Tempo and drivers of plant diversification in the European mountain system

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Tempo and drivers of plant diversification in the European mountain system

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Other materials for this manuscript include the following:

main SI document

Dataset S1 – Accession table.

Dataset S2 – Genomic regions.

Dataset S3 – Maximum credibility species trees.

Dataset S4 – Ecological and geographic information.

Dataset S5 – Alternative maximum credibility species trees.

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Abstract

There is still limited consensus on the evolutionary history of the species-rich temperate alpine floras due to a lack of comparable and high-quality phylogenetic data covering multiple plant lineages. Here we reconstructed when and how European alpine plant lineages diversified, i.e., the tempo and drivers of speciation events. We performed full-plastome phylogenomics and used multi-clade comparative models applied to six representative angiosperm lineages that have diversified in the European mountains. The diversification rates remained surprisingly steady for most clades, even during Pleistocene glaciations, with speciation events being mostly driven by geographic divergence and bedrock shifts. Interestingly, we inferred asymmetrical historical migration rates from siliceous to calcareous bedrocks, and from higher to lower elevations, likely due to repeated shrinkage and expansion of high elevation habitats during the Pleistocene. This may have buffered climate-related extinctions, but prevented plant speciation along elevation gradients as are often documented for tropical alpine floras.

Introduction

Mountain regions across the world are important biodiversity hotspots, owing to their high species richness, endemism and faster pace of species diversification compared to lowlands[1–4], with tropical mountain regions harboring by far the highest concentrations of plant diversity[1,5]. The diversity of temperate mountain floras is less prominent at the global scale, but still remarkably high compared to surrounding lowlands, specifically when considering the stressful climatic and edaphic conditions prevailing at high elevations and the dramatic climate and glacial oscillations of the Pleistocene period[6–8]. The exceptional plant diversity and endemism of the European mountains was early recognized, and its emergence has challenged the understanding of botanists and biogeographers since the pioneering work of von Haller 250 years ago[9,10]. Yet, how and when plant lineages have diversified in temperate mountains still remains insufficiently documented. While the drivers (how) and the tempo (when) of species diversification have already been explored for several European mountain plant lineages (e.g. [11–13]), no consensus has yet emerged on the evolutionary history of the species-rich European alpine flora. This is mostly due to previous clade-specific studies using both sparse and distinct genetic data, diverse analytical methods, and as a result, sometimes even yielding non-conclusive results[11–15]. To allow generalizations about the tempo and drivers of alpine plant diversification, we thus need to collect high-quality genomic data covering multiple plant lineages, estimate reliable and comparable phylogenies, and apply a multi-clade framework of modern phylogenetic comparative methods across study lineages.

Most theories aiming to explain why clades undergo rapid species diversification involve some form of ecological opportunity[16]. The tempo of species diversification of mountain biotas is indeed often described as a continuous, typically fast, process following the uplift of mountain ranges[17–19], which slows down after saturation of the whole available physical and ecological space (e.g. [20]), eventually being modulated by Pleistocene climatic oscillations[11,18,19,21]. This modulation can be positive in terms of net diversification rates with speciation being stimulated by climate-induced range dynamics (speciation pump mechanism, [22,23]), or negative with decreased speciation or increased extinction rates due to the impact of Pleistocene glacial cycles. The latter perspective is motivated by the observation of greater plant endemism in areas that have experienced relative climatic stability[7,24–26]. Contrary to subtropical and tropical mountains such as the Northern Andes where massive plant diversification occurred during the Pleistocene (e.g. [27,28]), it has long been considered that temperate mountains such as the European Alps were so severely glaciated during Pleistocene cold periods that this posed limits to recent plant diversification[15,29]. However, the evidence for refugia at the peripheries of glaciated European mountain ranges[7,8,30,31] and also on ice-free refugia within glaciated areas (so called nunataks, [32–35]) suggests that unglaciated mountain habitats persisted during glacial periods and may even have triggered plant speciation. Whether Pleistocene climatic oscillations slowed down or spurred the diversification of European mountain plants remains unclear, with most recent reliable analyses concerning *Primulaceae* only[11]. Documenting the tempo of alpine plant diversification requires extensive phylogenomic data that allow estimating relatively accurate divergence times for distinct plant clades. Such data allow to assess carefully how diversification rates have varied through time and between regions and environments that have been differently impacted by Pleistocene climate and glacial oscillations.

