others along the first axis (25.9 % of the total variance) and the Parviflora Group was separated from the Grandiflora Group along the second axis (9.9 % of the total variance). All individuals inferred

to occupy intermediate positions in the neighbour-net (EM75-2, EM75-3, EM76-2, EM66-6, EM29-1, EM87-1 and EM79-2) were positioned between the Alpina Group and the Parviflora Group.

The Delta K method suggested $K = 2$ as the optimal number of groups [see Supporting Information—Fig. S2B], corresponding to the Alpina Group versus the Grandiflora Group plus Parviflora Group (Fig. 3C). All individuals of *E. minima* inferred to occupy intermediate positions in the neighbour-net (tetraploid EM75-2, EM75-3; diploid EM29-1, EM66-6, EM76-2, EM79-2 and EM87-1) showed strong admixture. Additionally, individuals of *E. alpina* s. l. were inferred to be admixed as well, with the proportion of the minority cluster being between 21 and 36 % (Fig. 3C). If taking the likelihood distribution over different values of K into account, $K = 3$ was suggested by a stable likelihood maximum and a relatively high ΔK value [see Supporting Information—Fig. S2]. Under $K = 3$, the Grandiflora Group and the Parviflora Group were separated (Fig. 3D). Patterns of inferred admixture were the same for $K = 2$ and $K = 3$, with all individuals inferred to occupy intermediate positions in the neighbour-net being admixed in roughly equal proportions between the Parviflora and the Alpina Groups and the individuals of *E. alpina* s. l. being admixed between the Alpina and the Grandiflora Groups (Fig. 3D).

Discussion

On the basis of morphology, Ehrendorfer and Vitek (1984) proposed *E. inopinata* and *E. sinuata* as diploid representatives of *Euphrasia* series Parviflorae, including the tetraploid *E. minima*. However, molecular data are inconsistent with their hypothesis. Instead, both ITS sequence and AFLP fingerprint data clearly indicate that *E. inopinata* and *E. sinuata* are not closely related to the morphologically very similar *E. minima*, but instead to *E. alpina* s. l. (Figs 2 and 3). Morphological traits in *Euphrasia* species are highly variable and prone to ecological convergence (Vitek 1998; Twyford *et al.* 2018), explaining the taxonomic misplacement of *E. inopinata* and *E. sinuata* in series Parviflorae. In the light of our results, *E. inopinata* and *E. sinuata* need to be taxonomically placed in *Euphrasia* series Alpinae.

The phylogenetic placement of *E. inopinata* and *E. sinuata* and their distribution close to the north-eastern edge of the distribution area of *E. alpina* s. l. (Fig. 1; Vitek 1985) suggest that they are peripheral segregates of *E. alpina* s. l. This is in line with the hypothesis of Vitek (1986) that *E. inopinata* and *E. sinuata* are dwarfish derivatives of a more widespread large-flowered diploid, even if this is without elevational differentiation as originally envisioned by Vitek (1986). Although

currently lacking from the Austrian Alps (the species is found in the adjacent Italian parts of the Ötztal Alps: www.florafauna.it, assessed on 10 October 2018), *E. alpina* s. l. or its ancestor has at one point reached the Tyrolean Alps. In peripheral populations, lack of pollinators, especially in the alpine zone, and lack of mates trigger the evolution of self-pollination (Kalisz *et al.* 2004), resulting in the reduction of corolla size (<5 mm in *E. inopinata* and *E. sinuata*; Ehrendorfer and Vitek 1984). Although no pollination data are available for *E. inopinata* and *E. sinuata*, small flower size is a good indicator of increased selfing in *Euphrasia* (French *et al.* 2005). Fixation of thus deviating morphological traits and, in case of *E. sinuata*, also ecological traits with respect to substrate type (siliceous in *E. alpina* s. l. and *E. inopinata* versus limestone and dolomites in *E. sinuata*) is expected to have been fostered by small population sizes, enhancing genetic drift, and by geographic isolation, reducing gene flow from core populations. These shifts and thus the origin of *E. inopinata* and *E. sinuata* might be as recent as the postglacial as suggested for *Euphrasia* species endemic to the British Isles (French *et al.* 2008). As only one population of *E. alpina* s. l. was included, further sampling will be necessary to fully address the evolutionary path from the widespread allogamous *E. alpina* s. l. to the narrowly distributed autogamous *E. inopinata* and *E. sinuata*.

