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ORIGINAL ARTICLE

Genetic divergence of *Clematis alpina* in the Swiss Prealps: a tale of the margins

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Abstract
The alpine clematis (*Clematis alpina* L., Ranunculaceae) is a liana growing in mountain regions of northern Eurasia. In the European Alpine region, where the subspecies *C. alpina* subsp. *alpina* occurs, a few populations are isolated from the main range in the peripheral north-western Prealps, a relatively understudied but biogeographically important region. It has been shown that such edge populations could potentially contain a source of unique genetic variability that reflects past biogeographical and microevolutionary processes. We tested this hypothesis using sequence capture data and a large population sampling across the species range. We show that individuals from the north-western Prealps form a genetic cluster that is clearly distinct from the other individuals of the European subspecies *C. alpina* subsp. *alpina.* This cluster adds to two other geographically driven clusters with a larger spatial extent, that include populations from the rest of the Alps and from the Carpathians/Balkan Peninsula mountains, respectively. Genetic diversity indices such as inbreeding and nucleotide diversity are the highest and lowest, respectively, for the Prealps populations indicating a possible loss of diversity. Our results demonstrate the biogeographical importance of isolated, marginal populations as sources of distinctive lineages, and highlight the conservation value of the north-western Prealps populations of alpine clematis. They also point out the promising use of sequence capture of gene selected for studies at high phylogenetic level for studies at intraspecific level.

Keywords Ranunculaceae · North-western peripheral Alps · Genetic diversity · Range edge populations · Conservation · DNA targeted capture

Introduction

The north-western Prealps (hereafter Prealps), the north-western periphery of central Alps, from Haute-Savoie in France to Lake of Thun in Switzerland, form a well-defined biogeographical and geological unit dominated by sedimentary rocks (Gilomen 1941; Becherer 1972; Caron 1973). They are composed of an archipelago of peripheral small chains and isolated summits (maximally 2400 m in altitude), and occupy an area which is ca. 120 km long and 35 km wide (Gerber et al. 2010). The Prealps region has been described as an important area of plant diversity in Switzerland due to its role as a peripheral refugium during the Quaternary glaciations (Gerber et al. 2010). Its flora is characterized by unique genetic and species composition (Parisod 2022) and includes several endemic plant taxa (Eggenberg and Landolt 2006; Naciri and Gaudeul 2007; Parisod 2008; Berthouzoz et al. 2013). Yet, compared to the Inner Alps, the flora of the Prealps and its genetic diversity remain relatively understudied (Landolt 2006; Gerber et al. 2010; Parisod 2022).

The alpine clematis (*Clematis alpina* L., Ranunculaceae) is one of the many emblematic species of the alpine region

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(including the Prealps), where it grows at altitudes from 700 up to 2000 m a.s.l. (Nechwatal 2004). The geographical range of the alpine clematis extends from the Western Alps in Europe to northeast Asia (Yang et al. 2009). According to the latest revision of the Clematis section *Atragene* (Yang et al. 2009), *C. alpina* comprises three allopatric subspecies: *C. alpina* subsp. *alpina*, *C. alpina* subsp. *sibirica* and *C. alpina* subsp. *ochotensis*. *Clematis alpina* subsp. *alpina*, the focus of our study, can be identified by its violet-blue flowers (typically composed of four tepals), feathery fruits and opposite, threefold pinnate leaves (Lauber et al. 2018). *Clematis alpina* subsp. *sibirica* is similar to the subspecies *alpina*, with the exception of its yellow or white flower. The shape of the staminodes and leave margins are morphological characters that allow to distinguish subsp. *alpina* from subsp. *ochotensis*. In Europe, the distribution of subsp. *alpina* exhibits a disjunct distribution (Fig. 1): In the Alps, its range includes two disjunct parts in the Western Alps (France) and the Eastern Alps (Austria, Germany, Slovenia, Italy and the Swiss cantons of Graubünden and Ticino). Outside these regions, few isolated populations also occur at the northern edge in the Swiss cantons of Fribourg and Bern in the Prealps (Aeschimann et al. 2004; Nechwatal 2004). Apart from the Alps, *C. alpina* subsp. *alpina* also occurs eastwards across the Carpathians (Mirek et al. 2020) and in the mountains of the Balkan Peninsula, e.g., in the Stara Planina and Rhodopes (Bulgaria, Markova 1970; Tashev and Starokina 1998).

Plant populations located at the range edges of species have been shown to be of high conservation value (Channell 2004; Suchan et al. 2019; Caissy et al. 2020). They are possibly adapted to different environmental conditions than the core populations and therefore represent an important source of genetic variability (Rehm et al. 2015). Genetic diversity is essential for the ability of a species to persist during periods of rapid climate change such as global warming (Savolainen et al. 2004; Jump et al. 2009; Geml et al. 2010). Hence, studying the genetic diversity of range-edge populations is an important subject in plant conservation research (Caissy et al. 2020). Such populations can also serve as models for understanding the biogeographic history of a taxon (Hampe and Jump 2011). Indeed, although the species diversity of marginal areas in the northern Alps is lower than in the marginal areas of the southern Alps (Aeschimann et al. 2011; Kadereit 2024), the genetic diversity has been shown to be higher for several species in the former (Gugerli et al. 2008; Taberlet et al. 2012). The origin of this pattern is however not well understood and the population dynamics and recolonization process in the northern margins of the Alps has been less studied.

In Switzerland, *C. alpina* subsp. *alpina* is under total protection in the cantons of Bern, Fribourg, and Ticino (InfoFlora 2023). According to the regional red list of the Swiss vascular plants, it is classified as vulnerable in the northern Alps and near threatened in the southern Alps (Bornand et al. 2019), but it is treated as least concerned at the national level (Bornand et al. 2016). Species, such as the alpine clematis, that are threatened regionally but not nationally, are often given less consideration in Swiss conservation projects. Moreover, besides a single morphological study on *C. alpina* (Yang et al. 2009), little is known about its ecological preferences and the genetic diversity and distinctiveness of its range-edge populations. To fill this knowledge gap, we decided to study the genetic diversity of *C. alpina* subsp. *alpina* by addressing the following research questions: (1) What is the genetic structure of the European *C. alpina* subsp. *alpina* populations? (2) Do the marginal populations from the Prealps show genetic particularities compared to the rest of the species' range? (3) What is the heterozygosity and inbreeding levels of these isolated populations? Our results on the distribution and genetic diversity of the alpine clematis contribute to a better understanding of the isolated, marginal plant populations. They also provide the Swiss federal and cantonal authorities with the necessary information to take appropriate measures to preserve the isolated Swiss populations of the alpine clematis.

Materials and methods

Fieldwork for population inventory

Fieldwork was conducted between May and September 2022. Our main objectives were to assess the distribution of *C. alpina* subsp. *alpina* in the Prealps and to gather data about the species in Switzerland. We consulted the National Data and Information Centre of the Swiss Flora (InfoFlora), which includes all reported occurrences as well as historical records, some dating back to the nineteenth century. A portion of these historical data originates from herbarium collections preserved at the Natural History Museum of Fribourg (MHNF). Occurrence data were also communicated by local botanists. At the beginning of the project, occurrences of *C. alpina* subsp. *alpina* were only known from eight sites in the western Prealps: the Boltigen region, Dent de Vounetse, Euschelspass, Gantrisch, Hürlisbode, La Gissetta, La Tzintre and Les Recardets. To further assess the distribution of the alpine clematis populations in the western Prealps, a comprehensive survey was conducted. This involved visiting all known sites with historical records of this taxon dating back to the nineteenth century, and prospecting nearby habitats with similar characteristics. The individuals from the Prealps are predominantly found on cliffs with northern and northwestern exposures. We thus

