ORIGINAL ARTICLE

Introgression of an isolated *Primula* lineage suggests the existence of a glacial refugium in the Écrins range (Southwestern French Alps)

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Abstract

The species-richness of the flora in the European Alps results from complex interactions between geographical, climatic and environmental factors. In this study, we focus on a complex of closely related Alpine plants: *Primula hirsuta*, *P. pedemontana* and their relatives. Using a large DNA dataset of thousands of single-nucleotide polymorphisms sequenced across 149 individuals spanning all the western Alps, we refine phylogenetic relationships in this clade and explore the evolutionary origins of a mysterious lineage found in one valley of the Écrins range (France): the Valgaudemar. In particular, we demonstrate that this lineage did not originate from a simple allopatric divergence, but from an isolated lineage related to *Primula pedemontana*, which later got introgressed by *P. hirsuta*. This leads us to develop a phylogeographic scenario explaining the origins of the Valgaudemar lineage, and shed light on a potential glacial refugium in the south of the Écrins range. We believe this study takes part in the deep understanding of the origins of endemism in the European Alps and more generally of the maintaining of species diversity.

Keywords Speciation · Alpine flora · Phylogeography · Écrins national park · *Primula pedemontana* · Phylogenomics · ddRAD-seq

Introduction

Wild floras in all alpine areas (i.e., high-elevation habitats above the climatic treeline; Körner 2003) are known to be species rich (Barthlott et al. 2005; Kier et al. 2005; Mamantov et al. 2021; Spehn et al. 2011), and have attracted the attention of naturalists since the expeditions of Villars, Lapeyrouse or Saussure in European mountains in the nineteenth century. Distributed all over the world (Körner et al. 2011), these places are characterized by a varied history of climate change and strong microhabitat differentiation (Patsiou et al. 2014), and include global hotspots of biodiversity such as the Himalayas or the Andes. While the richness of these high-elevation zones remains an "enigma" for biogeography and evolutionary biology (Rahbek et al. 2019), several factors driving speciation have been identified including geographic isolation and fine-scale environmental heterogeneity (Boucher et al. 2016a, b; Favre et al. 2015; Hughes and Atchison 2015; Smyčka et al., 2022). In the European Alps in particular, the main driver of plant diversity is considered to be topography (Körner 2003; Marini et al. 2008). The combination of a rugged topography with alternating glacial and interglacial periods resulted in temporal habitat fragmentation and led to major range movements of alpine species (Pauli and Halloy 2019; Schonswetter et al. 2005). This enhanced the isolation of populations, leading to genetic differentiation and eventually speciation in cases where re-colonization following glacier retreat did not lead to the merging of diverging populations (Feliner 2011; Flantua et al. 2018). Spatial isolation during glacial cycles is, however, not the only process at play and lineage diversification in mountain plants implies a grasp of complex interactions between factors such as dispersal barriers, phenology, substrates, elevation gradients, etc. (Boucher et al. 2016a, b; Kadereit 2017).

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Here, we focus on a clade of four closely related primrose species growing in the Alps, Apennines and Pyrenees: *Primula apennina*, *P. cottia*, *P. hirsuta* and *P. pedemontana*. In the following of this article, we refer to this clade as /Hirsuta following Boucher et al. (2016a, b). All of these species grow in similar habitats in the alpine and subalpine elevation belts: rock crevices or scratched meadows on siliceous substrates (Boucher et al. 2016a, b; Zhang and Kadereit 2004) are particularly interested in a primrose population from the Valgaudemar valley in the Écrins range that displays ambiguous morphological characters. Despite sharing some similarities with *P. hirsuta*, this population, which we refer to as 'the Valgaudemar lineage' in the following of this article, was described as *P. pedemontana* in the past (Arvet-Touvet 1873), but is sometimes considered as a putative hybrid between *P. hirsuta* and *P. pedemontana*. The only phylogenetic study that included the Valgaudemar lineage suggested that it could be a new taxon or a subspecies of *P. pedemontana* (Boucher et al. 2016a, b), but due to insufficient sampling no firm conclusion could be reached.

Understanding the origins of cryptic lineages in /Hirsuta may provide more general insights into the origins of plant endemism and persistence through ice ages in the Alps (Gaston 2000). In addition, this knowledge could be used to protect *Primula* diversity, especially in our study area, the Écrins range, which is covered by a National Park. Indeed, protected areas like the Écrins National Park are getting increasingly involved in scientific research to take the evolutionary history and potential of lineages into account in their conservation actions (Forest et al. 2007; Nielsen et al. 2020).

In this study, we used thousands of Single-Nucleotide Polymorphisms (SNPs) from 149 *Primula* individuals obtained by double-digest restriction-site-associated DNA sequencing (ddRAD-seq; Peterson et al. 2012) to clarify the phylogeographic origin of the Valgaudemar lineage. Our aims are (i) to accurately delimit the distribution of this lineage in the Écrins range; (ii) to test whether this lineage is of hybrid origin or not and (iii) to develop a phylogeographic scenario for the origins of this lineage that takes into account the role of potential local glacial refugia. Our molecular data clarify phylogenetic relationships and genetic diversity within /Hirsuta, and reveals a complex scenario for the origin of the Valgaudemar lineage, which is restricted to a single valley. By comparing several alternative phylogeographic scenarios, we show that the Valgaudemar lineage originated from an isolated lineage of *P. pedemontana*, which later got introgressed by neighboring populations of *P. hirsuta*. Finally, this phylogeographic scenario reveals the possible existence of a glacial refugium in the south of the Écrins range.