Euphrasia minima was hypothesized to be of allo-tetraploid origin (Yeo 1978; Ehrendorfer and Vitek 1984; Vitek 1986). One of the suggested parental species is *E. hirtella* (Vitek 1986). This is in agreement with our AFLP data (Fig. 3), which indicates a closer relationship between series Parviflorae and *Euphrasia*, the latter including *E. hirtella*. As second suggested parental species either *E. inopinata* and *E. sinuata* (Ehrendorfer and Vitek 1984) or *E. alpina* s. l., all from series Alpinae in its emended circumscription, were suggested, but neither finds support from the molecular data. After re-examination of the voucher specimen (from Goldberggruppe in the Hohe Tauern range, ~67 km south-east of Kitzbühler Horn), the single accession of *E. minima* used by Gussarova *et al.* (2008) grouping with *E. alpina* in the ITS tree (Fig. 2) might actually be *E. sinuata*. Thus, *E. sinuata* may be more widespread on base-rich soils in the central Alps of Austria, but has remained overlooked due to confusion with the morphologically extremely plastic *E. minima*. The distinctness of a clade of *E. minima* in the ITS tree (Fig. 2) suggests that the origin of this species might be old relative to the species divergence in European *Euphrasia* (including the origin of *E. inopinata* and *E. sinuata*), although peculiarities of ITS evolution (Álvarez and Wendel 2003) warrant caution in inference of (absolute or relative)

temporal evolution. Another complicating factor is the high frequency of hybridization in *Euphrasia* (Yeo 1978), outcrossing species of series *Alpinae* (*E. alpina* s. l.) and ser. *Euphrasia* (*E. officinalis* s. l., *E. hirtella*) being no exception (Yeo 1976; Liebst and Schneller 2005). Hybridization and introgression in conjunction with concerted evolution likely explain the phylogenetic position of EM88-1, EM91-1 and EM91-4 off all other accessions of *E. minima* as inferred from ITS data and the two admixed individuals of *E. minima* (EM75-2, EM75-3) inferred from AFLP data. The identity of diploid individuals referred to as *E. cf. minima* 2x (EM29-1, EM66-6, EM76-2, EM87-1 and EM79-2) remains unclear, as introgression from *E. minima*, as suggested by AFLP data, appears unlikely given the implied directionality of gene flow from a tetraploid into a diploid (but see Yeo 1956). Instead, these individuals may be hybrids involving any of the widespread and allogamous diploid species *E. alpina*, *E. hirtella* and *E. officinalis* s. l., whose intraspecific genetic diversities certainly are insufficiently covered in this study.

Conclusion

Molecular data provide clear evidence that diploid *E. inopinata* and *E. sinuata*, narrowly distributed in the central eastern Alps, are peripheral autogamous segregates of the widespread allogamous *E. alpina* s. str. instead of close relatives or ancestors of the morphologically very variable allotetraploid autogamous *E. minima*. This indicates that the shift to autogamy in *E. inopinata* and *E. sinuata* on the one hand and in *E. minima* on the other hand has happened independently, rendering this a well-suited system to study shifts in pollination (allo- to autogamy) in the context of different ploidy levels. The origin of species in formerly glaciated areas, if not associated with major range shifts (colonization from periglacial refugia and subsequent extirpation in those refugia, as has been suggested for members of the *Galium pusillum* complex/Rubiaceae: Kolář *et al.* 2015), commonly involves allopolyploidy and/or hybridization (e.g. *Saxifraga opdalensis* /Saxifragaceae and *Arabidopsis suecica* /Brassicaceae: Steen *et al.* 2000; Jakobsson *et al.* 2006). The herein investigated species *E. inopinata* and *E. sinuata* deviate from this pattern, as both are diploid and show no signs of hybridization with other taxa, rendering them, together with a few other groups, such as *Odontites vernus* and *O. litoralis* (Orobanchaceae), excellent model systems for (possibly) postglacial speciation at the diploid level in formerly heavily glaciated areas.

Data

Internal transcribed spacer (ITS) sequences are available from GenBank under accession numbers MK040308 – MK040324 and MK040326 – MK040328. Sequence alignments (as nexus file) and amplified fragment length polymorphism (AFLP) data (as fasta file) are available from Dryad under doi:10.5061/dryad.2pm2j8j.

