

Mechanisms of speciation in the Alpine flora: study of the origins of a primrose taxon from Valgaudemar (Massif des Écrins, Western Alps).

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Context

The Écrins National park aims at clarifying the taxonomic status of a primrose population growing on its territory, which looks morphologically intermediate between *Primula pedemontana* Thomas ex Gaudin and *Primula hirsuta* All.. Stakes are high given that the conservation status of this taxon depends on its taxonomic assignment.

Following previous genetic analyses (Boucher et al. 2016 Molecular Phylogenetics and Evolution 104, 60-72), we decided to undertake larger sampling of this taxon across the Écrins National park but also to sample closely related species in order to understand both the origins and taxonomic status of primroses from the Écrins National park. For these analyses, a research convention was signed between the Écrins National park and the Laboratoire d'Écologie Alpine of Grenoble (LECA), for an amount of 10 000 \in .

Sampling has been completed in the summers of 2016, 2017, 2019 and 2020. This sampling has involved many people, which are cited at the end of this document in Table A1. It has targeted wild populations of the following taxa:

- *Primula sp.* across the Écrins National park (France)
- Primula hirsuta All. across all of the French Alps (France)
- Primula pedemontana Thomas ex Gaudin in the Vanoise range (France)

- *Primula cottia* Widmer in the Cottian Alps (Italy) and Queyras range (France)
- Primula apennina Widmer in the Northern Apennines (Italy)

Genetic analyses have then been undertaken in 2020 and 2021 on all collected samples with the aim of sequencing thousands of single nucleotide polymorphisms, which can help to discriminate between closely-related lineages. In order to do so we used the high-throughput genotyping technique known as double-digest RAD-sequencing (Peterson et al. 2012 PlosOne, https://doi.org/10.1371/journal.pone.0037135). Genetic analyses have been done by Delphine Rioux and Florian C. Boucher at LECA.

Data yielded by this genotyping procedure were then analyzed by Camille Voisin during his first year Master's internship in the spring of 2021. The main results that were found is that the Écrins National park actually hosts a distinct taxon, which is confined to a single valley of its territory: the Valgaudemar, and that this taxon is most likely of hybrid origins with *Primula pedemontana* Thomas ex Gaudin and *Primula hirsuta* All. as parents. The following of this report is made of Camille Voisin's masters thesis.

1. Introduction

Wild floras in all alpine areas (i.e. high-elevation habitats above the climatic treeline; Körner 2003) are known to be rich (Kier & al., 2005; Barthlott & al., 2005; Spehn & al., 2011), and have attracted lots of naturalists since the expeditions of Villars, Lapeyrouse or de Saussure in European mountains in the 19th century (Pépy, 2020). Covering 3% of the land outside Antarctica, alpine areas are distributed all over the world (Körner & al., 2011; Testolin & al., 2020; Leist, 2000). These places are characterized by a varied history of climate change and strong microhabitat differentiation (Körner 2003), and include hotspots of biodiversity like in the Himalayas or the Andes. The richness of these high elevation zones is in contrast with the general latitudinal diversity gradient (Testolin & al., 2021). Until now, the explanation for this richness remains a challenge for biogeography and evolutionary biology.

In the European Alps, the main driver of plant diversity is accepted to be topography (Marini & al., 2008; Körner 2003). In fact, the high summits and deep valleys promote habitat fragmentation and thus population isolation. Moreover, the history of alpine areas, with alternating warm periods and ice ages, created alpine refugia (Schönswetter & al., 2005). The combination of these two factors (geographical and temporal) enhance the isolation of populations, and can lead to genetic differentiation. Following recolonization after glacial retreat, if the genetic differentiation between populations is important enough, it leads to an increase of biodiversity richness (Flantua & al., 2018; Feliner 2011). Nevertheless, the understanding of lineage diversification in mountain plants imply a grasp of complex interaction between geographical, climatic processes and environmental factors (Ebersbach & al., 2017; Kadereit & al., 2017) through time and space.

In fact, in the European alps, *in situ* diversification implies different kinds of drivers with more or less relative importance (genetic barriers, phenology, ecology..., Boucher & al., 2016b; Kadereit & al., 2017). The emergence of molecular analyses creates new approaches to explain the change in the diversification rate and the causes it implies. The major accepted explanation of population divergence is gene flow restriction (Slatkin 1985).

Here we chose to focus on the largest endemic group of European alpine system: *Primula sect. Auricula* (Ozenda 1995) and more precisely on the youngest group of the section (Kress, 1989), the /Hirsuta clade (Boucher & al., 2016a). The /Hirsuta clade is composed of four species: *Primula apennina*, *P. cottia*, *P. hirsuta* and *P. pedemontana*. All of these species are perennial, with pink or purple corollas, and grow mainly on acid soils in the alpine and subalpine altitudinal belts (Zhang & al., 2004a).