Materials and methods

Taxon sampling

Our study focused on four species that form the /Hirsuta clade (Boucher et al. 2016a, b): *Primula apennina* Widmer, *P. cottia* Widmer, *P. hirsuta* All., and *P. pedemontana* Thom. ex. Gaudin. They are part of the largest endemic clade of the European alpine system: *Primula sect. Auricula* (Ozenda 1995), which contains over 25 species spanning all mountains of Europe. Species of /Hirsuta are long-lived perennials forming rosettes or loose mats. Their pink or violet flowers bloom in spring, usually only a few weeks after snowmelt. Being heterostylous, they are obligate outcrossers (Kadereit et al. 2011). With the exception of a few populations, they are all specialized on siliceous substrates (mainly granite, gneiss, quartzite) and grow on rocky habitats of the alpine and subalpine belts (e.g., crevices, rocks and stony meadows c. 1400–3000 m a.s.l.). While *P. hirsuta* has a wide distribution from the central to the western Alps and in the central Pyrenees, other species in /Hirsuta have much more restricted distributions. *P. apennina* grows in the northern Apennines, *P. cottia* in the Cottian Alps (south-eastern France, north-western Italy), and *P. pedemontana* has a disjunct distribution between the Cottian and Graian Alps (France and Italy) and the Cantabrians (Zhang and Kadereit 2004).

We sampled a total of 149 individuals from wild populations during spring and summer in 2016, 2017, 2019 and 2020 (see list of samples in Table A.1.). All four species were represented by at least 10 samples each and were collected in a minimum of two localities (Fig. 1). We also added eight samples of the enigmatic Valgaudemar lineage, sampled in the highest part of the Valgaudemar valley. Two individuals of this lineage had already been included in a previous phylogenomic study of *Primula* sect. *Auricula*, in which its locality was mentioned as 'the Lauzon valley' (a part of Valgaudemar), but results regarding its origins remained inconclusive (Boucher et al., 2016a, b). Finally, we included a sample of *P. marginata* Curtis as an outgroup for phylogenetic analyses. Figure 1 shows a map of all sampling locations.

Genotyping protocol

To gather robust information on thousands of SNPs, a double-digestion restriction site-associated DNA sequencing experiment (ddRAD-seq) was conducted. This strategy was adopted following previous studies which demonstrated how reduced-representation libraries provided deeper insights into fine-scale phylogenetic relationships

compared to traditional sanger sequencing in *Primula* sect. *Auricula* (Boucher et al. 2016a, b) or in the closely related *Androsace* sect. *Aretia* (Boucher et al. 2021).

We used a modified version of the original ddRAD-seq protocol (Peterson et al. 2012). DNA was first extracted from silica-dried leaves using the DNEasy Plant mini kit (Qiagen, Germany). A total of 150 ng of DNA template from each individual was then double-digested with 10 units each of PstI and MspI (New England Biolabs Inc.) at 37 °C during 2h30 in a final volume of 36 µl using the CutSmart buffer provided with the enzymes. Digestion was further continued together with ligation of P1 and P2 adapters (see Peterson et al. 2012) by adding 10 units of T4 DNA ligase (New England Biolabs Inc.), adapters P1 and P2 in tenfold excess (compared to the estimated number of restriction fragments) and 1 µl of 10 mM ribo-ATP (New England Biolabs Inc.) in each sample. This simultaneous digestion-ligation reaction was performed on a thermocycler using a succession of 60 cycles of 2 min at 37 °C for digestion and 4 min at 16 °C for ligation. An equal volume mixture of all the digested-ligated fragments were purified with NucleoMag NGS clean-up and Size select (Macherey Nagel Germany). Fragments were size-selected between 200 and 600 bp on a BluePippin. The ddRAD libraries obtained were amplified using the following PCR mix: a volume of 20 µl with 2 µl of DNA template, 10 mM of dNTPs, 10 µM of each PCR probe (Peterson et al. 2012) and 2 U/µl of Taq Phusion-HF (New England Biolabs Inc.); and the following PCR cycles: an initial denaturation at 98 °C for 30 s; 15 cycles of 98 °C for 10 s, 66 °C for 30 s and 72 °C for 1 min; followed by a final extension period at 72 °C for 10 min. The four amplified ddRAD libraries were purified NucleoMag NGS clean-up and Size select (Macherey Nagel Germany) and then sequenced on a full lane of a NovaSeq 6000 sequencer by Fasteris SA (Switzerland).

Bioinformatics pipeline

All sequenced ddRAD-seq tags (c. 350 million reads) were assembled using the program ipyrad (https://github.com/dereneaton/ipyrad) allowing a maximum of five heterozygous sites and five SNPs per *locus* and using default parameters otherwise. Rather than relying on de novo assembly of these *loci*, we preferred to align them on the reference genome of another primrose species, *Primula vulgaris* Huds. (Cocker et al. 2018). While aligning to a rather distant reference inevitably loses *loci*, we preferred to rely on this reduced dataset in order to avoid paralogs as much as possible, as had already been done in a previous study of *Primula* sect. *Auricula* which used short reads (Boucher et al. 2016a, b). This procedure yielded a full DNA matrix of 1,362,766 bp, including a total of 40,917 SNPs.

To check for the directionality of hybridization in the Valgaudemar lineage (see below), we realized another assembly

of chloroplastic DNA only (hereafter, cpDNA), which is maternally inherited in most angiosperms. We followed the same procedure as above but aligned ddRAD-seq tags to the reference chloroplast of *P. pedemontana* (S. Lavergne, unpublished data), producing a DNA matrix of 131,182 bp with 4303 SNPs.

Phylogenetic inference

Phylogenetic relationships within the /Hirsuta clade were inferred using maximum-likelihood inference carried out by IQ-TREE (Minh et al. 2020; Nguyen et al. 2015) on the concatenated SNP matrix. We first kept individuals with less than 60% missing data. We then removed loci with more than 50% missing data, according to some studies showing similar results for samples with less than 50% missing data and less than 1% (Altermann et al. 2014). Preliminary phylogenetic analyses (not shown here) enabled the removal of some problematic individuals, which appeared to be recent hybrids between *P. hirsuta* and *P. pedemontana*. We finally had a total of 123 samples and 18,545 SNPs for phylogenetic inference. The best model of DNA substitution for this dataset, TVM + F + R3 was selected thanks to ModelFinder (Kalyaanamoorthy et al. 2017) among 153 possible ones according to the Bayesian information criterion (BIC), and 1000 ultrafast bootstrap replicates (Hoang et al., 2018) were used to measure node support on this phylogeny.

Another phylogenetic inference was later realized using cpDNA only and on the concatenated cpDNA matrix. In this case, the best substitution model that was used for inference was TPM3 + F + I + G4.