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Contributions by the Authors

Conceptualization: D.P., G.M.S.; formal analysis: D.P.; investigation: D.P., P.S., T.M.; supervision: G.M.S.; writing: D.P., P.S., T.M., E.V., G.M.S.

Conflict of Interest

None declared.

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Supporting Information

The following additional information is available in the online version of this article—

Figure S1. Relative DNA amount of *Euphrasia minima*, *E. inopinata*, *E. sinuata* and *E. cf. minima* 2x.

Figure S2. Plots of (A) mean Log₁₀(X|K) and standard deviation over 10 runs and (B) Delta K for different K values.

Table S1. Details of samples: sampling regions, ploidy level, GenBank accession numbers and voucher information.

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Figure S1 Relative DNA amount of *E. minima*, *E. inopinata*, *E. sinuata* and *E. cf. minima* 2x individuals. In each panel one taxon is highlighted in black.

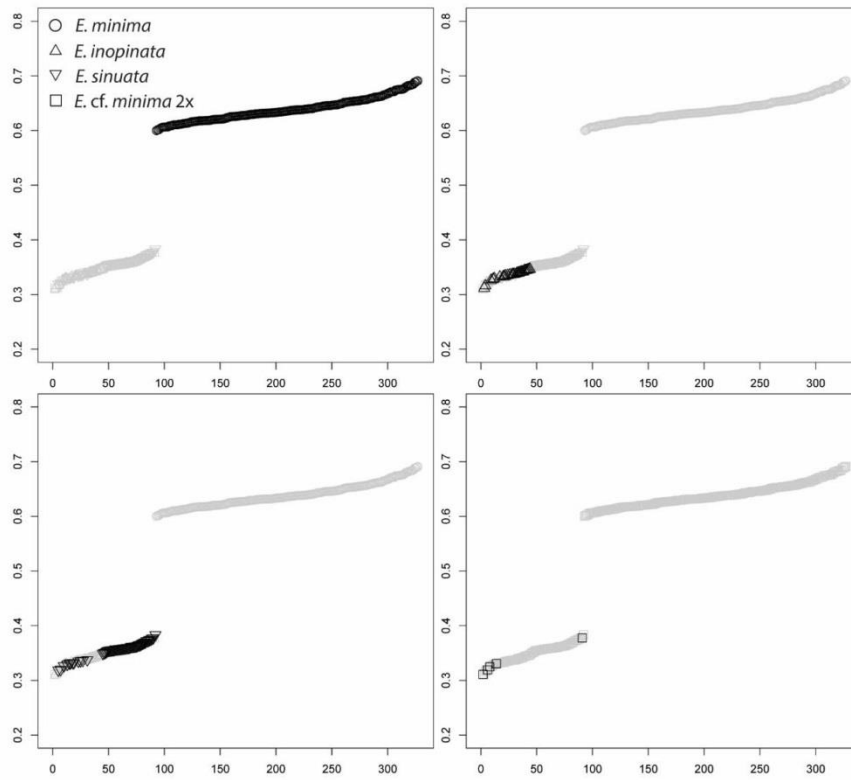


Figure S2 Plots of (a) mean $\text{Log}_e(X|K)$ and standard deviation over 10 runs and (b) Delta K for each K value.

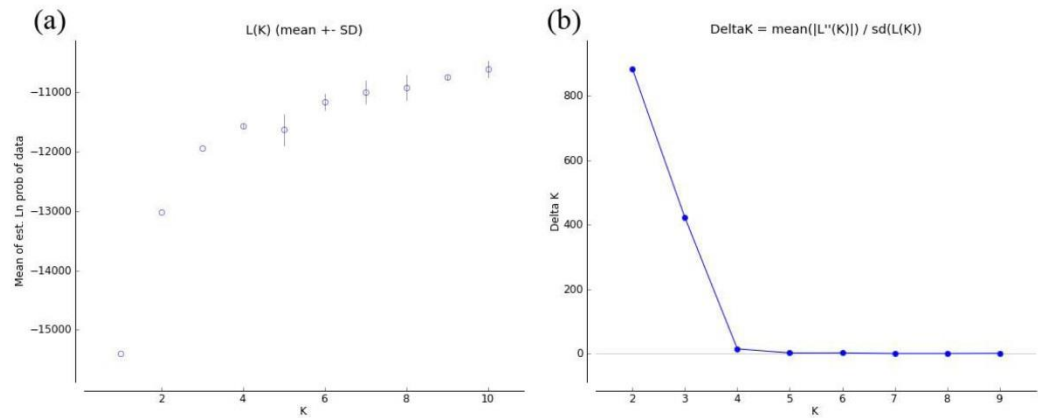


Table S1 Detail of samples: sampling regions, ploidy level, GenBank accession numbers and voucher information.