Previously observed by botanists in the Ecrins National Park (hereafter referred to as 'ENP'; Boucher & al. 2016a), a primrose population from the Valgaudemar valley presents unclear morphological characters. Despite sharing some similarities with *P. hirsuta*, it was mainly described as *P. pedemontana* in the past. But, this population is disjunct from the rest of the species distribution, the closest *P. pedemontana* population growing in the Vanoise range, 50km away. The last phylogenetic analysis defined the Valgaudemar lineage as a potential new taxon, or a subspecies of *P. pedemontana* (Boucher & al., 2016a), but this conclusion awaits further investigation.

Understanding the origin of this isolated lineage would increase our understanding of the functioning of endemism in the Alps and more generally of the maintaining of species diversity.

Identifying and explaining *Primula* history could also help to obtain the required knowledge for their conservation (Gaston 2000). In face of ongoing global warming, protected areas like the ENP need the support of scientific research to support them in the maintaining of ecosystem biodiversity, by the protection of species and their evolutionary potential (Forest & al., 2007; Nielsen & al., 2020) for a species long-term persistence (Saura & al., 2019). More specifically, a good understanding of the origin of the Valgaudemar lineage could lead to a long-term conservation plan in the ENP.

In this study we used thousands of Single Nucleotide Polymorphisms (SNPs) from 149 samples from the French and Italian Alps obtained by double-digest restriction-site-associated DNA sequencing (ddRADseq; Peterson & al., 2012) to clarify the historical and phylogeographic origin of the Valgaudemar primrose and explain its origin. Our aims are (i) to determine whether different lineages of /Hirsuta clade coexist in the ENP; (ii) refine the phylogeny of this group; and (iii) understand and explain the complex origin and biogeographic history of this lineage as a potential key of diversification in European Alps.

Our molecular data clarifies the genetic diversity of the /Hirsuta clade, and reveals a complex hybridization or admixture origin of the Valgaudemar lineage leading to a potential sub-taxon in the Ecrins National Park. Finally, resolving the evolutionary history of this *Primula* from the Valgaudemar has the potential to yield insights into the diversification of the Alpine flora and thus bring new insights into high mountain evolutionary patterns.

2. Material and methods

2.2 Taxon sampling

Our study focused on /Hirsuta, a clade including *P. hirsuta* All., *Primula apennina* Widmer, *P. cottia* Widmer, and *P. pedemontana* Thom. ex. Gaudin (the last three species forming a clade named after /Pedemontana). These species are long-lived perennials forming rosettes or loose mats. The flowers are pink or violet and occur in early spring, usually only a few weeks after snowmelt (Kadereit & al., 2011). They are all specialized on acidic subtracts, grow on rocky habitats (e.g. crevices, rocks and stony pastures), only in the alpine and subalpine belt (1,400-3,000 m a.s.l.). *Primula apennina* grows only in the north of the Apennine mountains. *P.cottia* grows in the Cottian Alps (south-eastern France, north-western Italy). *P. hirsuta* has a wide distribution from the central to the western Alps and in the central Pyrenees. Finally, *P. pedemontana* has a disjoint distribution too, in the Cottian & Graian Alps (France & Italy) and in the Cantabrian Mountains (Zhang & al., 2004a).

We sampled a total of 141 individuals from /Hirsuta clade (see list of samples in Table A.1), including the four species presented above and samples from the ENP. All taxa were represented by at least 10 accessions each and were collected in a minimum of two localities (Fig. 1).

We also added eight accessions of the Valgaudemar lineage, a putative hybrid between *P. hirsuta* and *P. pedemontana*, or subspecies of the later (Boucher et al. 2016a) sampled in the highest part of the Valgaudemar valley in the ENP (France, 05). All samples included in the present studies were collected from wild populations during spring or summer in 2016, 2017, 2019 and 2020. Finally, we included an accession of *P. marginata* Curt. as an outgroup for phylogenetic analyses. Fig. 1 shows a map of all sampling locations.



Fig. 1. Distribution of the four species of /Hirsuta clade in the western Alps. The Valgaudemar lineage is labeled as 'P. Valgaudemar' and represented with red triangles. Symbols correspond to sampling location, see legend. Approximate distributions are drawn according to Zang & al. (2004a) and INPN (https://inpn.mnhn.fr/).