The unique flora of alpine environments was likely assembled through a complex interaction between spatial and ecological drivers that have influenced the divergence and migration of mountain plant lineages, but the relative influence of these drivers requires further investigation. It has long been considered that allopatric divergence was the main speciation driver in European mountains[11,15,36,37] However, this view is now challenged by the evidence that genetic structure and local adaptation across different elevation belts and bedrocks is pervasive in European mountain plants[38,39], and that parapatric speciation have occurred along ecological gradients in mountains across the world[1,5,28,40–43]. In addition, the Pleistocene climatic oscillations may have unevenly impacted the rates of migration, speciation and extinction of plant lineages across the major ecological gradients (e.g. bedrocks, elevation) and between mountain ranges, depending on whether the particular areas were heavily glaciated or remained ice-free during most of the Pleistocene – e.g. high elevation, siliceous areas of central Alps vs. low and mid-elevation, calcareous areas of peripheral Alps, respectively[44]. Disentangling the effects of geography and environmental heterogeneity on the evolutionary assembly of mountain floras thus requires simultaneously estimating past rates of migration and cladogenesis within and between mountain ranges, elevation belts and bedrock types, which has never been performed so far on any temperate mountain flora.

In our study, we aimed at providing a window into the evolutionary history of European mountain plants by inferring the tempo and drivers of speciation of six study plant lineages considered as representative cases of *in situ* diversification within European mountains (Figure 1). These clades are: *Androsace* sect. A*retia* (hereafter *Androsace*), *Campanula* sect. *Heterophylla* (hereafter *Campanula*), *Gentiana* sections *Gentiana*, *Ciminalis* and *Calanthianae* (hereafter *Gentiana*), *Phyteuma*, *Primula* sect. *Auriculata* (hereafter *Primula*) and *Saxifraga* sect. *Saxifraga* (hereafter *Saxifraga*). We reconstructed their phylogenies using a full-plastome phylogenomic dataset obtained by low coverage shotgun sequencing. Using a newly developed likelihood-based multi-clade comparative model approach, we estimated past rates and tempo of species diversification across all study lineages, and also lineage-specific deviations from general trends. Further, we investigated the evolutionary assembly of these plant lineages across different bedrocks, elevational belts and geographic regions using a comparative state-dependent diversification model framework. Finally, we examined the importance of allopatric and other modes of speciation based on sister species comparisons.

Results

Tempo of species diversification. The six study lineages started to diversify in Europe at variable dates within the last 40 Ma (Fig. 2A). The oldest lineage of our collection was likely *Saxifraga* (95% highest probability density of crown age 12.5-38.1 Ma BP), followed by *Phyteuma* (13.7-24.5 Ma BP), *Gentiana* (6.4-28.5 Ma BP) and *Campanula* (10.1-16.5 Ma BP). Both Primulaceae clades, namely *Androsace* (4.7-10.7 Ma BP) and *Primula* (3.2-7.9 Ma BP), had by far the youngest crown ages. All lineages accumulated considerable amounts of diversity both before and during the Pleistocene (2.6 Ma BP) so that none of them results from exclusively Tertiary nor exclusively Quaternary diversification. Our estimates integrate sources of uncertainty stemming from both molecular and fossil data in conservative way and seem to be robust to alternative interpretations of fossil record – for details about dating analyses and handling of various sources of uncertainty, see Methods and Supplementary methods SM1.