Species	Location No.	Sampling region ¹	Latitude/longitude	Herbarium	Population	Individual	Ploidy level	GenBank accession number
<i>E. inopinata</i>	8	A, Ötztaler Alpen	47°51'57"/11°01'24"	NHM2014-0014157	EM45	EM45-1	2x	MK040313
						EM45-2	2x	
						EM45-3	2x	
						EM45-4	2x	
						EM45-5	2x	
						EM45-6	2x	
						EM45-7	2x	
						EM45-8	2x	
						EM45-9	2x	
						EM45-10	2x	
						EM45-11	2x	
						EM45-12	2x	
	7	A, Ötztaler Alpen	46°49'07"/10°54'02"	WU:GMS-249	EM12	EM12-1	2x	MK040308
						EM12-2	2x	
						EM14-1	2x	
						EM15-1	2x	
						EM15-2	2x	
						EM66-4	2x	
						EM69-1	2x	
						EM69-2	2x	
						EM69-3	2x	
						EM70-1	2x	
						EM70-2	2x	
						EM70-3	2x	
			46°48'18"/10°53'26"	WU:GMS-250	EM69	EM70-4	2x	MK040315

<i>E. sinuata</i>	13	A, Kitzbüheler Alpen	46°48'57"/10°53'52"	WU:GMS-250 NHM2014- 0014161	EM71 EM37	EM70-5	2x	MK040310
						EM70-6	2x	
						EM71-1	2x	
						EM37-1	2x	
						EM37-2	2x	
						EM37-3	2x	
						EM37-4	2x	
						EM37-5	2x	
						EM37-6	2x	
						EM37-7	2x	
						EM37-8	2x	
			47°28'26"/12°25'41"	NHM2014- 0014161	EM38	EM38-1	2x	MK040311
						EM38-2	2x	
						EM38-3	2x	
						EM38-4	2x	
						EM38-5	2x	
						EM38-6	2x	
						EM38-7	2x	
						EM38-8	2x	
						EM38-9	2x	
						EM38-10	2x	
						EM38-11	2x	
						EM38-12	2x	
						EM38-13	2x	
						EM38-14	2x	
						EM38-15	2x	
						EM38-16	2x	
						EM38-17	2x	
						EM38-18	2x	

11	A, Rofangebirge und Brandenberger Alpen	47°28'26"/12°25'41"	NHM2014- 0014161	EM41	EM41-1	2x	MK040312
					EM41-2	2x	
					EM41-3	2x	
					EM41-4	2x	
					EM41-5	2x	
					EM41-6	2x	
					EM41-7	2x	
					EM41-8	2x	
					EM41-9	2x	
					EM41-10	2x	
					EM41-11	2x	
					EM41-12	2x	
					EM41-13	2x	
					EM41-14	2x	
					EM41-15	2x	
			EM61	EM61-4	2x		
		47°28'22"/12°25'44"	NHM2014- 0014161	EM46	EM46-1	2x	MK040314
					EM46-4	2x	
				EM49	EM49-1	2x	
					EM49-2	2x	
					EM49-3	2x	
					EM49-4	2x	
					EM49-5	2x	
					EM49-6	2x	
					EM49-7	2x	
					EM49-8	2x	
		47°27'16"/11°47'08"	WU:GMS-252	EM96	EM96-1	2x	
	EM96-2			2x			