2.3 DNA extraction, sequencing

In order to gather robust information on thousands of SNPs, a double-digestion restriction site associated DNA sequencing (ddRADseq; Peterson & al., 2012) was conducted by F. Boucher and D. Rioux similarly to Boucher & al., 2021. The double-digestion was realized with the two enzymes PstI and MspI. At the end, the fragments were size-selected between 200 and 600 bp. The final four libraries were processed using Illumina technology to prepare libraries, which were then sequenced on a NovaSeq 6000 sequencer by Fasteris SA (Switzerland). This protocol allows to obtain a large number of loci (Peterson & al., 2012) throughout the genome, which has already proven useful to conduct robust genetic analysis, and especially for short time phylogenetic histories (e.g. Boucher & al., 2016a, 2021).

2.4 Bioinformatics pipeline

All sequenced ddRAD-seq tags (c. 350 million reads) were assembled using the program ipyrad (<u>https://github.com/dereneaton/ipyrad</u>) using default parameters except for a maximum of 5 heterozygous sites and 5 SNPs per *locus*. 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). Finally, after filtering, we had a total of 12,162 aligned loci for 40,915 SNPs.

2.5 Phylogenetic inference

Phylogenetic relationships within the /Hirsuta clade were inferred using maximum-likelihood inference carried out by IQ-TREE (Nguyen & al., 2015) on the concatenated SNP matrix, using one accession of *P. marginata* for outgroup rooting. We first kept individuals with less than 60% of missing data. We then removed loci with more than 50% of missing data, according to some studies showing similar results for samples with less than 50% missing data and less than 1% (Altermann & al., 2014). Preliminary phylogenetic analyses (not shown here) enabled the removal of some problematic individuals, probably recent hybrids. We finally had a total of 123 samples and 18545 SNPs for phylogenetic inference.

The best model of DNA substitution for this dataset, TVM+F+R3 (a transversion model with unequal base frequencies), was selected thanks to ModelFinder (Kalyaanamoorthy & al., 2017) among 153 possible ones according to the bayesian information criterion (BIC), and 1000 ultrafast bootstrap replicates (Hoang & al., 2018) were used to measure node support on this phylogeny.

2.6 Genetic diversity and differentiation

We first visualized genetic variation among individuals using PCA in the "factoextra" R package (Kassambara & al., 2017), in R version 4.0.5 (R core team, 2021). In all cases, 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 "LEA" R package (Frichot & al., 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 & al., 2014). Given that /Hirsuta clade comprises four species and since we expected the Valgaudemar lineage to be genetically different, 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 value of K (Frichot & al., 2014).

Genetic diversity was estimated using observed heterozygosity (H_0), population genetic diversity, allelic richness, inbreeding coefficient (F_{IS}) and population-specific F_{ST} using the hierfstat r package (Goudet, 2005). This package was also used to estimate pairwise F_{ST} (Weir & Cockerham, 1984) between populations.

2.7 Test of the hybrid origin of two populations

Given the observed patterns of multivariate analyses and genetic structure, we decided to test the putative introgression in the lineage of Valgaudemar (05) by measuring the hybrid index (Buerkle, 2005). This test was run using the introgress R package (Gompert & al., 2010) with *P. hirsuta* and *P. pedemontana* as parents and Valgaudemar population as the introgressed population.

Given that this analysis confirmed that these individuals were significantly introgressed (see Results), we then tested the possibility that the Valgaudemar lineage is of hybrid origin. We did so using the Approximate Bayesian Computation software diyABC (Cornuet & al., 2014). We compared three scenarios: (i) a pure phylogenetic one in which the Valgaudemar lineage was sister to *P. pedemontana*, (ii) one in which it resulted from hybrid speciation between *P.hirsuta* and *P. pedemontana*, and (iii) one in which the Valgaudemar lineage resulted from introgression from *P.hirsuta* into a "ghost" sister lineage of *P. pedemontana*, extinct today (Fig. 2). The second scenario is a strict hybrid origin of the Valgaudemar lineage, while the third is more closely related to an introgression scenario.



Fig. 2. Sketches of the three historical scenarios tested using diyABC. In each of them the blue lineage ('Pop 1' on the sketches) represents *P. hirsuta*, the green lineage ('Pop 2') represents *P. pedemontana*, the red lineage ('Pop 3') represents the Valgaudemar lineage, and the turquoise line 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.