Genetic diversity and differentiation

We first visualized genetic variation among individuals using PCA (Principal Component Analysis) using the package factoextra in R (Kassambara and Mundt 2017). All PCAs were performed after removing individuals and loci with more than 60% and 50% missing data, respectively.

We then used the nonparametric K-means clustering procedure implemented in the package LEA in R (Frichot and François 2015) to identify genetic groups among individuals. Genetic clusters were inferred with no a priori grouping using an efficient algorithm aimed at estimating individual ancestry: sNMF (Frichot et al. 2014). Given that /Hirsuta comprises four named species and since we expected the Valgaudemar lineage to be genetically distinct, we ran the program for a number of genetic clusters K ranging from 1 to 10 with 1,000 iterations and 20 repetitions per value of K. The optimal number of clusters was chosen based on the cross-entropy of the best run for each K value (Frichot et al. 2014).

Genetic diversity was estimated using observed heterozygosity (H_O), population genetic diversity, allelic richness, inbreeding coefficient (F_{IS}) and population-specific F_{ST} using the package hierfstat in R (Goudet 2005). This package was also used to estimate pairwise F_{ST} (Weir and Cockerham 1984) between populations.

Test of the hybrid origin of the Valgaudemar lineage

Following results of phylogenetic and genetic structure analyses (see below), we decided to test for putative introgression in the Valgaudemar lineage by measuring the hybrid index (Buerkle 2005). This test was run using the package introgress in R (Gompert et al. 2010) with *P. hirsuta* and *P. pedemontana* as parents and the Valgaudemar lineage as the introgressed population. In addition to this method, we tested for directional introgression either from *P. hirsuta* or from *P. pedemontana* in the Valgaudemar lineage using the ABBA/BABA test in the package evobiR in R (Blackmon et al. 2013), using *P. marginata* as outgroup with 1000 jack-knife repetitions.

Given that these analyses confirmed that individuals from the Valgaudemar lineage were significantly introgressed (see Results), we then tested if the Valgaudemar lineage is of hybrid origin. We did so using the Approximate Bayesian Computation software diyABC (Cornuet et al. 2014) and compared three scenarios: (i) a simple phylogenetic one in which the Valgaudemar lineage was sister to *P. pedemontana,* (ii) one in which the Valgaudemar lineage resulted from hybrid speciation between *P. hirsuta* and *P. pedemontana*, and (iii) one in which the Valgaudemar lineage resulted from the long term isolation of a lineage of *P. pedemontana*, followed by introgression from *P. hirsuta* (Fig. 4). We did not test the symmetrical scenario of introgression from *P. pedemontana* into a lineage closely related to *P. hirsuta* due to the absence of *P. pedemontana* in the areas surrounding the Valgaudemar valley, to the greater morphological similarity between the Valgaudemar lineage and *P. pedemontana*, and to our ABBA/BABA results that rejected this direction of introgression. In other words, the second scenario captures a strict hybrid origin of the Valgaudemar lineage, while the third scenario captures an introgression scenario.

Because of the high computational requirements of this approach, we reduced our genomic dataset by randomly sampling two individuals from each population of *P. hirsuta* (12 individuals in total) and each population of *P. pedemontana* (8 individuals in total). On the contrary, all individuals from the Valgaudemar lineage were included (8 individuals). This random sampling was repeated twice to check that it did not influence the inference. Effective population sizes were kept constant on each branch and given uninformative priors in all simulations (all log-uniform with minimum 10 and maximum 150,000). We used a strict calibration at

3.45 Myrs for the initial split between *P. hirsuta* and the other species (Boucher et al. 2016a, b) and log-uniform priors between 10,000 years and 3.45 Myrs for all subsequent divergence or reticulation events. In scenarios that included hybridization, the prior on the proportion of genetic input from *P. pedemontana* was uniform between 0.5 and 0.7, following measures of the hybrid index (see Results).

The fit of alternative scenarios was compared using all available summary statistics for within population genetic diversities, pairwise F_{ST} values, Nei's distances and admixture estimates except for the means of non-zero values. We generated 1,000,000 data sets for each scenario and calculated the posterior probabilities of all scenarios using a logistic regression on the 1% of simulated data closest to the observed data (Cornuet et al. 2010, 2014). Confidence in scenario choice was evaluated by generating 1,000 newly simulated data sets from priors and by computing Type I and Type II errors.

Results

Phylogenetic inference

Maximum-likelihood analysis of our SNP dataset produced a highly resolved phylogenetic tree, with all species forming clades supported by 100% bootstrap (Fig. 2). The ingroup was separated into two main clades. The first one included all populations of *P. hirsuta*. The other contained *P. apennina*, *P. cottia*, *P. pedemontana,* and the Valgaudemar lineage, corresponding to a clade named /Pedemontana (Boucher et al. 2016a, b). While phylogenetic structure appeared to be weak within *P. hirsuta*, all populations of *P. apennina*, *P. cottia*, and *P. pedemontana* that we sampled formed distinct clades. This was not the case for individuals from the Valgaudemar lineage, however, which formed a paraphyletic group at the base of /Pedemontana in spite of their strong spatial isolation from any other population in this clade.

The cpDNA phylogeny recovered the same general structure with a clade made up almost exclusively by individuals of *P. hirsuta* and another made up almost exclusively by those of /Pedemontana, but much weaker phylogenetic structure within both of these clades. All individuals of the Valgaudemar lineage but one formed a cpDNA clade within /Pedemontana that also included one *P. hirsuta* individual from a neighboring location in the Écrins range. The last individual of the Valgaudemar lineage that we studied had its cpDNA nested within *P. hirsuta* (Fig. A.1.).

Genetic diversity and differentiation

The genetic PCA with all populations showed proximity between *P. pedemontana*, *P. cottia* and *P. apennina* (Fig. 3a),

in agreement with phylogenetic results (Fig. 2). More precisely, the first axis (explaining 5.3% variance) distinguished *P. hirsuta* from /Pedemontana. The second axis (explaining 3.1% variance) showed variability within /Pedemontana (Fig. 3a.). Individuals from the Valgaudemar lineage were intermediate between *P. hirsuta* and *P. pedemontana* with no overlap with either of these two species.