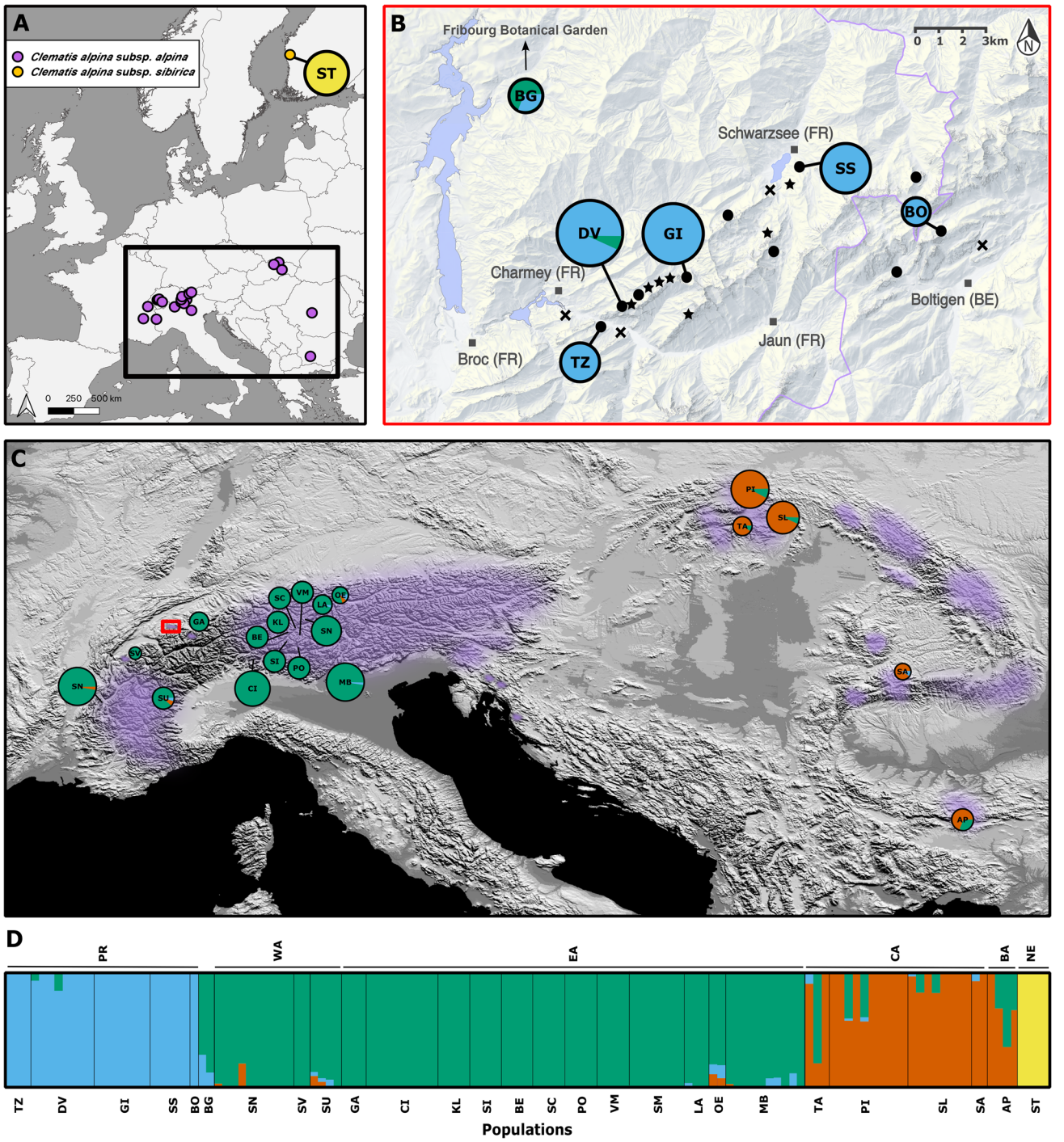

Fig. 1 **A** Map showing the sampling sites of *C. alpina* subsp. *alpina* across its european range as well as the sampled *C. alpina* subsp. *sibirica* population. **B** Distribution of *Clematis alpina* subsp. *alpina* in the Prealps, corresponding in panel C to the red rectangle. Locations obtained from *Infoflora* and confirmed are represented by a circle, those not confirmed by a cross and the newly discovered sites by a star—*map by map.geo.admin***C** Geographical distribution of *C. alpina* subsp. *alpina* obtained consulting GBIF occurrence data, iNaturalist and literature (purple area) with populations analyzed in this study represented as pie charts colored according to FastStructure results and size according to number of sampled individuals. **D** FastStructure results based on the genetic data set with K = 4. The colors indicate different clusters distribution for each individual represented by vertical bars, the y axis showing the Q-value. Abbreviations for populations grouping and populations name are presented respectively on the top and bottom of the chart. See Table 1 for population abbreviations

conducted surveys in the surrounding valleys of known locations, focusing on shaded cliffs with similar exposures in search of undiscovered populations.

Tissue sampling for genetic analyses

Leaf samples of *C. alpina* subsp. *alpina* individuals were collected in the field and stored individually in paper coffee filters hermetically enclosed in plastic zip bags filled with silica gel. In the Alpine region, we sampled five populations in the Swiss Prealps (Boltigen, Dent-Vertes, Schwarzsee, La Gissetta, La Tzintre), one population in the Bernese Alps (Gasterental), three populations in the Western Alps, in France (Saint-Nizier-du-Moucherotte, Salève), and Italy (Susa), and twelve populations in the Eastern Alps, in Switzerland (Bergün, Cimadera, Klosters, Poschiavo, Samnaun, Scuol, Sils, Val Müstair), Austria (Ötztal, Ladis), and Italy (Monte Baldo). For a wider biogeographical context, we also included four populations of *C. alpina* subsp. *alpina* from the Carpathians (Poland, Slovakia and Romania) and one from the Stara Planina Mts. in the Balkan Peninsula (Bulgaria), as well as one population of *C. alpina* subsp. *sibirica* from Finland. A population was defined as a group of individuals occurring in a radius of two kilometers. Additionally, two individuals cultivated at the Botanical Garden of the University of Fribourg, Switzerland, were included to verify the genetic affinity of the only existing ex situ material close to the region of interest. In total, samples from 27 alpine clematis populations were collected in 8 countries (Fig. 1, Table 1). In order to maximize the information to answer our study questions, we decided to reduce the number of selected individuals to 3 to 4 individuals for a set of populations to be able to retrieve the general structure and in the same time to estimate more confidently genetic diversity and differentiation measures in the population with more individuals. Moreover, this the unbalanced sample size was due to the difficulties to collect individuals, either because of the reduced size of the populations (Ötztal, Salève, Boltigen) or the inaccessibility of plants (Schwarzsee).

Genome size and ploidy level

Genome size was determined for one to two randomly selected individuals per population (45 samples covering the whole distribution area of the study taxon) by the Plant Cytometry Services (Didam, The Netherlands, www.plantcytometry.nl) using propidium iodide (PI)-based flow cytometry (Doležel et al. 1998). Each individual was analyzed once. The number of nuclei measured for each sample was large enough in order to determine the ploidy and ranged between 100 and 1000 nuclei per sample. The samples were measured together with the internal standard of *Clivia miniata*, whose genome size is known (2C = 35.77 pgy), to obtain the absolute DNA ratio (Plant Cytometry Services 2023). From this ratio, the genome sizes of the *C. alpina* samples were calculated (Plant Cytometry Services 2023). Ploidy level was estimated based on comparison against previously published 2C values and chromosome counts (Šmarda et al. 2019; Zonneveld 2019).

DNA extraction and library preparation

To prevent contamination, DNA extraction was performed in a dedicated room and all equipment was exposed for a minimum of 30 min to UV light. We ground approximately 30 mg of dried tissue per plant sample with a TissueLyser II (QIAGEN, Hilden, Germany). Subsequently, genomic DNA was extracted using a protocol based on the standard CTAB method with a DNA silica matrix recovery (SILEX; Vilanova et al. 2020). The DNA quantity per extraction was estimated using a spectrophotometer Nano-100 (Hangzhou Allsheng Instruments Co., Hangzhou, China) and a Qubit™ 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc., Waltham, USA). We also checked the integrity and quality of the extracted DNA with agarose gel electrophoresis (Biotium, Hayward, USA). After quality control, 150 samples were selected for further analyses.

Genomic libraries were prepared according to a modified protocol based on Meyer and Kircher 2010 and the original documentation provided for the KAPA HyperPrep kit (Roche, Switzerland). First, 1000 ng of genomic DNA from each sample was sheared into fragments of approximately 500 bp by sonication with a Q800R3 Sonicator (Qsonica L.L.C, Newtown, USA). The size of the resulting fragments was then controlled using a bio-fragment analyzer Qsep100™ (Bioptic Inc., Palm Springs, USA). After an end repair and A-tailing step, adapters were ligated to the DNA fragments with a T4 DNA ligase provided in the KAPA HyperPrep kit and single strained part of the fragments completed by a BST DNA polymerase, Large Fragment (NEB, USA). Different P5 (binds to the 5' end) and P7 (binds to the 3' end) adapters composed of an indexing part of 7 bp nucleotides, forming a specific combination for each sample, and the Illumina specific sequence, were added and amplified by polymerase chain reaction (PCR) using the following protocol: 98 °C for 30 s, followed by 8 cycles of 98 °C for 10 s and 60 °C for 10 s. During library preparation, libraries were purified after each step using ethanol and Sera-Mag™ beads (Cytiva, Marlborough, USA) with free carboxyl groups on the surface, which helped to remove proteins and polysaccharides (Cytiva 2023). We adapted the ratio of beads and Polyethylene glycol (PEG) during the purification step to keep fragments around 500 bp and remove unbound primers and adapters. DNA