To compare these three scenarios we randomly sampled two individuals from each population of *P*. *hirsuta* (Pop 1, 12 individuals in total), two individuals from each population of *P*. *pedemontana* including *P*. *cottia* because of it genetic proximity (Pop 2, 10 individuals in total), and add all individuals from the Valgaudemar lineage (Pop 3, 8 individuals in total). This random sampling was repeated twice to verify that it did not influence our inference. We then chose settings for simulations:

- constant effective population sizes on each branch
- wide, uninformative priors for effective population sizes (all log-uniform with minimum 10 and maximum 150,000). A strict calibration at 3.45 Myrs for the initial split between *P*. *hirsuta* and /Pedemontana clade (Boucher et al. 2016b) and log-uniform priors between 10,000 years and 3.45 Myrs for all subsequent divergence or reticulation events. The

proportion of genetic input from /P. pedemontana clade in the hybrid speciation scenario was uniform between 0.5 and 0.7, following results from 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 and 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 their posterior probabilities using a logistic regression on the 1% of simulated data closest to the observed data (Cornuet & 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.



3. **Results**

3.2 Phylogenetic inference

Fig. 3. Maximum likelihood phylogenetic tree for /Hirsuta clade 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 ENP are colored in light blue and individuals from outside the ENP in dark blue.

Maximum-likelihood analysis of our SNPs dataset produced a highly resolved phylogenetic tree, with all species forming clades supported by 100% bootstrap (Fig. 3). *Primula apennina* and *P. cottia* are sister clades, and together form a clade sister to *P. pedemontana*. This result corroborates



the /Pedemontana clade supported by Boucher & al., 2016a. Unlike other populations which form well supported clades in the tree, individuals from the Valgaudemar lineage form a grade (i.e., a paraphyletic group), which is located at the base of /Pedemontana clade.

3.3 Genetic diversity and differentiation

Fig.4. Genetic structure within /Hirsuta clade. (a, b) Principal component analysis (PCA) results on the two first PCs including all populations (a) and focus on the Valgaudemar population (b). (c) 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. APE = *P. apennina*, COT = *P. cottia*, HIR = *P. hirsuta*, ENP = Sample from Ecrins national park, HV = Sample from Haut Valgaudemar.

The genetic PCA with all populations shows proximity between *P. pedemontana*, *P. cottia* and *P. apennina* (Fig.4a), in agreement with phylogenetic results (Fig. 3). The PCA restricted to French Alps show that all populations of *P. hirsuta* completely overlap, individuals from the Mont Blanc range being the most distant (Fig.4b). More importantly, individuals from the Valgaudemar population are intermediate between *P. hirsuta* and *P. pedemontana* with no overlap with either of these two species.

The nonparametric K-means clustering procedure shows bayesian information criterions (BIC) really close for K = 4, 5 & 6. For K=4, all species are assigned to a distinct genetic cluster, and the Valgaudemar lineage shows an equal genetic proportion between *P. hirsuta* and *P. pedemontana* (Fig. 4c). K=5 shows the same genetic pattern except for the Valgaudemar lineage, which forms a distinct cluster. Finally, for K=6, *P. pedemontana* is separated into two genetic groups corresponding to populations from the Maurienne and Tarentaise valleys respectively.

Genetic diversity was roughly similar in all populations, except for populations of *P. apennina* that are twice less diverse (Tab. 1a). The pairwise F_{ST} between all /Hirsuta populations is shown in Tab. 1b, the Valgaudemar lineage showing maximum proximity with the *P. hirsuta* of the ENP range, with an $F_{ST} = 0.166$.

(a)	(a) Genetic diversity (Hs)												
PSA	ENP	HV	AB	AN	СР	HCF	HCN	HL	HT	PE	PF	PGU	PHP
0.21			0.12				0.21			0.19			
2	0.258	0.223	4	0.117	0.212	0.283	7	0.173	0.251	2	0.210	0.248	0.254
(b)	(b) Pairwise F _{st}												
	ENP	HV	AB	AN	СР	HCF	HCN	HL	HT	PE	PF	PGU	PHP
HV	0.166	-	-	-	-	-	-	-	-	-	-	-	-
AB	0.413	0.403	-	-	-	-	-	-	-	-	-	-	-
AN	0.420	0.415	0.13 5	-	-	-	-	-	-	-	-	-	-
СР	0.314	0.223	0.31 2	0.301	-	-	-	-	-	-	-	-	-
HCF	0.105	0.253	0.49 7	0.504	0.336	-	-	-	-	-	-	-	-
HCN	0.116	0.276	0.52 4	0.524	0.356	0.075	-	-	-	-	-	-	-
			0.54				0.13	_	_	_	_	_	_
HL	0.101	0.304	3	0.547	0.383	0.087	1						
нт	0.191	0.326	0.55 4	0.567	0.376	0.079	0.19 0	0.227	-	-	-	-	-

Tab.1. Genetic diversity (a) and pairwise F_{ST} values (b) measured between all /Hirsuta populations. Population labels refer to the sampling name (see Table A.1). Population labels are color refer to the species color in PCA and phylogenetic tree.