The nonparametric K-means clustering procedure showed close BIC values for $K = 4$, 5 & 6. For $K = 4$, all species were assigned to a distinct genetic cluster, and the Valgaudemar lineage showed roughly equal genetic proportions between *P. hirsuta* and *P. pedemontana* (Fig. 3b). $K = 5$ showed the same pattern except for the Valgaudemar lineage forming a distinct cluster. Finally, for $K = 6$, *P. pedemontana* was separated into two genetic groups corresponding to populations from the Maurienne and Tarentaise valleys of the Vanoise range, respectively.

Genetic diversity was roughly similar in all populations, except for populations of *P. apennina* that were twice less diverse (Table 1). The pairwise F_{ST} between all /Hirsuta populations is shown in Table 1b, the Valgaudemar lineage

Table 1 Genetic diversity measured between all /Hirsuta populations

Genetic diversity (Hs)													
PSA	ENP	HV	AB	AN	CP	HCF	HCN	HL	HT	PE	PF	PGU	PHP
0.212	0.258	0.223	0.124	0.117	0.212	0.283	0.217	0.173	0.251	0.192	0.210	0.248	0.254

Population labels refer to the sampling name (HV refers to the Upper part of Valgaudemar and ENP to the Écrins national park; see Table A.1.). Population labels are color refer to the species color in PCA and phylogenetic tree

Fig. 1 Distribution of the four species of /Hirsuta clade in the western Alps. The Valgaudemar lineage is represented with red dots. Symbols correspond to sampling location, see legend. Approximate distributions are drawn according to Zhang and Kadereit 2004) and INPN (https://inpn.mnhn.fr/)
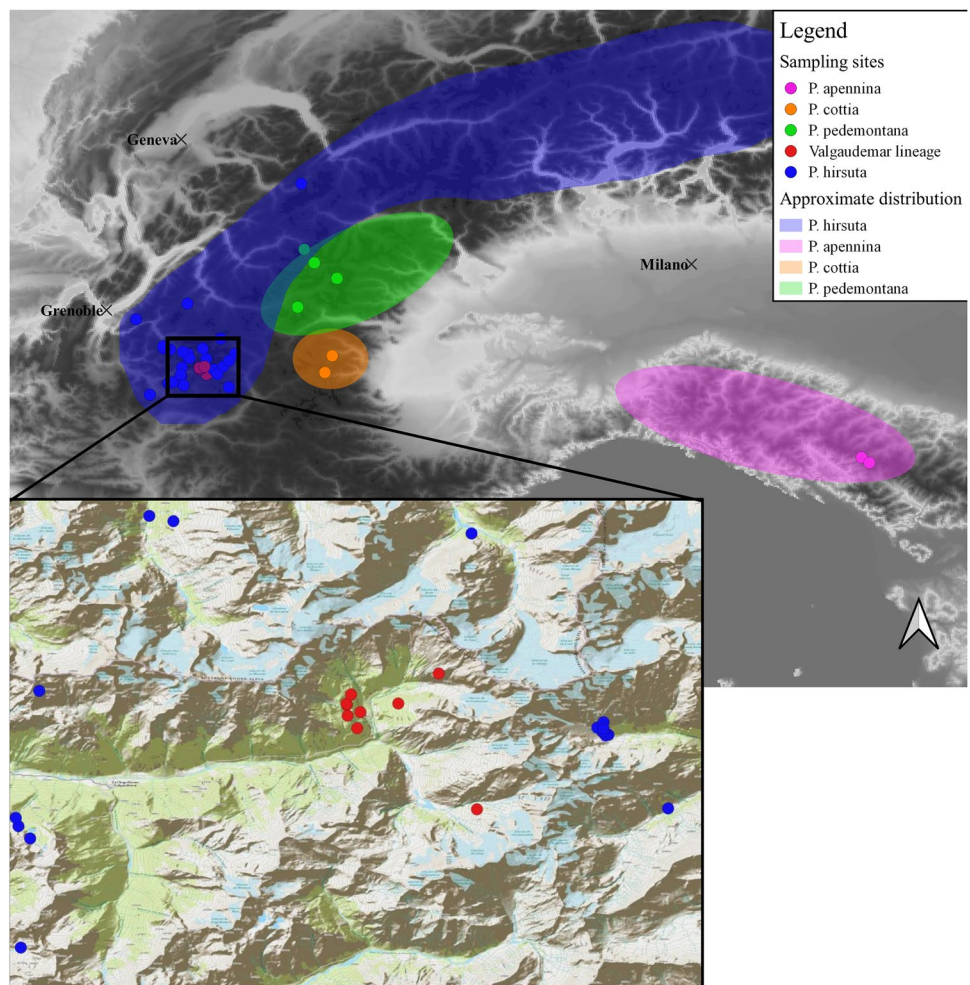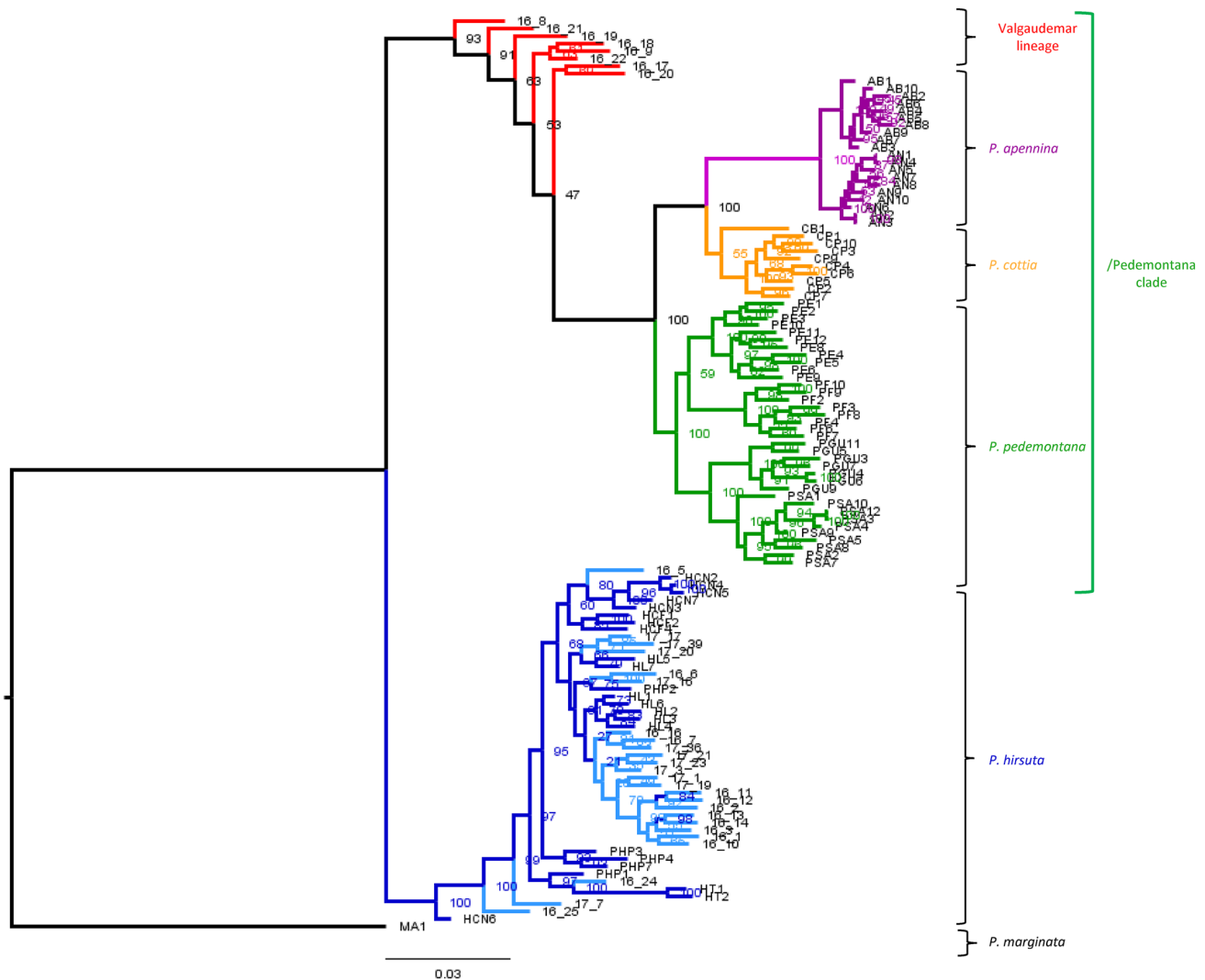
Fig. 2 Maximum-likelihood phylogenetic tree for /Hirsuta obtained from the SNPs dataset (individuals 60% NA max., loci 50% NA max.) using IQ-TREE. Bootstrap support is shown on the crown node of each clade. Each clade was colored and labeled with the species name. The clade /Pedemontana is labeled by a green bracket. Within *P. hirsuta*, individuals from the Écrins range are colored in light blue and individuals from outside the Écrins range in dark blue