Table 1 Information and statistics for the 27 populations used in this study

Group	ID	Country	Mountain range	Locality	Lat. (N)	Long. (E)	Alt.	Sample	Ho	Ho stdev	Avg. π (all genes)	Avg. π (all genes) stdev	Averaged F	Averaged F stdev
PR	PR_GI	CH	Prealps	Gissetta	46.631	7.232	1390	7	0.09	0.01	0.0007	0.0018	0.19	0.05
	PR_DV	CH	Prealps	Dents-Vertes	46.618	7.203	1471	8	0.08	0.01	0.0007	0.0018	0.27	0.09
	PR_SS	CH	Prealps	Schwarzsee	46.665	7.300	1300	5	0.08	0.01	0.0007	0.0018	0.27	0.09
	PR_TZ	CH	Prealps	Tzintre	46.611	7.175	860	3	0.06	0.00	0.0004	0.0018	0.43	0.02
	PR_BO	CH	Prealps	Boltigen	46.644	7.371	1572	1	0.05	–	0.0004	0.0022	0.53	-
								24	**0.07**	**0.01**	**0.0006**	**0.0019**	**0.34**	**0.06**
ALP	EA_SM	CH	Eastern Alps	Samnaun	46.961	10.412	1578	6	0.12	0.01	0.0010	0.0021	-0.07	0.10
	EA_MB	IT	Eastern Alps	Monte Baldo	45.797	10.894	1665	10	0.12	0.01	0.0012	0.0022	-0.10	0.12
	EA_LA	AT	Eastern Alps	Ladis	47.077	10.643	1375	3	0.11	0.02	0.0010	0.0021	0.00	0.16
	EA_PO	CH	Eastern Alps	Poschiavo	46.322	10.083	1517	4	0.11	0.01	0.0010	0.0022	-0.01	0.07
	EA_KL	CH	Eastern Alps	Klosters	46.869	9.879	1199	4	0.11	0.00	0.0010	0.0022	0.01	0.04
	EA_OE	AT	Eastern Alps	Oetz	47.202	10.894	800	2	0.12	0.01	0.0011	0.0024	-0.09	0.05
	EA_SI	CH	Eastern Alps	Sils	46.429	9.770	1844	4	0.11	0.01	0.0009	0.0019	0.04	0.05
	EA_SC	CH	Eastern Alps	Scuol	46.786	10.285	1285	4	0.11	0.00	0.0010	0.0021	0.04	0.03
	EA_CI	CH	Eastern Alps	Cimadera	46.065	9.056	1456	9	0.10	0.01	0.0008	0.0020	0.14	0.06
	EA_BE	CH	Eastern Alps	Bergün	46.638	9.793	1717	4	0.10	0.01	0.0010	0.0021	0.05	0.09
	EA_VM	CH	Eastern Alps	Val Müstair	46.617	10.365	1728	4	0.11	0.00	0.0010	0.0022	0.03	0.02
	EA_GA	CH	Eastern Alps	Gasterental	46.458	7.696	1402	3	0.10	0.03	0.0008	0.0021	0.12	0.27
								57	**0.11**	**0.01**	**0.0010**	**0.0021**	**0.01**	**0.09**
	WA_SU	IT	Western Alps	Susa	45.108	7.075	1280	4	0.12	0.01	0.0011	0.0022	-0.04	0.08
	WA_SN	FR	Western Alps	Saint-Nizier-du-Moucherotte	45.157	5.637	1570	9	0.11	0.01	0.0009	0.0020	-0.03	0.09
	WA_SV	FR	Western Alps	Salève	46.114	6.154	960	1	0.08	–	0.0007	0.0027	0.30	–
								14	**0.10**	**0.01**	**0.0009**	**0.0023**	**0.08**	**0.08**
CAB	CA_TA	PL	Carpathians	Tatra	49.262	19.882	1020	2	0.13	0.00	0.0014	0.0024	-0.13	0.02
	CA_PI	PL	Carpathians	Pieniny	49.418	20.442	700	10	0.14	0.02	0.0013	0.0022	-0.23	0.15
	CA_SL	SK	Carpathians	Slovinky	48.878	20.776	850	7	0.13	0.01	0.0013	0.0023	-0.19	0.08
	CA_SA	RO	Carpathians	Sădurel	45.602	24.005	770	2	0.13	0.02	0.0013	0.0030	-0.18	0.15
	BA_AP	BG	Stara Planina	Apriltsi	42.740	24.975	1910	4	0.14	0.02	0.0013	0.0024	-0.25	0.19
								25	**0.13**	**0.01**	**0.0013**	**0.0023**	**-0.18**	**0.08**
	ES_BG	CH	–	Fribourg Botanical Garden	46.792	7.157	635	2	0.11	0.02	0.0009	0.0022	0.00	0.14
	NE_ST	FI	Northern Europe	Storgräspotten	62.303	21.636	50	4	0.18	0.01	0.0017	0.0029	-0.62	0.12
	Total							**126**						

Total number of individual and mean values is also given in bold by mountain ranges

Group group for demographic analysis, *ID* population code, *CountrySample* number of individual collected, *Ho* Observed heterozygosity, *Avg. π* averaged nucleotide diversity calculated for all loci, *Average F* averaged inbreeding coefficient

concentrations were measured repeatedly using a Qubit™ 3.0 Fluorometer. Libraries were pooled equimolarly in 6 pools of 25 samples according to their concentration after the last purification step. Excess water was removed with a Savant SpeedVac™ Vacuum Concentrator (Thermo Fisher Scientific Inc., Waltham, USA). To prevent false detection of population clustering, populations coming from the same geographic regions were not processed together as far as possible (Meirmans 2015). Information associated to laboratory steps, such as date of manipulations and DNA concentrations is available for each sample in Table S2.

Sequence capture

A genomic approach was used to obtain sufficient informative characters to quantify intraspecific genetic diversity. We adapted here the Angiosperm353 baits set targeting 353 single-copy loci present across all angiosperms (Johnson et al. 2019) and a selection of 1079 exons from an existing bait set specifically designed for the genus *Ranunculus* (Tomasello et al. 2020) to match *C. alpina* subsp. *alpina* sequences (see Baits selection in Supplementary Material). We chose to include the taxon-specific capture set because it allows for more detailed population genetic analyses (Yardeni et al. 2022). Off-target information such as regions flanking the target genes and partial recovery of chloroplast genome were also analyzed. We used myBaits® target capture kits (Arbor Biosciences, Ann Arbor, USA) to selectively isolate and amplify regions of interest from our DNA samples. The final bait panel was based on 1429 orthologous sequences in *C. alpina* subsp. *alpina* (615′266 bp) and consisted of 12′400 probes. After the capture and washing of unwanted sequences at 65°, the regions of interest were amplified via two separate PCRs of 12 cycles to avoid unbalanced amplification. This step was followed by further purification and quantification of double-stranded DNA (dsDNA). The size distribution and concentration of final DNA fragments were assessed using a Femto Pulse system (Agilent, Santa Clara, USA) and quantitative PCR at the genomic platform of the University of Bern. Ultimately, 148 samples were sequenced at the NGS platform at the University of Bern with an Illumina NextSeq 1000 P1 600 cycle kit (2 × 250 bp paired-end).

Reads processing

Illumina raw reads were processed using Yggdrasil, the high-performance computing cluster of the University of Geneva, and quality checked with FastQC as well as MultiQC (Andrews 2010; Ewels et al. 2016). We then used the preprocessing tool Trimmomatic to remove Illumina adapters and reads as follows: ILLUMINACLIP:adapters.fa:2:30:10:8:true SLIDINGWINDOW:4:18 LEADING:20 TRAILING:20 MINLEN:20 (Bolger et al. 2014). The program HybPiper was then run to generate a consensus sequence of the genes and supercontig for each sample (Johnson et al. 2016). HybPiper was run again with the post-processing command paralog_retriever in order to detect potential paralogs. Loci were removed from downstream analyses when either more than 1 contig was found in more than 5% of individuals (paralogs) or no contigs were retrieved for more than 50% of individuals. The gene consensus for every individual was aligned with MAFFT (Katoh et al. 2002) and concatenated before being filtered with TrimAl (Capella-Gutiérrez et al. 2009) and TAPER (Zhang et al. 2021). The same procedure was done for the supercontig.

The longest sequence per gene for the supercontig was selected before any filtering and taken as reference. BWA version 0.7.17 (Li and Durbin 2009) was used for mapping separately the paired and unpaired reads against the reference, then the files were sorted with Picard version 2.21.1 (Broad Institute 2023) and indexed with samtools version 1.9 (Li et al. 2009). After merging the paired and unpaired reads, duplicates were flagged with Picard version 2.21.1 (Broad Institute 2023) and reads realigned around indels with GATK version 3.8. Known sites were then called in order to produce a recalibration table with GATK 4.1.3. The calling of genotypes was done with GATK 4.1.3 HaplotypeCaller and produced a variant call format (VCF) file. An all-sites VCF file was produced and filtered as following: removal of genes identified as having too many paralogs, indel, site with mapping quality < 30, missing data > 30%, depth = < 5 > 200 and loci with strong HWE violations (–hwe 0.001). A variant-only VCF file was produced and filtered the same way as for the all-sites VCF, removing all monomorphic loci in addition.

For the two VCF files, using the information from Hybpiper, three files were produced, with exons only, introns only, and both together. The different files were used to compare our results for regions affected by different selection regimes. The complete file was also separated for the Angiosperms353 loci and the ones from the *Ranunculus* set from Tomasello et al. (2020) in order to compare the efficiency of the Angiosperms353 kit at the intraspecific level. For the variant site VCF files only, a linkage disequilibrium-based SNP pruning was applied to remove sites in approximate linkage equilibrium with each other, following the method implemented in PLINK v1.90b6.17 (Purcell et al. 2007;–indep 50 5 2) which recursively removes SNP exceeding the variance inflation factor (VIF; 2), within a sliding window (50 kbp) shifted at each step by a number of SNP (5).