			0.41				0.37						
PE	0.363	0.290	3	0.414	0.232	0.360	9	0.419	0.389	-	-	-	-
			0.44				0.37			0.10			
PF	0.382	0.331	1	0.443	0.271	0.346	7	0.435	0.369	8	-	-	-
			0.44				0.33			0.15			
PGU	0.342	0.292	3	0.444	0.273	0.318	5	0.411	0.332	9	0.153	-	-
			0.49				0.05			0.36			
PHP	0.082	0.243	4	0.497	0.334	0.003	5	0.082	0.116	7	0.361	0.319	-
			0.37				0.34			0.15			
PSA	0.317	0.211	3	0.373	0.177	0.322	1	0.381	0.349	2	0.202	0.191	0.324

3.4 Test of hybrid origin

The 'introgress' analysis showed a proportion between 51 to 65% of alleles derived from *P*. *pedemontana* in the genetic background of the height individuals from the Valgaudemar lineage, confirming the possible hybrid origin of this lineage.

The diyABC analysis showed clearcut support for the introgression scenario, its posterior probability being > 0.99 for both replicates (Tab. 2). Parameter estimations showed a drastic reduction in the effective population size of the ghost lineage that later gave rise to the Valgaudemar lineage (size reduced by 85 in comparison to the population size of *P. pedemontana*). Base on a split between *P. hirsuta* and the rest of /Hirsuta clade at 3.47 Mya (Boucher & al., 2016b), the ABC analysis estimated the ghost lineage emergence to be 5.4 times more recent (replicate 1: 659 kyrs [95% CI: 115-1360 kyrs] ; replicate 2: 596 kyrs [95% CI: 103-1270 kyrs]), and the admixture event between this ghost lineage and *P. hirsuta* to be 74.5 times more recent (replicate 1: 57.8 kyrs [95% CI: 10.4-395 kyrs] ; replicate 2: 34.8 kyrs [95% CI: 10-213 kyrs]).

Tab. 2. Results of the ABC procedure with the three scenarios for each of the two random sampling. For each analysis, the posterior probability and confidence in scenario choice are given (95% confidence intervals in brackets [CI95].

Scenario (Sample1)	Posterior probability [CI95]	Type I error	Type II error
Simple phylogenetic split	0.0000 [0.0000,0.0000]		
Hybrid origin	0.0003 [0.0001,0.0004]		
Isolation and introgression	0.9997 [0.9996,0.9999]	0.285	0.142
Scenario (Sample2)	Posterior probability [CI95]	Type I error	Type II error
Simple phylogenetic split	0.0000 [0.0000,0.0000]		
Hybrid origin	0.0001 [0.0000,0.0001]		
Isolation and introgression	0.9999 [0.9999,1.0000]	0.278	0.15

4. Discussion

Our large DNA dataset allows us to produce a stronger support phylogenetic hypothesis for the /Hirsuta clade (Boucher & al., 2016a; Fig. 3). Our analyses confirm that *P. hirsuta* is sister to the /Pedemontana clade, which comprises *P. pedemontana* and the two Italian lineages *P. cottia* and *P. apennina*.

4.2 Clarification of Primula diversity in the Ecrins national park

The sampling protocol realized in ENP is characterized by individuals regularly sampled across the ENP, rather than a few focal populations as done elsewhere. This protocol allows us to explore the genetic structure of /Hirsuta clade across the park at a fine spatial scale. We found here that two taxa coexist at a small scale in the ENP, one limited to a small valley, and the other widespread in all the park (Fig. 1).

In fact, the PCA shows an overlap between the ENP sampling and all other *P. hirsuta* populations (Fig. 4b). Adding to this result, the clustering analysis groups *P. hirsuta* individuals from the ENP with all other individuals of *P. hirsuta*, regardless of the number of genetic clusters chosen (K= 4,5,6; Fig. 4c). According to these two analyses, we can assert that the whole diversity of /Hirsuta clade in the ENP is composed of *P. hirsuta* species with only one exception. This distinct population is located only in the highest part of the Valgaudemar valley.

To summarize, the Valgaudemar lineage is restricted to a small valley (c. 6 x 5 km) and is surrounded by *P. hirsuta* in all the park territory. The two lineages grow less than 4km away from each other, separated only by a ridge line. They share the same habitat (siliceous rocks of the subalpine and lower alpine elevation belts), and yet, one of the two is reduced to a single valley. We face here to a narrow endemic lineage, a subject long studied for the European Alpine flora (Bresinsky, 1965; Smyčka & al., 2017), even in the Primulaceae family (Boucher & al., 2021).