46°52'05"/10°52'40"	WU:GMS-257	EM9	EM9-1	4x
			EM9-2	4x
46°52'02"/10°53'01"	WU:GMS-257	EM10	EM10-1	4x
46°51'20"/10°54'39"	WU:GMS-258	EM11	EM11-1	4x
46°49'07"/10°54'02"	WU:GMS-258	EM13	EM13-1	4x
			EM13-2	4x
			EM13-3	4x
			EM13-4	4x
46°50'15"/10°54'48"	WU:GMS-258	EM16	EM16-1	4x
46°47'21"/10°51'69"	WU:GMS-259	EM66	EM66-1	4x
			EM66-2	4x
			EM66-3	4x
			EM66-5	4x
46°47'37"/10°52'09"	WU:GMS-259	EM67	EM67-1	4x
			EM67-2	4x
			EM67-3	4x
			EM67-4	4x
46°47'59"/10°52'53"	WU:GMS-259	EM68	EM68-1	4x
			EM68-2	4x
			EM68-3	4x
			EM68-4	4x
			EM68-5	4x
			EM68-6	4x
46°48'18"/10°53'26"	WU:GMS-259	EM69	EM69-4	4x
46°49'15"/10°54'02"	WU:GMS-259	EM72	EM72-1	4x
			EM72-2	4x
			EM72-3	4x
			EM72-4	4x
			EM72-5	4x
			EM72-6	4x
46°49'57"/10°54'34"	WU:GMS-259	EM73	EM73-1	4x

MK040319

46°52'05"/10°52'40"	WU:GMS-257	EM9	EM9-1	4x
			EM9-2	4x
46°52'02"/10°53'01"	WU:GMS-257	EM10	EM10-1	4x
46°51'20"/10°54'39"	WU:GMS-258	EM11	EM11-1	4x
46°49'07"/10°54'02"	WU:GMS-258	EM13	EM13-1	4x
			EM13-2	4x
			EM13-3	4x
			EM13-4	4x
46°50'15"/10°54'48"	WU:GMS-258	EM16	EM16-1	4x
46°47'21"/10°51'69"	WU:GMS-259	EM66	EM66-1	4x
			EM66-2	4x
			EM66-3	4x
			EM66-5	4x
46°47'37"/10°52'09"	WU:GMS-259	EM67	EM67-1	4x
			EM67-2	4x
			EM67-3	4x
			EM67-4	4x
46°47'59"/10°52'53"	WU:GMS-259	EM68	EM68-1	4x
			EM68-2	4x
			EM68-3	4x
			EM68-4	4x
			EM68-5	4x
			EM68-6	4x
46°48'18"/10°53'26"	WU:GMS-259	EM69	EM69-4	4x
46°49'15"/10°54'02"	WU:GMS-259	EM72	EM72-1	4x
			EM72-2	4x
			EM72-3	4x
			EM72-4	4x
			EM72-5	4x
			EM72-6	4x
46°49'57"/10°54'34"	WU:GMS-259	EM73	EM73-1	4x

MK040319

8	A, Ötztal Alps	46°51'57"/11°01'24"	NHM2014-0014158	EM42	EM73-2	4x	MK040316
					EM73-3	4x	
					EM73-4	4x	
					EM42-1	4x	
					EM42-2	4x	
					EM42-3	4x	
					EM42-4	4x	
					EM42-5	4x	
					EM42-6	4x	
					EM42-7	4x	
					EM43-1	4x	
					EM43-2	4x	
					EM43-3	4x	
					EM43-4	4x	
9	I, Alpi Venoste/Ötztal Alps	46°51'23"/11°05'37"	WU:GMS-261	EM17	EM43-5	4x	MK040317
					EM43-6	4x	
					EM43-7	4x	
					EM43-8	4x	
					EM43-9	4x	
					EM17-1	4x	
					EM17-2	4x	
					EM18-1	4x	
					EM18-2	4x	
					EM19-1	4x	
					EM19-2	4x	
					EM20-1	4x	
					EM20-2	4x	
					EM21-1	4x	
					EM21	4x	