Genetic structure and diversity

First, we visualized the whole dataset with a network using SplitsTree (Huson and Bryant 2006) using the concatenated gene alignment and with a principal component analysis (PCA) using smartPCA (Patterson et al. 2006) on the thinned dataset. This allowed us to detect the general pattern of variation in our dataset as well as potential outlier samples due to high level of missing data or contamination. We performed again the same analyses after removing few outlier samples (low coverage/high missing data). We ran the program FastStructure 100 times with the logistic prior that return more accurate ancestry estimates when structure is difficult to resolve, with the number of genetic clusters (K) ranging from one to twelve (Raj et al. 2014). The software StructureSelector was used to estimate the optimal K that minimizes the genetic variability within groups and maximizes the genetic variability between groups (Li and Liu 2018). The R package pophelper (Francis 2017) was used to visualize the data, and the run with the best likelihood was drawn for each K. We also produced a coalescent-based phylogenetic tree on the hybrid mode weighed ASTRAL part of ASTER v. 1.16 (Zhang and Mirarab 2022). For this, first, trimmed supercontig alignment were used to generate a maximum likelihood single tree using IQ-TREE v. 2.2.2.6 (Minh et al. 2020) with the default GTR + GAMMA substitution model and an UFboot of 1000 to estimate branch support. Resulting trees were then concatenated and branches with a bootrap support lower than 10 were collapsed using Newick_Utils (Zhang and Mirarab 2022; Junier and Zdobnov 2010). The final tree was visualized in iTOL v. 5 (Letunic and Bork 2021). Nucleotide diversity was calculated with the script pixy version 1.2.5 beta1 (https://doi.org/10.5281/zenodo.4432293; Korunes and Samuk 2021) for each site and averaged by genes within each population on the filtered all-sites VCF file. Observed heterozygosity and inbreeding (F) was calculated with PLINK v1.90b6.17 (Purcell et al. 2007) on a filtered and prunned variant sites VCF. The software Arlequin (Excoffier and Lischer 2010) was used to compute the pairwise fixation index (FST), an analysis of molecular variance (AMOVA), and two Mantel tests to test for isolation by distance (Mantel 1967; Wright 1943, 1949), Due to the unequal sample size between populations, we also computed these analyses on a set of 10 populations with > 6 individuals.

Plastome recovery

Even if the targeted capture method is very efficient in amplifying targeted genes, some sequences belonging to the chloroplast genome are present in the sequenced reads (Maurin et al. 2021; Schneider et al. 2021). We mapped the trimmed reads against the closest relative of *Clematis alpina* with a reference chloroplast genome publicly available at the moment of the study, *C. montana* (MT292622, Mao et al. 2020), following the same procedure as for the nuclear genes until the calling step. Variable recalibrated sites were called for each individual with GATK 4.1.3 before producing a combined VCF file. This procedure was done two times separately, once with a ploidy level of 2 and a second time with a ploidy level of 1. The chloroplast genome is haploid hence a ploidy level of 1 is appropriate for the calling. However, due to nuclear transfer, some regions can be very similar between the nuclear and the chloroplast genome (Scarcelli et al. 2016). This can bias the analyses, including nuclear sequences in the final analyses. Calling the variant with a ploidy level of 2 allowed to detect heterozygous variant that should not be included in the chloroplast analyses. It also helped to detect the potential presence of contamination at the individual level. The final VCF used in the subsequent analyses was the one called with a ploidy of one. VCFtools version 0.1.16 (Danecek et al. 2011) was used to filter the variants in order to remove indels, and mask information when less than 5 reads were present, individuals with more than 50% of missing data were removed as well as singletons and monomorphic sites. VCF was inspected visually to carefully check for correlation of variant with presence of heterozygous sites in the VCF called with a ploidy of two. Then the VCF was converted into plink bed files with PLINK v1.90b6.17 (Purcell et al. 2007). Principal Coordinate Analyses (PCA) of genetic data were computed with the package smartPCA (Patterson et al. 2006) and visualized with R (R Core Team 2023).

Demographic analysis

We used the software GADMA2 (Genetic Algorithm for Demographic Model Analysis; Noskova et al. 2020, 2023) to infer the evolutionary trajectory of the Prealps populations respectively to the other main genetic clusters detected in *C. alpina* subsp. *alpina* from the previous analyses (see Results). The software GADMA employs a global genetic algorithm in conjunction with diverse pre-existing local optimization "engine", including δaδi (Gutenkunst et al. 2009), moments (Jouganous et al. 2017), and momi (Kamm et al. 2020), to generate anticipated joint alleles-frequency spectra (JAFS) across multiple populations under varying demographic models and compare it with the observed JAFS. Specifically, the genetic algorithm integrated within GADMA facilitates the determination of temporal frameworks (epochs) that exhibit distinct patterns in effective population size (Ne) over time, such as linear or exponential growth or decline during specific periods. A local optimization algorithm further refines the estimation of demographic

parameter values within these established structures. This approach enhances the likelihood of identifying the most probable demographic scenario responsible for the observed genetic diversity, even with minimal prior knowledge, thereby necessitating the exploration of an extensive parameter space.

First, we split a supercontig SNP dataset by the three clustering regions found in *C. alpina* subsp. *alpina* to remove the loci not in Hardy–Weinberg equilibrium within each region. The resulting VCF was pruned with the same method as described in the read processing section in order to keep statistically non-linked sites with the exception that singletons were kept, as they are informative for demographic inferences. As JAFS cannot include missing data in order to give coherent results, we downprojected the JAFS in identifying the number of samples maximizing the number of segregating sites using the easy SFS python script (https://github.com/isaacovercast/easySFS). A folded 3D JAFS for each pair of sites was then generated with the same script. We conducted two 3D demographic models, in order to take into account two possible historical scenarii 1) with Carpathians/Balkan Peninsula (CAU; CA_TA, CA_PI, CA_SL, CA_SA, BA_AP) populations as first region to split, with Prealps (PR; PR_GI, PR_DV, PR_SS, PR_TZ, PR_BO) and Alps (ALP; WA_SU, WA_SN, WA_SV, EA_SM, EA_MB EA_LA, EA_PO, EA_KL, EA_OE, EA_SI, EA_SC, EA_CI, EA_BE, EA_VM, EA_GA) regions splitting in a second time and 2) with Prealps (PR) populations as first region to split, then Carpathians/Balkan Peninsula (CAU) and Alps (ALP) regions splitting in a second time. For each model run the initial and final structures were set to [1, 1, 1] which define the number of time periods before the first split, after the first split and the after the second split. We allowed migration between the populations. Sequence length was inferred from the all-sites VCF at the filtering step where only good-quality SNPs are present (no genes with sign of paralogy, GQ > 30, depth > 5 < 200; 637′786 sites). The local search algorithm optimization was the BFGS method with log transformation (BFGS_log). The average genomic mutation rate for the *Clematis* genus being unknown, we used a mutation rate of 7e-9 based on estimated nuclear mutation rate in other Ranunculaceae and Ranunculales (Shang et al. 2024). A generation time of 7 years was selected as a previous study in *C. alpina* subsp. *sibirica* and subsp. *ochotensis* found that the reproductive phase of development was starting between 5 and 10 years (Chubatova and Churikova2016). Parameters for the genetic algorithm (GA) were left as default as recommended (Noskova et al. 2020). For demographic inference, we used 32 replicates (4 processes of 8 repeats) for each scenario with the engine δaδi (Gutenkunst et al. 2009), to observe convergence and infer parameter values, as well as statistical robustness. Finally, we compared and ranked the models using the Akaike Information Criterion (AIC) and draw the schematic plot of the best model with demedraw 0.4.0 API in Python.

Results

Distribution of *C. alpina* subsp. *alpina* in the Prealps

Our field survey showed that the geographical distribution of alpine clematis is restricted to a small portion of the of the Prealps. The distribution range extends from La Tzintre (Val-de-Charmey) along the Dent de Vounetse (Val-de-Charmey) to Schwarzsee (Plaffeien) (Fig. 1b), all three in the Swiss canton of Fribourg. We have also confirmed the presence of the species near the Euschelspass (Jaun, canton of Fribourg), in Gantrisch (canton of Bern) and in the Boltigen region (canton of Bern). The largest *C. alpina* subsp. *alpina* population in terms of number of individuals (> 100) was located along the Dent de Vounetse. We were unable to relocate the species at several historical sites where it had previously been observed, based on data recorded in Infoflora. Overall, we located 17 populations in the Prealps, (Fig. 1b). We also gained a more comprehensive understanding of their ecological preferences. For example, we found that *C. alpina* subsp. *alpina* populations in the Prealps, grow on cliffs, unlike the other Alpine populations that grow on the ground and on trees (S. Stefani, personal observations).

Genome size and ploidy level

The flow cytometry results showed that all analyzed *C. alpina* samples were diploid, a finding that is consistent with reports of diploidy in the *Clematis* genus in the literature (Šmarda et al. 2019; Zonneveld 2019). The median 2C-value was 17.28 pg of DNA (Table S1), which is consistent with the 2C-values of other diploid *Clematis* species like *C. vitalba* (17.49 and 20.2 pg), *C. viticella* (18.32 and 20.6 pg), and *C. montana* (19.1 pg) (Šmarda et al. 2019; Zonneveld 2019).

Sequencing and variant recovery

Twelve individuals failed during sequencing and were removed at this step. On average 1′327′707 (± 647′659) reads were retrieved for each individual (Table S2). The percentage of reads on target was 38.6% (± 18.3%) on average. After running Hybpiper, on the 1429 loci targeted, an average of 1280 (± 147) loci with a recovery length > 50% were retrieved. The mean length of the total retrieved exonic and intronic flanking regions was 1′343′438 bp ± 282′021 bp

(634′437 ± 141′748 bp and 709′000 ± 141′033 bp for Angiosperms353 and Ranunculaceae specific loci respectively). The mean length of targeted exonic regions only was 516′019 bp ± 58′841 bp (221′791 ± 30′460 bp for Angiosperm353 and 294′229 ± 28′912 bp for Ranunculaceae specific loci; Table S2). A total of 261 loci were removed after the inspection of number of contigs reported for each individual by paralog_retriever (195 potential paralogs and 66 loci not present in > 50 of individuals; Table S3). After the mapping and the filtering steps a total of 126 individuals remained.