The major factors leading to the existence of narrow endemics in the Alps are range fragmentation by recent climate changes and long-distance dispersal (Šingliarová & al., 2008; Lutz & al., 2000). According to Singliarova & al. (2008), the endemic population resulting from vicariance or long-distance dispersal should present some decrease of the genetic diversity. In our case, the Valgaudemar lineage doesn't show any decrease in genetic diversity (Tab. 1). But this genetic signal can be hidden by admixture events which enhance genetic diversity (Szövényi & al., 2009; Casazza & al., 2013a). In fact, allopatric divergence does not necessarily result in the rapid establishment of strong barriers to reproduction between the diverging populations (Kastally & al., 2019). Moreover, the rapid glacial cycles of the Pleistocene in Europe resulted in a short time population fragmentation followed by the emergence of multiple hybrid zones between formerly isolated lineages when they came back into contact (Hewitt, 2000; Taberlet & al., 1998).

4.3 Hybridization history of the Valgaudemar lineage

The Valgaudemar lineage was observed as a "strange *P. pedemontana*" population for several decades¹. According to Boucher & al. (2016a), the Valgaudemar lineage presents the same general habit as *P. hirsuta* (i.e. scapes shorter than leaves), but leaf pubescence similar to that of *P. pedemontana*. In fact, genetic clustering (Fig. 4c) shows a probability of assignment nearly equal for the two species (K=4). In the same way, the PCA analysis situated the Valgaudemar lineage between the two other species. Specific analyses confirmed introgression of *P. hirsuta* in the background of the Valgaudemar lineage.

But explaining the origin of the Valgaudemar lineage remains complicated because the closest population of *P. pedemontana* is located 50km away, a significant distance for this kind of organism

¹ <u>https://www.ecrins-parcnational.fr/actualite/lenigme-de-la-primevere-du-valgaudemar</u>

(Crema & al., 2013; Kropf & al., 2006). The ACP (Fig. 4b), 'introgress' results and also its phylogenetic position as a grade at the base of the /Pedemontana clade (Fig. 3) leads us to think of an introgression hypothesis more than a hybrid origin of the Valgaudemar lineage. This was confirmed by the diyABC analysis, the introgression scenario receiving unequivocal support (posterior >0.99; Tab. 2). In this scenario, after the initial split between *P. hirsuta* and the /Pedemontana clade, another divergence event occurred within the /Pedemontana clade, separating one lineage from the rest of the clade. This could correspond to the geographic isolation of a small population of *P. pedemontana* because of climatic events for example. In a later stage, this isolated lineage was introgressed by *P. hirsuta*, which could have occurred after secondary contact between these two lineages, due for example to the post glacial migration of *P. hirsuta*.

This kind of secondary contact after glacial cycles was well illustrated for several plants and animals in European Alps like *Carex curvula* (Choler & al., 2004), *Gonioctena* beetles (Kastally & al., 2019), with sometimes the emergence of hybrid lineages (Casazza & al., 2012; Capblancq & al., 2015; Boucher & al., 2021). Consequently to this secondary contact, if reproductive isolation is not complete, gene flow can act again. In fact, although hybridization has played a role in the emergence of a few species in *Primula* (Casazza & al., 2012; Zang & al., 2004b) this situation remains rare in nature (Kadereit & al., 2011).

Surprisingly, unlike in the examples cited above, we found in the Valgaudemar valley a strong pattern of gene flow, showing about 40% of *P. hirsuta* in the genetic background of the Valgaudemar lineage (Fig. 4c). In contrast to the few studies of hybridization in *Primula* (Casazza & al., 2013a; Kadereit & al., 2011), we found a unidirectional introgression pattern. In fact, the closest individuals of *P. hirsuta* do not show any signs of *P. pedemontana* alleles in their genetic background, while they are growing less than 4km apart from the Valgaudemar lineage (Fig. 1, Fig. 4c). 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 (Lepais & al., 2009; Reutimann & al., 2020), and thus saturates pollen flow (Minder & al., 2007). In our case, *P. hirsuta* is widely distributed across the ENP, surrounding the Valgaudemar lineage.

Finally, despite strong introgression or hybridization patterns, species can keep their integrity (Minder & al., 2008; Reutimann & al., 2020). We are probably facing a crucial event here, at the crossroads between two possibilities: the future disappearance of the Valgaudemar lineage, erased by introgression from *P. hirsuta*; or the maintenance of the lineage, with perhaps speciation in the long term.