showing maximum proximity with the *P. hirsuta* population of the Écrins range ($F_{ST} = 0.166$).

Test of the hybrid origin of the Valgaudemar lineage

The 'introgress' analysis showed that between 51 and 65% of alleles in the genetic background of the height individuals from the Valgaudemar lineage derived from *P. pedemontana* (hence between 35 and 49% derived from *P. hirsuta*), confirming the potential hybrid origin of this lineage. The ABBA/BABA test strongly supported introgression from *P. hirsuta* in the Valgaudemar lineage (D statistic = 0.2186; Z-score = 25.626).

The diyABC analysis provided clearcut support for the introgression scenario (Fig. 4). Its posterior probability

was > 0.99 for both replicates including different random samples of individuals (Table A.3.). Parameter estimates (Table A.4.) showed a drastic reduction in the effective population size (N_e) of the Valgaudemar lineage (N_e divided by 93 in comparison to that of *P. pedemontana*). The estimated proportion of genetic input from *P. pedemontana* into the Valgaudemar lineage was around 60% [replicate 1: 0.664 (95% CI 0.597–0.690); replicate 2: 0.656 (95% CI 0.585–0.690)], the rest coming from introgression from *P. hirsuta*. Based on a split between *P. hirsuta* and the rest of /Hirsuta at 3.47 Mya (Boucher et al. 2016a, b), the diyABC analysis estimated that the Valgaudemar lineage diverged from *P. pedemontana* around 1,000,000 years ago [replicate 1: 1030 kyrs (95% CI 186–1830 kyrs); replicate 2: 1210 kyrs (95% CI 252–2020 kyrs)], and the admixture event between
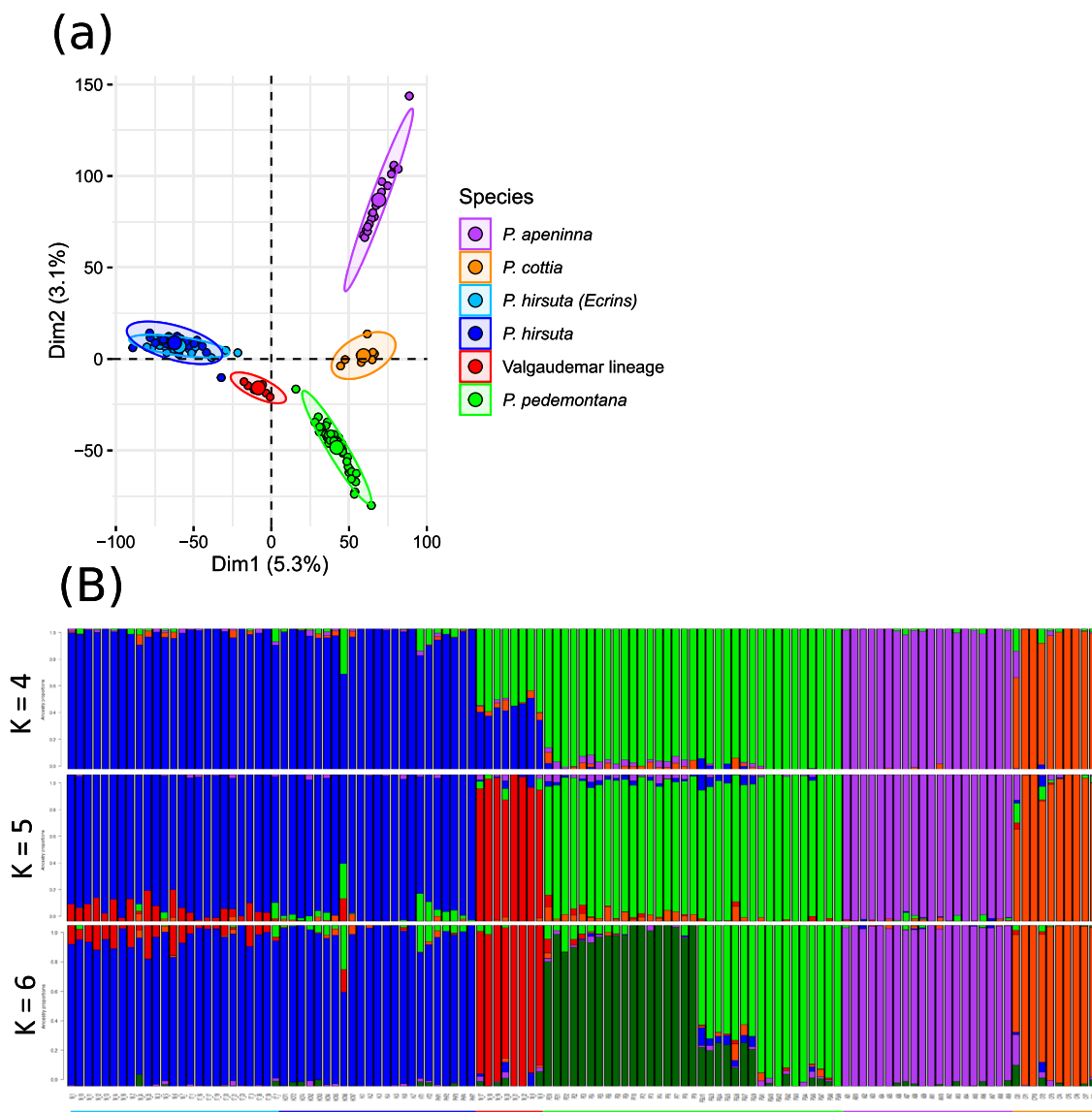
Fig. 3 Genetic structure within /Hirsuta. **a** Principal component analysis (PCA) results on the two first PCs including all populations. **b** admixture barplots for all populations. Individuals are represented by vertical bars along the plot and admixture coefficients represent the probability of assignment of each individual to a group. Individual labels refer to the sampling name (see Table A.1.). Color bars refer to the species color in PCA and phylogenetic tree

this Valgaudemar lineage and *P. hirsuta* to be much more recent [replicate 1: 65,3 kyrs (95% CI 10,3–417 kyrs); replicate 2: 33,2 kyrs (95% CI 10–195 kyrs)].

Discussion

Primula sect. *Auricula* is the largest clade of plants endemic to the European Alpine System (Ozenda 1995) and as such it has already attracted much attention (Boucher et al. 2016a, b; Boucher et al. 2016a, b; Kadereit 2017; Kadereit et al. 2011; Kress 1989; Zhang et al. 2004; Zhang and Kadereit 2004). However, some aspects of its systematics and evolutionary history remained unclear (Boucher et al. 2016a, b). Using a large DNA dataset of thousands of SNPs as well as dense sampling of individuals within species allowed us to produce a strongly supported phylogenetic hypothesis for a particular clade within *Primula* sect. *Auricula*: /Hirsuta (Fig. 2). This phylogeny clearly confirms that all currently named species are monophyletic and that *P. hirsuta* is sister to the /Pedemontana clade (Boucher et al. 2016a, b) including *P. apennina*, *P. cottia*, *P. pedemontana*, and the Valgaudemar lineage, as already suggested by a previous study (Boucher