We present the results deriving from the all-sites full dataset (nucleotide diversity) or from the full dataset trimmed in order to avoid linkage dequilibrium between loci, resulting in 5072 SNPs. The number of SNPs retrieved for each separated dataset is summarized in Table S4. For plastome data, after careful filtering, 42 SNP could be retrieved for 83 individuals.

Genetic structure

Population structure analysis using FastStructure aims at determining the most likely number of populations (K) given the genetic data. According to FastStructure choosing algorithm, model components used to explain structure in data was K = 4 in 86% of the runs. Based on this analysis, we obtained four genetically delineated populations (Fig. 1d). One group included the individuals belonging to the subspecies *sibirica,* and three other segregated the individuals of subspecies *alpina* from: (2) the Prealps, (3) the remaining parts of the Alps (both eastern and western populations) and the two individuals collected in the Botanical Garden of Fribourg, and finally (4) the Carpathians and the Balkan Peninsula mountains. Some populations presented signs of admixture, two populations collected in the Carpathians and Balkan Peninsula (CA_PI and BA_AP) showed introgression from the Alpine genetic group. When looking at a hierarchical division with increasing number of K (Supp. Fig. S1), the division of the Prealps, observed from K = 3, against all the remaining populations (Alps, Carpathians, Balkan Peninsula, subsp. *sibirica*) remains the same (K = 2 featuring the division of the group representing Carpathians/Balkan populations). The division of the subspecies *sibirica* appears at K = 4. The division of the Western Alps appears at K = 5. The two individuals from ex-situ collections are assigned in majority to the same cluster as the Eastern Alps individuals at K > 4. The genetic network based on concatenated exon sequences (Fig. S4) identifies groups of individuals consistent with the population structure analysis found by Fastructure. The coalescence-based phylogenetic tree also largely agrees with the population structure, although most of the relationships have low bootstrap support (< 80%; Supp. Fig. S2). The individuals of *C. alpina* subsp. *Sibirica*, set out as outgroup, as well as the samples from the Western Alps, Eastern Alps and the Prealps are monophyletic. Samples collected in the Carpathians are found in three clades, one of which containing samples from the Balkans. The two samples collected in the Botanical Garden of the University of Fribourg are found in different parts of the tree: BG1 is found sister to the Prealps clade whereas BG2 is sister to the Eastern Alps clade.

The PCA of *C. alpina* individuals revealed two genetic clusters corresponding to the different subspecies *alpina* and *sibirica* (Fig. S3). The first principal component explained 12.58% of the variability and the second 6.29%.

After excluding samples belonging to the subspecies *sibirica*, we obtained a PCA visualizing the patterns of genetic variation within the subspecies *alpina* (Fig. 2). The first principal component, which accounted for 7.01% of the variability, revealed a gradient of genetic distance from the Eastern Alps through the Western Alps to the Carpathians/Balkans. The individuals sampled in the Prealps were clearly separated from the rest of the individuals along the second principal component, which explains 5.02% of the variability. In particular, we found that the population of the Bernese Alps (EA_GA) was closer to the other alpine populations than to the Prealps ones despite the close geographical proximity of the Bernese Alps to the Prealps (Fig. 1C and Fig. 2). The alpine clematis individuals cultivated in the Botanical Garden of the University of Fribourg are found genetically related to samples from the Eastern and Western Alps (Fig. 2). We found that the PCA of individual datasets corresponding to exons, intronic flanking regions, Angiosperms353 loci, and Ranunculaceae-specific loci (Fig. S6) yielded very consistent results with the PCA of the total dataset (Fig. 2).

Finally, the PCA of the 42 SNP retrieved from the chloroplast genome (Fig. S5) complements the results based on the nuclear dataset. The first axis representing 21.5% of the variance separates all individuals from the Prealps from the rest of the dataset. The population of *C. alpina* subsp. *sibirica* is close to the group of samples collected in the Eastern and Western Alps and some samples collected in the Carpathians. The second axis (14.6%) separates the samples collected in Bulgaria (population BA_AP). The third axis (11.8%, data not shown) isolates the samples collected in the Western Alps.

Genetic divergence

The pairwise FST values among the populations were not significant for many pairs due to the small samples size of some populations (Table S5 and 1). Considering only significant values, we identified four genetically differentiated

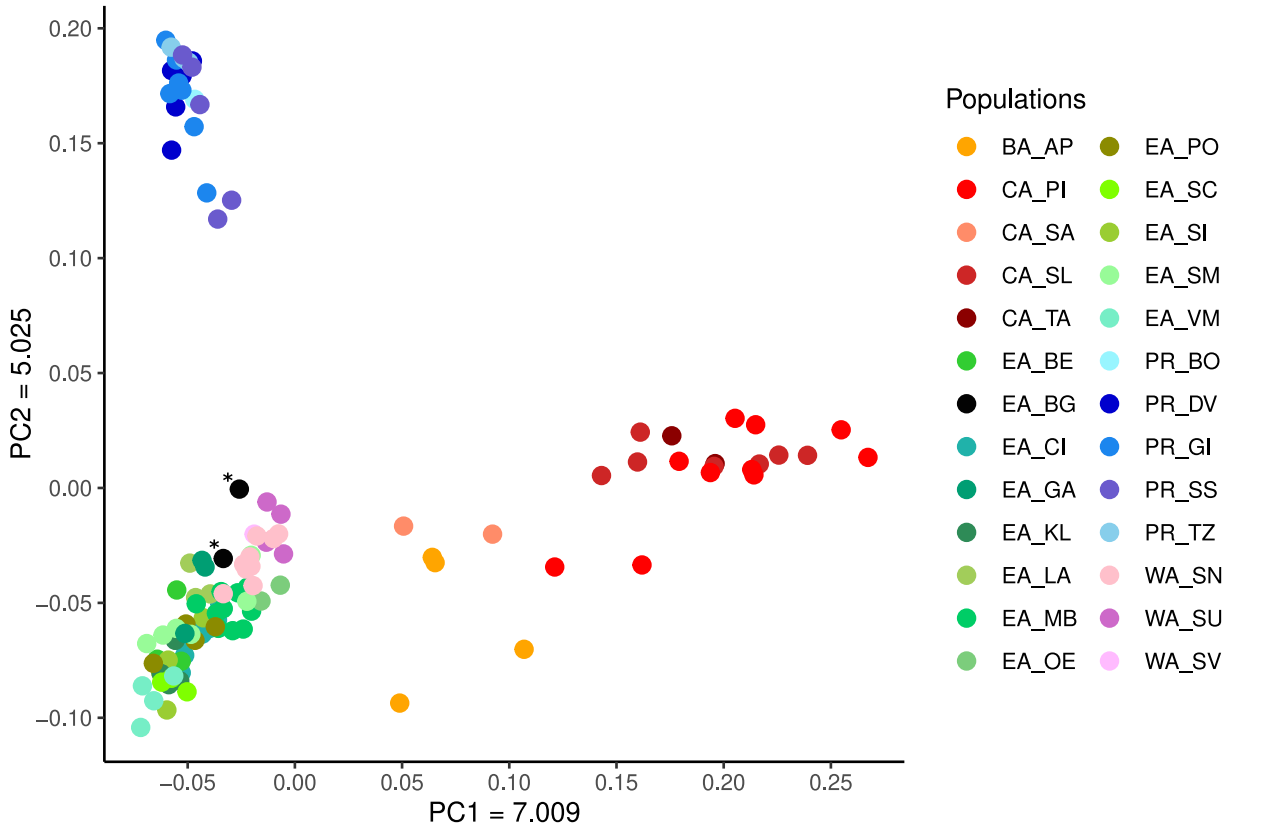

Fig. 2 Principal component analysis for samples of *Clematis alpina* subsp. *alpina* representing the genetic variation at multiple loci for each sample, using the first (7.009%) and second (5.025%) principal components. The distance between the points represents the genetic similarity or dissimilarity between individuals. Samples were colored according to the population to which they belong and color hues match mountain ranges (Prealps: blue, Western Alps: pink, Eastern Alps: green, Carpathians and Balkans: red) and asterisk highlight the two samples collected in Fribourg botanical garden. See Table 1 for population abbreviations

groups (Table S5): 1) the population belonging to the subspecies *sibirica* was clearly distinct from the others with pairwise FST values from 0.356*** (NE_ST-BA_AP) up to 0.489** (NE_ST-PR_DV), 2) the populations from the Prealps, which have a pairwise FST between them from 0.054** to 0.117**, 3) the populations from the Carpathians, with a FST within the group from 0.002* to 0.191**, and 4) the populations from the Eastern and Western Alps with pairwise FST within the group ranging from 0.0196* to 0.253**, with the highest values corresponding to the FST between Eastern Alps and Western Alps populations. Higher FST values were found between these groups with similar minimal and maximal values; between Prealps and Carpathians/Balkan Peninsula populations with FST between 0.173*** and 0.349***, between Prealps and Alps populations with FST between 0.156 *** and 0.349** and between the Carpathians and Balkan Peninsula and Alps populations with FST between 0.111*** and 0.339***. The results of AMOVA and the Mantel test computed within the selected 10 populations with more than 6 individuals (data not shown) and the total dataset were very similar and we present here the results for the complete dataset. The AMOVA performed for all populations indicated a moderate but significant variation among populations (FST = 0.124, Table S6a). Additionally, we performed an AMOVA with populations grouping according to the geographical regions (Table S6b). The variation among groups (FCT = 0.103) is lower than the variation among populations within groups (FSC = 0.142). We performed the isolation by distance test twice, once with the populations from the Prealps (r = 0.29, p = 0.019) and once without them (r = 0.59, p < 0.001). The correlation coefficient is higher and significant for the comparison excluding the populations from the Prealps compared to the comparison including them.