4.4 Implications for biogeographic history in the ENP

Long-distance dispersal is considered to be a rare event, although of great biogeographic importance (DeQueiroz, 2005; Piñeiro & al., 2007). In alpine primroses, long-distance dispersal of seeds could be due to animals, like the wallcreeper (*Tichodroma muraria*) or other birds as hypothesized for *P*. *allionii* (Casazza & al., 2013a). Unfortunately we can't investigate the true origin of the Valgaudemar lineage isolation. But, 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 659 kyrs ago [95% CI: 115-1360 kyrs], during glacial cycles of the Pleistocene.

This isolated lineage most likely grew on acid soils, according to the ecology of all the /Hirsuta clade. But the closest known glacial refugia on this kind of substrate were more than 50km away in Mercantour or the Cottian Alps (Schönswetter & al., 2005), separated from the ENP by a large territory of calcareous habitats. In fact, peripheric refugia between the western Alps and Apennines seems to have had a great importance for other species in *Primula* (Schorr & al., 2012; Crema & al., 2013; Moore & al., 2013), but these species show a present distribution confined near the Cottian Alps or Apennines. Moreover, it has been shown that during post glacial expansion, species could be limited by competition with other co-generic taxa already present (Taberlet & al., 1998; Casazza & al., 2013b). These results lead us to hypothesize the existence of a glacial refugium in the southern part of the ENP near the Valgaudemar valley, an area never before considered as a potential refugium. To confirm this refugium hypothesis further investigations are necessary, like the rate of rarity and private alleles (Schorr & al., 2012; 2013), a good indicator of former refugium survival.

Following this isolation, and during the post glacial expansion, *P. hirsuta* likely colonised the ENP from nunatak (i.e. ice-free localities above the glaciers) and/or peripheral refugia (Schorr a al., 2012; Schonswetter & al., 2005) and could then have established a secondary contact with the Valgaudemar lineage (57.8 kyrs ago [95% CI: 10.4-395 kyrs] based on our analysis). This secondary contact led to genetic introgression as our results indicate. But in order to figure out the possible future of this endemic lineage, one needs to test the strength of current reproductive barriers between the Valgaudemar lineage and *P. hirsuta*, using experimental crossbreeding for example. Moreover further analyses could refine our understanding of the history of the Valgaudemar lineage, especially testing multiple introgression events, or long-time introgression duration, more than a unique event as we choose to test.

Finally, in front of this specific lineage, maybe in a crucial moment for his persistence, we hope this work will provide useful information to facilitate the work of field botanists and officers, and will help the ENP in his long-time management.

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Appendix

Table A.1. Sampling of /Hirsuta clade from the Western Alps. Individual code (Code), species name (Taxon), collect place (Locality), longitude and latitude (Longitude & Latitude), name of the collectors (Collector) and number of samples in a location (No. of individuals). All samples were collected during spring or summer in 2016, 2017, 2019 and 2020.

Code	Taxon	Locality	Longitud e	Latitude	Collector	No. of individual s
16_17	P. sp.	Haut Valgaudemar, Ecrins, FR	6.278518	44.8416 3	C. Dentant	1
16_18	P. sp.	Haut Valgaudemar, Ecrins, FR	6.277454	44.8362 8	C. Dentant	1
16_19	P. sp.	Haut Valgaudemar, Ecrins, FR	6.274271	44.8404 6	C. Dentant	1
16_20	P. sp.	Haut Valgaudemar, Ecrins, FR	6.273984	44.8441 3	C. Dentant	1
16_21	P. sp.	Haut Valgaudemar, Ecrins, FR	6.273857	44.8444 1	C. Dentant	1
16_22	P. sp.	Haut Valgaudemar, Ecrins, FR	6.275406	44.8475 0	C. Dentant	1
16_8	P. sp.	Haut Valgaudemar, Ecrins, FR	6.317412	44.8092 1	D. Vincent	1
16_9	P. sp.	Haut Valgaudemar, Ecrins, FR	6.291150	44.8445 0	D. Vincent	1
16_(11, 12, 13, 14)	P.hirsuta	Ecrins national park, FR	6.359702	44.8352 0	C. Dentant	4
16_1	P.hirsuta	Ecrins national park, FR	6.361284	44.8340 9	C. Albert	1
16_10	P.hirsuta	Ecrins national park, FR	6.360372	44.8338 3	C. Dentant	1
16_15	P.hirsuta	Les Bans, Ecrins, FR	6.359633	44.8361 2	C. Dentant	1
16_16	P.hirsuta	Ecrins national park, FR	6.208063	44.9070 9	E. Ollieu	1
16_2	P.hirsuta	Ecrins national park, FR	6.359292	44.8350 4	C. Albert	1
16_23	P.hirsuta	Ecrins national park, FR	6.074998	44.9660 9	N. Bertrand	1
16_24	P.hirsuta	Ecrins national park, FR	6.061500	44.9625 0	C. Dentant	1
16_25	P.hirsuta	Ecrins national park, FR	6.165178	44.7630 8	R. Bonet	1
16_27	P.hirsuta	Ecrins national park, FR	6.181543	44.7440 2	R. Bonet	1
16_3	P.hirsuta	Ecrins national park, FR	6.357594	44.8364 7	C. Albert	1
16_4	P.hirsuta	Ecrins national park, FR	6.171329	44.8487 1	B. Merhan, C. Albert	1
16_5	P.hirsuta	Ecrins national park, FR	6.101974	44.9559 3	inconnu	1
16_6	P.hirsuta	Ecrins national park, FR	6.315605	44.9012 0	inconnu	1
16_7	P.hirsuta	Ecrins national park, FR	6.168300	44.7994 9	inconnu	1
17_1	P.hirsuta	Ecrins national park, FR	6.092379	44.7549 7	R. Bonet	1
17_16	P.hirsuta	Close to Lac des Fetoules, Oisans, FR	6.216100	44.9053 2	E. Ollieu	1
17_17	P.hirsuta	Ecrins national park, FR	6.176323	44.9422 3	S. Durix	1