Fig. 4 Sketches of the three historical scenarios tested using diyABC. The blue lineage ('Pop 1' on the black and white sketches) represents *P. hirsuta*, the green lineage ('Pop 2') represents *P. pedemontana*, the red lineage ('Pop 3') represents the Valgaudemar lineage and the black line on scenario 3 represents the ghost lineage. Parameters used: N1, N2, N3, and N4 are effective population sizes, NA represents ancestral population size; t2 is the ancestral divergence time; ta is the time of ghost lineage formation; t1 is the hybridization or divergence time; r1 is the relative genetic contribution from *P. pedemontana/*ghost lineage in the hybrid species. The parameter values in scenario 3 correspond to the median of the first analysis replicate (Table A.4.)
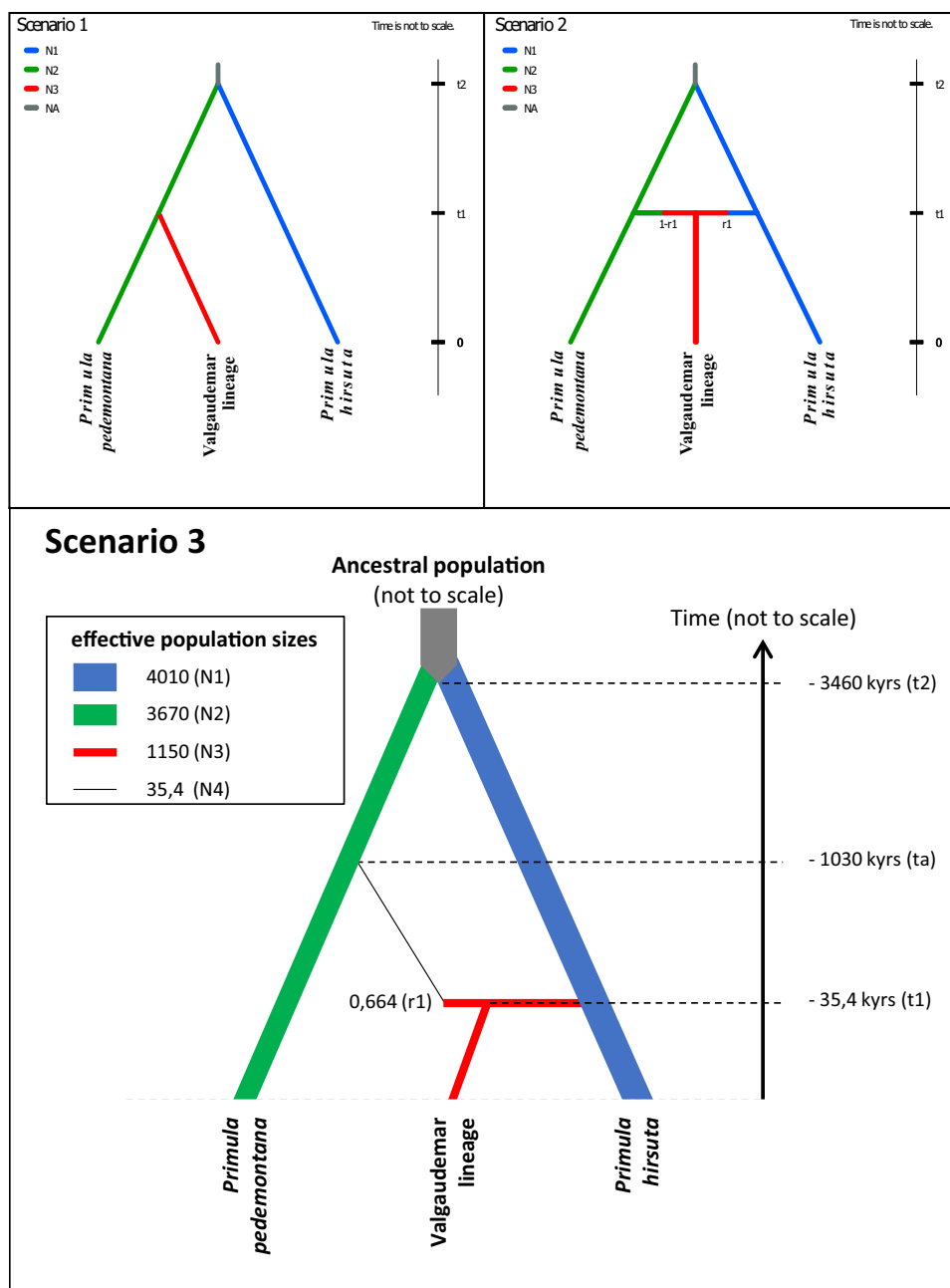
et al. 2016a, b). More importantly, these data allow us to elucidate the origins of the Valgaudemar lineage.

Identification of a narrow endemic Primula lineage

We chose to sample individuals regularly within the Écrins range rather than to sample a few focal populations as done elsewhere in order to explore the fine-scale genetic structure of /Hirsuta across this area. Our results clearly show that two taxa coexist in the Écrins range: one of them restricted to the uppermost part of the Valgaudemar valley and the other being widespread (Fig. 1). This is shown by phylogenetic

analyses displaying individuals from Valgaudemar forming a grade at the base of /Pedemontana while all individuals from other areas of the Écrins range were nested within *P. hirsuta* (Fig. 2). These two groups of individuals also belonged to different clusters of the genetic PCA (Fig. 3b). In addition, the clustering analysis grouped *P. hirsuta* individuals from the Écrins range with all other individuals of *P. hirsuta*, regardless of the number of genetic clusters chosen ($K = 4$, 5, 6; Fig. 3b). On the contrary, this analysis assigned individuals from Valgaudemar to a distinct genetic cluster for $K > 4$ (Fig. 3b), highlighting their genetic distinctiveness. In summary, our data show that the Valgaudemar lineage is

a narrow endemic lineage restricted to a single upper valley (c. 6×5 km) and surrounded by *P. hirsuta*, which is distributed across the rest of the Écrins range and beyond (Fig. 1). Both lineages grow less than 4 km away from each other, separated only by a ridge higher than 3000 m around most of the valley.

Endemism in the European Alpine flora has long been studied (Bresinsky 1965; Smyčka et al. 2017), and narrow endemics have recently been discovered in the *Primulaceae* family in particular, including *Primula albenensis* (Banfi and Ferlinghetti 2013), *Primula recubariensis* (Prosser and Scortegagna 1998) and *Androsace vesulensis* (Boucher et al. 2021). The Valgaudemar lineage is an important addition to this list of narrow endemics since its known range is even smaller than that of the aforementioned taxa. The major factors leading to the existence of narrow endemics in the Alps are hypothesized to be range fragmentation during glacial cycles and long-distance dispersal (Šingliarová et al. 2008; Smyčka et al. 2017). Both scenarios imply demographic bottlenecks and should lead to decreases in genetic diversity (Šingliarová et al. 2008). However, the Valgaudemar lineage does not show such a decrease compared with other populations in /Hirsuta (Table 1). This is probably due to the introgression event that has marked this lineage's history, which increased its genetic diversity and, thus, erased the signal of a former genetic bottleneck (Casazza et al. 2013; Szövényi et al. 2009). Demographic analyses actually recover this scenario with a drastic reduction in N_e in the ancestral Valgaudemar lineage (85-fold decrease, Table A.4.) before its introgression from *P. hirsuta* (Table A.2.).

Origins of the Valgaudemar lineage: introgression of an isolated lineage

Explaining the origin of the Valgaudemar lineage remains complex. While *P. hirsuta* grows in neighboring valleys, the closest populations of /Pedemontana are located more than 50 km away, a significant distance for primroses (Crema et al. 2013; Kropf et al. 2006). The absence of one of the two parent taxa in close proximity, plus the phylogenetic position of individuals from Valgaudemar at the base of /Pedemontana (Fig. 2) support an introgression scenario rather than a strict hybrid origin of the Valgaudemar lineage. This was confirmed by the diyABC analysis, in which both scenarios were statistically compared, the introgression scenario receiving unequivocal support (posterior > 0.99; Table A.3.). In this scenario, a divergence event occurred within /Pedemontana roughly 1 Myrs ago [95% CI 186–1830 kyrs] separating one lineage from the rest of the clade after the initial split between *P. hirsuta* and /Pedemontana c. 3.4 Myrs ago. This could correspond to the geographic isolation of a small population of /Pedemontana, due to range fragmentation following glacial cycles when the distribution