Genetic diversity

Heterozygosity (observed heterozygosity; Table 1) ranged from 0.05 in population PR_BO from the Prealps to 0.182 ± 0.013 in population NE_ST, the only population representing *C. alpina* subsp. *sibirica*. Within the subspecies *alpina*, the nucleotide diversity was the lowest in the Prealps populations (0.07 ± 0.01) and the highest in the Carpathians populations (0.13 ± 0.01). Within-population, the

pairwise nucleotide diversity averaged by gene (π; Table 1, Fig. 3) was the lowest for populations PR_TZ and PR_BO from the Prealps (0.0004 ± 0.0018) and the highest for population NE_ST (0.0017 ± 0.0029, subsp. *sibirica*). Within the subspecies *alpina*, the lowest nucleotide diversity was found in the Prealps (0.0006 ± 0.0019) and the in highest in the Carpathians populations (0.013 ± 0.0023). Inbreeding coefficients (F; Table 1, Fig. 3) values, averaged for each population, ranged from– 0.62 + /0.12 in population NE_ST (subsp. *sibirica*) to 0.53 in population PR_BO from the Prealps. Within the subspecies *alpina*, inbreeding coefficients were the highest in the Prealps (0.34 ± 0.06) and the lowest in the Carpathians (– 0.18 ± 0.08). Twelve of the twenty-seven populations had negative values. Inbreeding coefficient calculated specifically within each population were all negative, with associated non-significant p-values. Values of genetic diversity estimated from the different datasets (i.e., exons only, splash zone, Angiosperms353, Ranunculaceae-specific) were highly consistent across the populations (Supp. Table 4, Fig. 4). For example, nucleotide diversity values estimated from the exonic dataset was only slightly higher (from 0.0004 ± 0.0023 for PR_TZ to 0.0019 ± 0.0033 for NE_ST subsp. *sibirica*) than those estimated from the flanking regions dataset (from 0. 0003 ± 0.0019 for PR_TZ to 0.0010 ± 00.30 for NE_ST subsp. *sibirica*). We also found that the values of nucleotide diversity, heterozygosity and inbreeding coefficient estimated from the set of loci captured by the Angiosperms353 kit were highly similar to the values estimated from the set of loci capture by the Ranunculaceae-specific regions selected by Tomasello et al. (2020; Fig. 5, Supp. Table 4).

Demographic inference

We ran demographic inferences using a 3D JAFS with 40 × 96 × 26 cells (PR-ALP-CAB), composed by 266, 438 and 418 segregating sites for the Prealps, Western/Eastern Alps and Carpathians/Balkans populations, respectively. The model inferring the first split as the Carpathians/Balkan Peninsula populations, followed by the split between the Alps and the Prealps populations, had higher AIC values and a better convergence than the other scenario that inferred the split of the Prealps populations. For the first scenario (Carpathians/Balkans populations splitting first), one run for each process of converged. Other runs were very close to convergence (defined as no improvement during 100 iterations). For the second scenario (Prealps populations splitting first), only one run in total converged (Table S7). We present the best model (Fig. 4) based on the best AIC value among replicates. The presented run had nearly converged with < 0.3 difference in likelihood score over 100 iterations; Table S7). The residuals for this model were

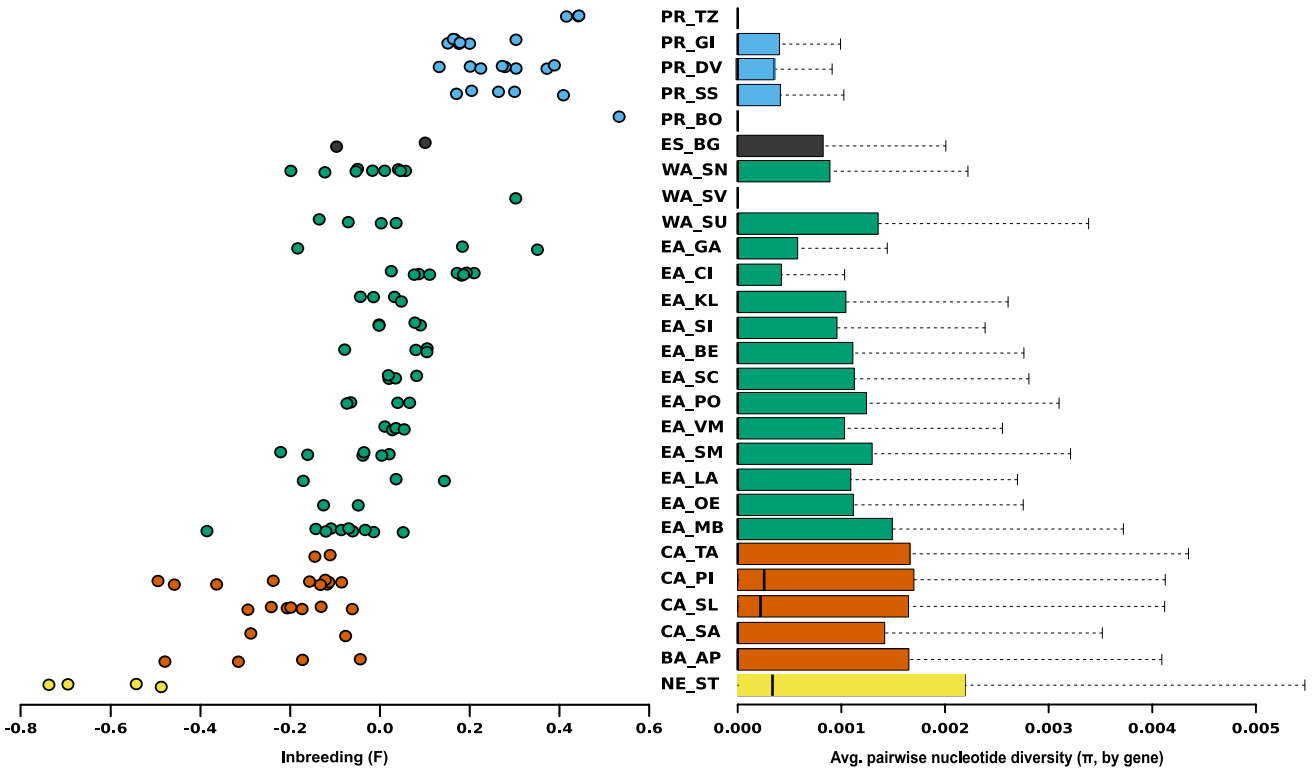

Fig. 3 Left: Inbreeding (F) calculated for each individual sorted by populations. Right: Nucleotide diversity (π) averaged by gene calculated by individual and sorted by populations. Each population is represented horizontally and colored according to the mountain range where it was sampled. See Table 1 for population abbreviations