1	I, Alpi Graie: Vallone di Laures	46°45'43"/10°48'56"	WU:GMS-265	EM76	EM76-1	4x	MK040327
					EM76-3	4x	
					EM76-4	4x	
					EM76-5	4x	
		46°45'32"/10°49'02"	WU:GMS-265	EM77	EM76-6	4x	
					EM77-1	4x	
					EM77-2	4x	
					EM77-4	4x	
		46°45'22"/10°49'04"	WU:GMS-265	EM78	EM78-1	4x	
					EM78-2	4x	
					EM79-3	4x	
					EM79-4	4x	
		45°41'25"/7°24'16"	WU:GMS-266	EM91	EM91-1	4x	
					EM91-3	4x	
					EM91-4	4x	
					EM92-1	4x	
45°41'38"/7°24'32"	WU:GMS-266	EM92	EM92-2	4x			
			EM92-3	4x			
			EM92-4	4x			
			EM93-1	4x			
45°41'44"/7°24'26"	WU:GMS-266	EM93	EM93-2	4x			
			EM93-3	4x			
			EM93-4	4x			
			EM87-2	4x			
46°15'27"/8°03'57"	WU:GMS-267	EM87	EM87-3	4x			
			EM88-1	4x			
			EM88-2	4x			
			EM88-3	4x			
46°15'14"/8°03'58"	WU:GMS-267	EM88	EM88-4	4x			

4	CH, Glarner Alpen	46°15'01"/8°03'47"	WU:GMS-267	EM89	EM89-1	4x	MK040326
					EM89-2	4x	
					EM89-3	4x	
					EM89-4	4x	
			WU:GMS-267	EM90	EM90-1	4x	
					EM90-2	4x	
					EM90-3	4x	
					EM94-1	4x	
			WU:GMS-268	EM94	EM94-2	4x	
					EM94-3	4x	
					EM94-4	4x	
					EM95-1	4x	
		46°58'24"/9°24'16"	WU:GMS-268	EM95	EM95-2	4x	
					EM95-3	4x	
					EM95-4	4x	
3	CH, Alpi Lepontine: Alpi Ticinesi	46°26'41"/8°30'15"	WU:GMS-269	EM85	EM85-1	4x	MK040324
					EM85-2	4x	
					EM85-3	4x	
					EM85-4	4x	
					EM86-1	4x	
					EM86-2	4x	
					EM86-3	4x	
					EM86-4	4x	
					EM86-5	4x	
5	I, Alpi Venoste/Ötztaler Alpen	46°49'12"/10°41'53"	WU:GMS-270	EM32	EM32-1	4x	MK040321
					EM33-1	4x	
					EM33-2	4x	
					EM34-1	4x	
					EM34-2	4x	
		46°49'07"/10°42'31"	WU:GMS-270	EM34			

			EM34-3	4x
			EM34-4	4x
			EM34-5	4x
			EM34-6	4x
		EM35	EM35-1	4x
			EM35-2	4x
		EM36	EM36-1	4x
			EM36-2	4x
		EM80	EM80-1	4x
			EM80-2	4x
			EM80-3	4x
			EM80-4	4x
			EM80-5	4x
			EM80-6	4x
		EM81	EM81-1	4x
			EM81-2	4x
			EM81-3	4x
			EM81-4	4x
			EM81-5	4x
		EM82	EM82-1	4x
			EM82-2	4x
			EM82-3	4x
			EM82-4	4x
			EM82-5	4x
			EM82-6	4x
			EM82-7	4x
			EM82-8	4x
		EM83	EM83-1	4x
			EM83-2	4x
			EM83-3	4x
		EM84	EM84-1	4x
46°49'12"/10°42'43"	WU:GMS-270			
46°49'09"/10°42'23"	WU:GMS-270			
46°49'08"/10°42'39"	WU:GMS-271			
46°49'07"/10°42'16"	WU:GMS-271			
46°49'11"/10°41'49"	WU:GMS-271			
46°49'06"/10°41'22"	WU:GMS-271			
46°49'39"/10°40'13"	WU:GMS-271			

12	A, Hohe Tauern: Venedigergruppe & Lasörlinggruppe	47°00'04"/12°15'10"	WU:GMS-272	EM59	EM84-2	4x					
					EM84-3	4x					
					EM84-4	4x					
					EM59-1	4x					
10	A, Stubai Alpen	47°06'57"/11°11'49"	WU:GMS-273	EM63	EM59-2	4x					
					EM63-1	4x					
					EM63-2	4x					
					EM63-3	4x					
					EM63-4	4x					
					EM64-1	4x					
					EM64-2	4x					
					EM64-3	4x					
11	A, Rofangebirge und Brandenberger Alpen	47°07'13"/11°12'21"	WU:GMS-273	EM64	EM64-4	4x					
					EM65-1	4x					
					EM65-2	4x					
					EM65-3	4x					
					EM65-4	4x					
					EM46-2	EM46	WU:GMS-274	47°26'37"/11°45'54"	4x		
					MK040318					EM46-3	4x
										EM46-5	4x
										EM46-6	4x
EM46-7	4x										
EM46-8	4x										
EM46-9	4x										
EM46-10	4x										
EM46-11	4x										
EM46-12	4x										
		EM46-13	4x								