of /Pedemontana was potentially much larger (see below). In a later stage, around 65.3 kyrs ago [95% CI 10.3–417 kyrs], this isolated lineage experienced introgression from *P. hirsuta*, in a phase of secondary contact between these two lineages probably due to the post-glacial expansion of *P. hirsuta*. This kind of secondary contact after glacial cycles has already been found in several plants and animals of the European Alps like *Carex curvula* (Choler et al. 2004) or *Gonioctena* beetles (Kastally et al. 2019), and sometimes led to the emergence of hybrid lineages (Boucher et al. 2021; Capblancq et al. 2015; Casazza et al. 2012; L. Zhang et al. 2004). Indeed, allopatric divergence does not necessarily result in the rapid establishment of strong reproductive barriers between diverging populations (Kastally et al. 2019) and the rapid glacial cycles of the Pleistocene resulted in temporary fragmentation only. This is reflected by the emergence of hybrid zones between formerly isolated lineages when they came back into contact (Hewitt 2000; Taberlet et al. 1998).

However, in contrast to the previous studies of hybridization in alpine *Primula* (Casazza et al. 2013; Kadereit et al. 2011), we found a strong unidirectional pattern of introgression. Whereas the Valgaudemar lineage has roughly 40% of *P. hirsuta* alleles in its genetic background, individuals of *P. hirsuta* growing less than 4 km away do not show any traces of alleles from the Valgaudemar lineage in their genetic background (Fig. 1, Fig. 3b). Such an asymmetric introgression pattern can occur when one of the two parental species is much more abundant at the local scale than the other one, and thus saturates pollen flow (Minder et al. 2007; Reutimann et al. 2020). In our case, this could have happened because *P. hirsuta* is widely distributed across the Écrins range while the Valgaudemar lineage has a small population size. This hypothesis is not only supported by diyABC but also by the cpDNA phylogeny, which shows that almost all individuals of the Valgaudemar lineage have kept their ancestral /Pedemontana chloroplast (Fig.A.1.) and thus suggests that introgression from *P. hirsuta* must have happened mainly through pollen.

A new glacial refugium in the Southwestern Alps?

Rather unexpectedly, our discovery of the origin of the Valgaudemar lineage has important phylogeographic implications. As already discussed, this lineage is isolated from other populations of /Pedemontana: the closest populations of *P. pedemontana* are located > 50 km to the North in the Vanoise range, while the range of *P. cottia* lies > 50 km to the East in Queyras and the Cottian Alps (Fig. 1). This isolation could be due to long-distance dispersal of seeds by animals like the wallcreeper (*Tichodroma muraria*) or other birds, as hypothesized for *P. allionii* (Casazza et al. 2013). Alternatively, the

Valgaudemar lineage could have originated from range fragmentation of a formerly widely distributed ancestor. The highly fragmented distribution of species in /Pede-montana across the Western Alps (*P. cottia, P. pedemon-tana*, plus the Valgaudemar lineage), the Apennines (*P. apennina*) and the Cantabrians (*P. pedemontana*) makes this last scenario plausible, but determining the cause of isolation of the Valgaudemar lineage is impossible at this stage. Nonetheless, whether it is long-distance dispersal or fragmentation of a formerly wide distribution across the Western Alps, our results suggest an isolation of the Valgaudemar lineage from the rest of the /Pedemontana clade 1030 kyrs ago [95% CI 186–1830 kyrs], spanning several glacial cycles of the Pleistocene.

This isolated lineage most likely grew on siliceous rocks, according to the ecology of all members of /Hir-suta (including the Valgaudemar lineage), except for a few populations. The closest known glacial refugia on this kind of substrate were situated more than 50 km away in Mercantour or the Cottian Alps (Schönswetter et al. 2005). This last one was hypothesized by Crema et al. (2013) to be the glacial refugium of *P. cottia* and of the ancestor of *P. apennina,* but it is separated from the Écrins range by a vast expense of calcareous terrain. For these reasons, it seems very unlikely that the Valgaud-emar lineage occupied known glacial refugia from the Southern Alps. This suggests the existence of a glacial refugium in the southern part of the Écrins range. This refugium had been hypothesized before (Marx et al. 2017) but had not been tested using genetic data at such a fine spatial resolution. Geological evidence indeed suggests that the valleys of the southern part of the Écrins range were not heavily glaciated during the Last Glacial Maxi-mum, with large ice-free areas above 1500 m a.s.l. that have persisted for long periods of time and could, thus, have supported *Primula* populations (van der Beek and Bourbon 2008; Delunel 2010). In addition, following a similar biogeographic pattern, an extremely rare species in the southwestern Alps, the alpine clubmoss (*Lycopo-dium alpinum*), has a strictly isolated population in the same area (Dhien 1962). However, further investigations are needed to confirm this refugium hypothesis. Stud-ies focusing on other plant taxa that potentially survived in the same refugium might add additional evidence to assess the generality of this hypothesis.

Finally, following this long phase of isolation, *P. hirsuta* likely colonized the Écrins range during its post-glacial expansion from nunataks (*i.e.,* ice-free localities above the glaciers) and/or peripheral refugia located in the central Alps (Schonswetter et al. 2005; Schorr et al. 2012) and established secondary contact with the Valgaudemar line-age [65.3 kyrs ago (95% CI 10.3–417 kyrs) based on our analysis].

Conclusion

In this article, we have proven the existence of an extremely narrow endemic *Primula* lineage in the Écrins range. Detailed analyses have then revealed that it origi-nated following an uncommon evolutionary scenario: via introgression of a lineage that had formerly been isolated in this area of the Southwestern Alps. The Valgaudemar lineage is probably at the crossroads between two pos-sibilities: (i) will this lineage keep its integrity despite introgression from the neighboring *P. hirsuta* (Minder and Widmer 2008; Reutimann et al. 2020)? Or (ii) will it slowly disappear, erased by introgression from this most widespread species in a scenario of Sisyphean evolution (McKay and Zink 2015)? Further analyses will try to elu-cidate these possibilities and to determine the taxonomic status that this lineage deserves.

Declarations

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