17_19	P.hirsuta	Ecrins national park, FR	6.206723	44.9309 3	E. Ollieu	1
17_2	P.hirsuta	Ecrins national park, FR	6.089706	44.7549 8	R. Bonet	1
17_20	P.hirsuta	Vallon des Etancons, Oisans, FR	6.294613	44.9613 4	M. Garnier	1
17_21	P.hirsuta	Ecrins national park, FR	6.449700	44.8894 4	T. Maillet	1
17_23	P.hirsuta	Ecrins national park, FR	6.485299	44.9300 4	C. Albert	1
17_24	P.hirsuta	Ecrins national park, FR	6.418016	44.8565 7	C. Albert	1
17_25	P.hirsuta	Ecrins national park, FR	6.448819	44.7333 6	C. Albert	1
17_3	P.hirsuta	Ecrins national park, FR	6.121750	44.7633 1	R. Bonet	1
17_36	P.hirsuta	Ecrins national park, FR	6.164385	44.8036 0	D. Vincent	1
17_39	P.hirsuta	Ecrins national park, FR	6.163486	44.8063 6	D. Vincent	1
17_5	P.hirsuta	Ecrins national park, FR	6.381214	44.8095 1	C. Albert	1
17_7	P.hirsuta	Chalet du Lauvitel, Oisans, FR	6.069334	44.9742 0	E. Ollieu	1
HCF	P. hirsuta	Col de la Croix de Fer, Grandes Rousses, FR	6.20345	45.2272 5	F. Boucher	5
HCN	P. hirsuta	Cliff above the Col du Noyer, FR	5.98127	44.6880 6	F. Boucher	7
HL	P. hirsuta	Ascent to Laurichard, Écrins, FR	6.40109	45.0205	F. Boucher / G. Casazza	8
нт	P. hirsuta	Jardin du Talèfre, Mont-Blanc, FR	6.878017	45.9371 9	S. Lavergne	3
PHP	P. hirsuta	Close to lac des Pourettes, Chamrousse, FR	5.89983	45.1347 8	F. Boucher	8
PE	P. pedemontana	GR above l'Écot, Vanoise, FR	7.08914	45.3767 5	F. Boucher / M. Jaunatre	12
PF	P. pedemontana	GR above la Fesse haut, Vanoise, FR	6.85664	45.2064 5	F. Boucher / M. Jaunatre	10
PGU	P. pedemontana	La Gurraz, path to refuge de Turia, Vanoise, FR	6.89472	45.5487	F. Boucher	12
PSA	P. pedemontana	Tignes, Vanoise, FR	6.955117	45.4689 2	F. Boucher	12
AB	P. apennina	close to Punta Buffanara, Appenins Nord, IT	10.19493	44.3168 9	F. Boucher / P. Dufour	10
AN	P. apennina	Ascent to la Nuda, Apennins Nord, IT	10.24093	44.2850 9	F. Boucher / P. Dufour	10
CB1	P. cottia	Under the Col de Bouchet, Queyras, FR	7.01765	44.8194 7	F. Boucher / S. Lavergne	1
СР	P. cottia	Prali road, locus classicus, Piémont, IT	7.06126	44.9186 5	F. Boucher / P. Dufour	10
MA1	P. marginata	Mercantour, FR	7.001372	44.1514 1	F. Boucher	1