				47°27'16"/11°47'08"	WU:GMS-275	EM96	EM96-4	4x	MK040320
				47°27'04"/11°46'47"	WU:GMS-275	EM97	EM97-4	4x	
				47°27'49"/11°46'30"	WU:GMS-275	EM98	EM98-1	4x	
							EM98-3	4x	
				47°26'43"/11°46'00"	WU:GMS-275	EM99	EM99-1	4x	
							EM99-2	4x	
							EM99-3	4x	
							EM99-4	4x	
							EM99-5	4x	
							EC-1	-	
							EC-2	-	
							EC-3	-	
							EA-1	-	
							EA-2	-	
							EA-3	-	
							EP-1	-	
							EP-2	-	
							EP-3	-	
							ER-1	-	
							ER-2	-	
							EH-1	-	
							EH-2	-	
							EH-3	-	
							EH-4	-	
							EH-5	-	
<i>E. christii</i>	1	I, Alpi Graie: Vallone di Laures		45°41'13"/7°24'25"	WU:GMS-276	EC			
<i>E. alpina</i> s. str.	1	I, Alpi Graie: Vallone di Laures		45°41'13"/7°24'25"	WU:GMS-277	EA			
<i>E. picta</i>	8	A, Ötztaler Alpen		46°51'57"/11°01'24"	NHM2014-0014160	EP			
<i>E. rostkoviana</i> s. str.	8	A, Ötztaler Alpen		46°51'57"/11°01'24"	NHM2014-0014158	ER			
<i>E. hirtella</i>	11	A, Rofangebirge und Brandenberger Alpen		47°26'37"/11°45'54"	NHM2014-0014155	EH			

^T I = Italy; CH = Switzerland; A = Austria

Conclusions

Many alpine plants have already been shifting their distribution ranges upwards in response to recent global warming (Dullinger et al. 2012; Hülber et al. 2016; Walther et al. 2005). Studies indicate that the warm limit of alpine plants is particularly sensitive to the threat from competitors that encroach from lower elevation due to climate change (Rumpf et al. 2018). Hence, although interglacial expansion indicates that alpine species actually profited from climate warming by allowing them to extend their ranges during the Holocene, the limited nature of available altitudinal space and the evident incapability to reach geographically distant yet ecologically suitable regions (such as the Arctic) renders species of the European temperate mountain systems highly threatened under current global warming.

In phylogeographic studies, many demographic processes may lead to similar genetic patterns. With explicit modelling, the genetic pattern can be explicitly linked to the specific phylogeographic history of a species, avoiding to be misled by alternative processes (He et al. 2013; Massatti and Knowles 2016). As shown in chapter two, we could explicitly demonstrate that *P. aspleniifolia* has survived on nunataks within the heavily glaciated areas during the LGM. Although the persistence of plants on nunataks during glacial periods has been debated and studied over decades (Gabrielsen et al. 1997; Schneeweiss and Schönswetter 2011; Westergaard et al. 2011), this is one of the first studies to explicitly test the hypothesis instead of solely using correlative evidence.

The origins of species in formerly glaciated areas are commonly associated with range shifts, polyploidization and hybridization (e.g. Jakobsson et al. 2006; Kolář et al. 2015; Steen et al. 2000). However, the origins of *Euphrasia inopinata* and *E. sinuata* deviate from this pattern. Molecular evidence supported that they are phylogenetically closely related to widespread diploid *E. alpina* s. l.. There is no evidence that they have participated in the

formation of tetraploid *E. minima*. This indicates that they are peripheral segregates of *E. alpina* s. l., rendering this an excellent model system to study speciation at diploid level in formerly heavily glaciated areas. Besides, our result indicates that the shift to autogamy in *E. inopinata* and *E. sinuata* on the one hand and in *E. minima* on the other hand has happened independently, rendering this also a well suited system to study shifts in pollination (allo- to autogamy) in the context of different ploidy levels.

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