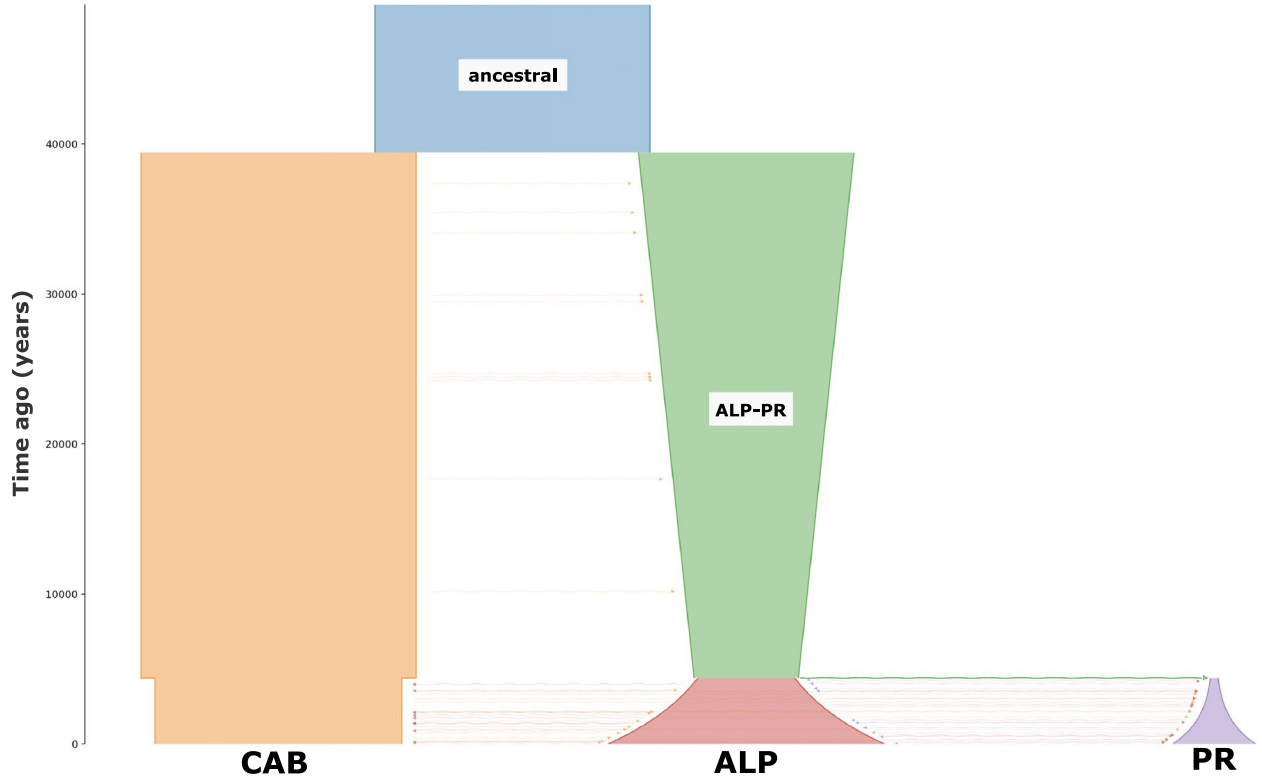

Fig. 4 Schematic plot of the best demographic scenario inferred by GADMA for the demographic history of Carpathians/Balkan Peninsula populations (CAB), Alps populations (ALP) and Prealps populations (PR). Past time is measured in years, zero being the present time

normally distributed around 0 with extreme values situated below– 3.2 and 3.2 (Fig. S7).

This model inferred a first split 35′033 years ago (with 7 years used as generation time) with an initial population size of 4814. It inferred an unbalanced split for population size between the Carpathians/Balkan Peninsula populations and the Alpine populations (78.26%, 4808 for CAB dataset; 21.74% 1823 for ALP/PR dataset). A linear population increase is inferred after the first split for the Alpine populations, and a sudden population increase for the Carpathians/Balkan Peninsula populations. The second event takes place 4410 year ago, with a very unequal split between the Alps populations with a population size of 1693 (92.88%) and the Prealps populations with a size of 129 (7.12%). After the second split, the model inferred a sudden population decrease for Carpathians/Balkan Peninsula populations (current inferred N = 4323), an exponential size expansion for the Alps populations (current inferred N = 4831) and the Prealps populations (current inferred N = 1458). The migration between the compared populations was very small in both directions at all periods (Table S7). The inferred inbreeding coefficient was low for all populations (0.093, CAB; 0.089, ALP; 0.001, PR).

Discussion

Distinction between *Clematis alpina* subspecies occurring in Europe

The genetic analyses conducted based on the nuclear loci indicated a clear distinction between *C. alpina* subsp. *alpina* and *C. alpina* subsp. *sibirica.* It is the primary genetic division in the PCA (Fig. S3—along the first PCA axis); in the FastStructure analysis, *C. alpina* subsp. *sibirica* individuals appear as a distinct group at K = 4 (Fig. S1), in the coalescent- based phylogenetic tree, the branch leading to the four samples of this subspecies is supported (Fig. S2) and differentiation values are the highest between all other collected populations (0.374 ± 0.056; Table S5). The information from the chloroplast genome that shows a proximity of subsp. *sibirica* to the populations collected in the main distribution range of *C. alpina* subsp. *alpina* (Eastern and Western Alps; Fig. S4, S5) would advocate for a recent diversification of these two entities. However, this genetic pattern could also result from a secondary contact during the Last Glacial Maximum, when populations could have expanded and met, leading to the phenomenon of plastid capture, as it is recorded for other species (Christe et al. 2014; Lammers et al. 2024). Altogether, our findings agree

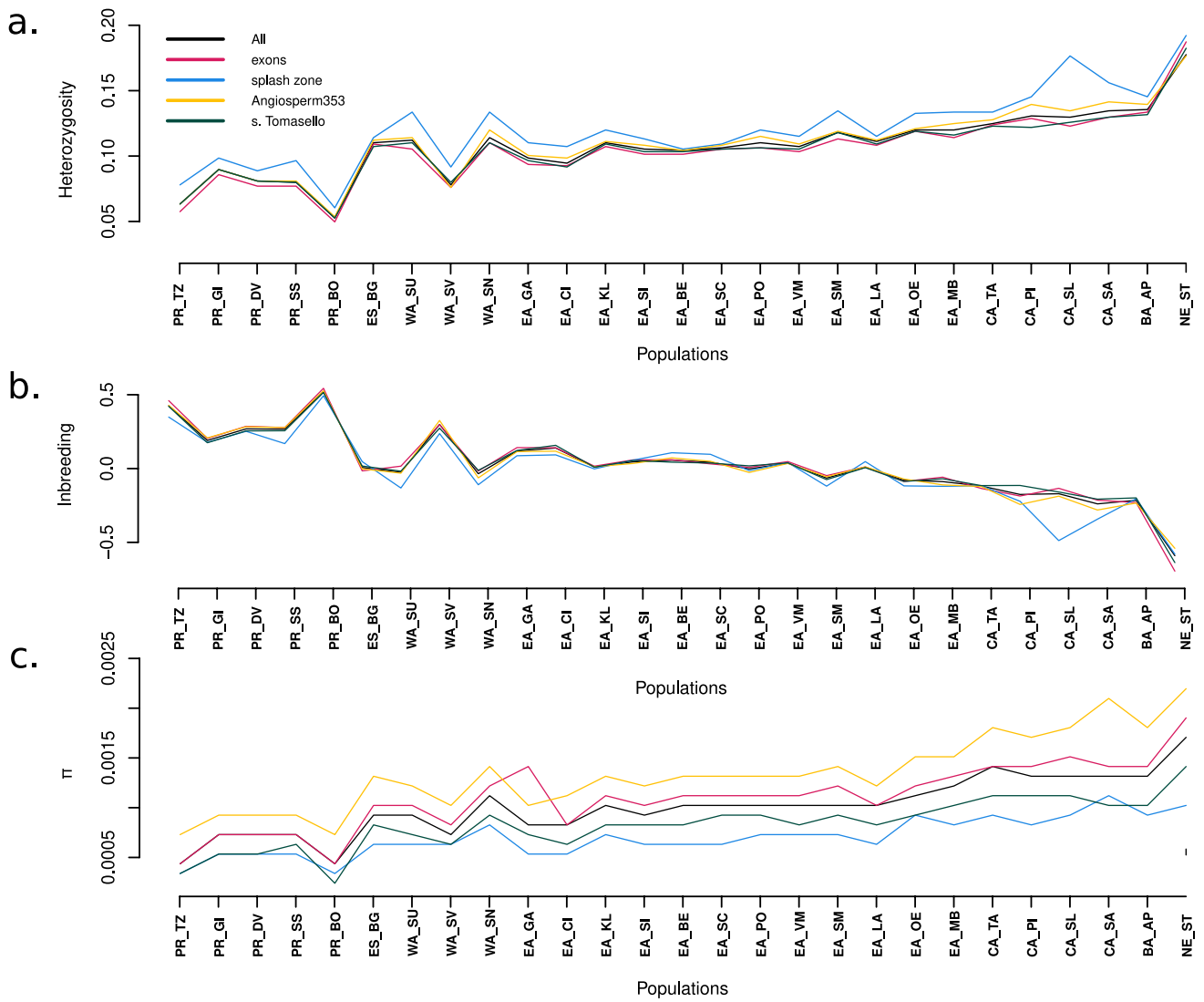

Fig. 5 Side by side comparison between the different datasets. all: black, exons: red, splash zone: blue, Angiosperms353: yellow, selection of loci coming from Tomasello et al. (2020) and represented for each population. **a** Average observed heterozygosity (Ho) calculated for each individual and averaged by population. **b** Average inbreeding (F) calculated for each individual and averaged by population. **c** Nucleotide diversity (π) averaged by gene calculated by individual and sorted by populations

with the studies that classify these two taxa as separate entities (Rydberg 1902; Serov and Jarvis 1988; Grey-Wilson 2000; Yang et al. 2009). Grey-Wilson (2000) distinguished *C. alpina* and *C. sibirica* as different species, whereas a more recent study by Yang et al. (2009) categorized them as subspecies based on field observations and morphometric analyses. We acknowledge that our sampling of *C. alpina* subsp. *sibirica* is not sufficient to conclude on this aspect. Additional research, including all *C. alpina* subspecies as well as the other species of the *Atragene* section, would be needed to further clarify the relationships in this *Clematis* complex (Yang et al. 2009; Xie et al. 2011).

Populations in the Prealps and the phylogeography of *Clematis alpina* subsp. *alpina*

The pattern of genetic variation within the subspecies *alpina* revealed three divergent and geographically segregated lineages that are consistently supported by FastStructure, PCA analyses and the coalescent-based phylogenetic tree (Figs. 1D, 2 and S2). The first lineage includes most populations from the Alps (both eastern and western parts of the range). The second widespread lineage includes all populations from the eastern part of the European range in the Carpathians and the Balkan Peninsula. Populations from the Western Carpathians form a genetically cohesive group, whereas the population from the Southern Carpathians

shows a closer affinity to the Balkan Peninsula (Figs. 2, S4), a pattern also observed also observed in several other mid-elevation species (Stachurska-Swakoń et al. 2020). One population in the northern Carpathians, as well as the population of the Balkan Peninsula, show signs of admixture from the Alpine group (Fig. 1). Finally, the third detected lineage includes the populations of the Swiss Prealps. These spatially restricted populations form a distinct cluster in both the nuclear and chloroplastic datasets (Figs. 1, S4, S5), a finding that aligns with previous genetic studies on plant taxa in the Prealps. For instance, *Eryngium alpinum* (Apiaceae), occurring in the North-Western Prealps, displays a distinct haplotype compared to neighboring populations from other parts of the Alps (Naciri and Gaudeul 2007). Particularly high genetic diversity in the Prealps has been also found in *Biscutella laevigata* (Brassicaceae;Parisod and Besnard 2007). In addition, *Papaver occidentale* (Papaveraceae), *Anacamptis pyramidalis* var. *tanayensis* (Orchidaceae), *Arenaria bernensis* (Caryophyllayeae) are all taxa that have been found to be endemic to the Prealps (Hess et al. 1977; Berthouzoz et al. 2013; Pittet et al. 2020). There are two main hypotheses as to why the Prealps exhibit genetically distinct populations: (1) During the Quaternary glaciations the Prealps themselves could have served as refugia allowing for plant taxa to persist and maintain their genetic diversity (Parisod and Besnard 2007; Berthouzoz et al. 2013; Pittet et al. 2020). (2) The presence of specific evolutionary lineages can also be a consequence of rapid spatial expansion (Excoffier and Ray 2008). Indeed, when occurring at the front of a spatial expansion in peripheral populations, drift can lead to strong structure but decreased genetic diversity as a result of repeated founder effects (Eckert et al. 2008).

The isolation by distance tests indicated that the genetic differentiation between populations was weakly correlated with geographic distance in our data set, and non-significant when the Prealps populations were included ($r=0.29$). After removing the populations from Prealps, this correlation doubled and was significant ($r=0.59$***). This result highlights the genetic distinctiveness of these Prealps populations despite their geographic proximity with the Alpine lineage populations. The population from Gasterental (Bernese Alps) offers a striking example of this pattern. Indeed, despite Gasterental is very close to the Prealps, this population belonged to the main Alpine phylogeographic cluster rather than to the Prealps lineage. This genetic affinity concurs with the fact that the Gasterental geologically and biogeographically belongs to the Inner Alps which are clearly delineated from the Prealps (Gerber et al. 2010).

The demographic reconstruction inferred the origin of Prealps populations as the split of an extremely small portion from an ancestral population. This inferred initial split followed by population increase highlights the important role of demographic processes on the origin of the differentiation of these populations. The time scale given by the demographic analyses places the first differentiation event between the Alpine and Carpathian/Balkan Peninsula populations at an older period than the split of the Prealps populations (Fig. 4). The transformed value in years depend on the generation time chosen, here equal to the age at the first reproduction (7 years), and should be taken with caution. It may however indicate that the Prealps populations are not the result of a long isolation history.

The genetic diversity indices in populations of *C. alpina* subsp. *alpina* throughout its range indicated that the Prealps population display a higher level of inbreeding and a lower proportion of heterozygotes and nucleotide diversity in the compared to other populations By contrast, the lowest level of inbreeding and higher proportion of heterozygotes and nucleotide diversity were found in the populations collected in the eastern part of the range (Carpathians, Stara Planina in Balkan Peninsula). Several populations display negative inbreeding coefficients, such as the populations of the Carpathians/Balkan Peninsula (Table 1). Negative inbreeding coefficients result from an excess of heterozygosity that can have different causes, such as technical bias, presence of several gene copies or signs of introgression (Stoeckel et al. 2006). As negative inbreeding does not concern all populations analyzed in this study (only Carpathians and Balkans populations, as well as EA_SM, EA_MB EA_OE) and no sign of polyploidy could be detected, either based on the genetic data (number of paralog genes) or cytologic results (Table S1), technical bias and presence of different ploidy level can be excluded. Alternative explanations for negative inbreeding are recent bottleneck or introgression of alleles from other populations (Cornuet and Luikart 1996). The second hypotheses is supported by the Q-value of the structure analyses that show gene flow from the Alps populations in at least three individuals collected in the Carpathians and Balkans populations.

The Carpathians and Balkan Peninsula populations deserve special attention due to their higher level of genetic diversity and the presence of a distinct evolutionary lineage documented in PCA and SplitsTree (Figs. 2, S4). The genetic diversity of the populations collected in the Eastern and Western Alps ranges between the values of the two clusters described above, leading to an East–West gradient (Fig. 3). This East–West gradient can be interpreted as an element to understand the dispersion of the alpine clematis in Europe. When populations rapidly colonize a new area, the gene flow between the front of the colonization and the core of the species is reduced, affecting the genetic diversity (Excoffier et al. 2009). Following a pattern already seen in other species with a montane European or Eurasian distribution (Mráz et

al. 2007; Hirsch et al. 2015), we could hypothesize an east to west colonization of the alpine clematis with an important role of genetic drift to explain the specificity of the Prealps populations both on the structure and diversity aspects. This pattern is also consistent with the center-periphery hypothesis that states that populations located at the periphery of a species' range should have lower levels of genetic variation than those at the center of the range (Hengeveld and Haeck 1982; Lira-Noriega and Manthey 2014).

Implications for the conservation of *Clematis alpina* subsp. *alpina* in the Prealps

The *C. alpina* subsp. *alpina* populations in the Prealps have unique genetic and ecological characteristics that make them particularly important for conservation efforts. Such geographically isolated populations are prone to inbreeding, which increases the risk of extinction (Young et al. 1996; Frankham 2003). The current distribution of the alpine clematis in the Prealps is confined to a small area and taxa with a limited geographical range are especially vulnerable (Purvis et al. 2000; Pearson et al. 2014), and indeed, these populations have already experienced a loss of diversity revealed by their higher level of inbreeding and lower heterozygosity and nucleotide diversity in comparison with populations from other regions (Fig. 3 and Table 1). Therefore, we strongly advocate for maintaining the current protection status of *C. alpina* subsp. *alpina* in the Swiss cantons of Fribourg and Bern. Including small reserves in conservation planning is crucial for effectively safeguarding biodiversity (Volenec and Dobson 2020). Another concrete conservation measure entails ex-situ culture of alpine clematis from the Prealps. As shown in this study, the specimens currently present in the Botanical Garden of the University of Fribourg do not belong to the genetic lineage of the Prealps. Given this finding, it is important to initiate a local collection, consisting of seeds and living individuals, which would include only individuals from the Prealps to ensure efficient conservation of the local diversity and, specifically, to prevent reinforcement or reintroduction based on populations of another provenance.

From a broader perspective, this study emphasizes the conservation value of the Prealps, corroborating earlier research findings, such as those by Berthouzoz et al. (2013). As the Prealps are located at the periphery of the Alps, they often harbor genetically distinct range-edge populations, as clearly exemplified by *C. alpina* subsp. *alpina*. Therefore, it is important not to neglect peripheral groups in conservation studies, especially if the species exhibits a disjoint distribution (Bunnell et al. 2004; Suchan et al. 2019; Caissy et al. 2020). We also observed that populations in the Prealps grow on cliffs, whereas those in the Western Alps almost never inhabit this type of terrain (S. Stefani, personal observations). Cliffs are known to support locally rare species due to their unique microclimatic conditions (Larson et al. 2005; Fragnière et al. 2024), reinforcing the importance to better protecting alpine clematis populations associated with this type of habitat.

Angiosperm353 at an intraspecific level and off target by-products in targeted capture

Angiosperm353 is a powerful tool now widely used to infer the angiosperms tree of life (Baker et al. 2021; Zuntini et al. 2024). The selection of conserved genes among all groups of Angiosperms was initially not meant to provide enough informative loci at low taxonomical level. However promising results have been obtained at the species and intraspecific levels (Slimp et al. 2021; Wenzell et al. 2021). The results presented here, deriving from the analyses of (1) the Angiosperm353 loci and (2) the Ranunculaceae-specific loci (Tomasello et al. 2020) were very similar (Figs. 5, S6, Table S4). Genetic structure, as well as diversity measures, are fully congruent and yield the same conclusions. Two off-target by-products accessible through the target capture method are the "splash zone" (the intronic and intergenic regions that flank target exons) and chloroplast sequences that are not washed away during the capture process. In this study, the proportion of SNP found in the splash zone was low (24% in splash zone and 76% in exon dataset, Table S4) compared to other studies (e.g., Ogutcen et al. 2021) and the recovery of chloroplast data was also not complete ($0.39 \pm 0.37\%$ missing data, 42 informative SNP recovered). These results are potentially due to technical issues (overshearing in the library preparation and lack of sequencing depth). However, this extra intronic information is in line with the results on the exonic part of the data only (Figs. 5, S6) and increased the power of the conclusion presented as it adds 24% more SNP. Overall, this means that angiosperms353 offer a powerful and versatile solution to investigate genetic diversity at the intraspecific level.

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Data availability Raw reads of all individuals are accessible in the European Nucleotide Archives (ENA) with the study accession PRJEB65714 and sample number for each individual is listed in Table S2.

Declarations

Conflict of interest The authors have no competing interests to declare.

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