To better understand the dynamics of clade diversification, we tested a range of hypotheses about diversification changes through time by contrasting five models depicting different diversification scenarios (Table 1). The model support was evaluated by difference in AIC between the focal model and a nested null model not containing the focal parameter(s) (referred to as AICdiff throughout the paper, see Methods for details). We first ran all the models in a multi-clade setup with parameters shared across the six lineages to seek for general patterns. We then separately re-ran the five models with lineage-specific parameters and compared their combined fit to the multi-clade model. Our multi-clade approach is based on calculating joint likelihood function as a product of individual likelihood functions of models fitted on each of the six lineages (see Methods for details). In order to validate the performance of multi-clade approach and to address general issues with identifiability in diversification models[45], we explored the models behavior with simulated data, showing that our approach can perform unbiased model selection and accurately estimate model parameters (Supplementary methods SM2).

The best performing multi-clade model assumed constant speciation and extinction rates (Table 1). Time- or temperature-dependent models (median AICdiff between -1.16 and -1.71 $_{[df=1]}$ across 100 sets of Bayesian posterior trees) cannot in principle be rejected with confidence, but our sensitivity analysis showed that the used modeling approach and dataset have sufficient statistical power to detect temperature-dependent scenarios where Quaternary speciation rate dropped to 63% of Tertiary rate or less (Supplementary methods SM3). The universal temperature-dependence of speciation across all clades in our dataset considered together was thus either absent or weaker than such drop. Importantly, the combination of lineage-specific models slightly outperformed the models with shared parameters (median AICdiff=9.68 $_{[df=10]}$), suggesting that the estimated diversification parameters in reality differed across lineages (Fig. 2B). In particular, the diversification dynamics of two lineages was better explained by models with non-constant rates (Table 1): *Primula* showed a slowdown of speciation either with time (median AICdiff=2.04 $_{[df=1]}$) or during colder periods (median AICdiff=2.09 $_{[df=1]}$); and *Androsace* showed support for a speciation slowdown in colder periods, although model performance was only slightly better than the constant rate model (median AICdiff=0.40 $_{[df=1]}$) in this clade.

Evolutionary assembly: the relative influence of bedrock, elevation and geography. We used cladogenetic state-dependent diversification models (ClaSSE, [46,47]) to analyze the evolutionary assembly of our study lineages across bedrock types (calcareous vs. siliceous), elevation belts (high elevation vs. mid-elevation habitats), and geographic regions (five major European mountain regions, see Fig. 1). Using AIC comparisons, we quantified the importance of speciation associated with splits between bedrock types, elevation belts, or regions (which we term state-change speciation), speciation within the same bedrock type, elevation belt or region (constant-state speciation), and anagenetic change of bedrock type, elevation belt or region (which we term migration). The ClaSSE models for bedrocks and elevation belts were equivalent to a GeoSSE model[46], while the model for geographic regions represents a generalization of GeoSSE for more than two regions (see Methods and Supplementary methods SM4 for details). The best models inferred from model selection were then re-run in a Bayesian framework to obtain credibility intervals around parameter estimates. As in the previous analyses, all models were evaluated in the multi-clade framework with a model sharing parameters between clades, and subsequently as lineage-specific models with distinct parameter set for each clade (see Supplementary methods SM4 for a validation of the state-dependent multi-clade model using simulations).

The state-change speciation between siliceous and calcareous bedrocks appeared to be an important driver of plant diversification (AICdiff=8.96 $_{[df=1]}$). Model parameters suggest that a bedrock generalist lineage split into descendant lineages specializing on calcareous or siliceous habitats on average cca 0.5 times per Ma and such speciation events constituted 19% of all speciation events (Table 2). However, we found no evidence for state-change speciation between elevation belts (AICdiff=-2 $_{[df=1]}$) or between the five major mountain regions of Europe (AICdiff=-1.62 $_{[df=1]}$). The latter result suggesting no allopatric speciation between mountain regions is however likely biased due to the coarse scale of the considered mountain regions caused by computational limitations (see Methods for details). For this reason we addressed the prevalence of allopatric speciation and its spatial scaling with complementary analysis of sister species (see below).

Constant-state speciation rates differed between bedrock types (AICdiff=3.51 $_{[df=1]}$) and regions (AICdiff=17.87 $_{[df=4]}$), but not between elevation belts (AICdiff=-1.02 $_{[df=1]}$). In particular, we inferred higher speciation rates on siliceous than on calcareous bedrock (mean estimate and credibility interval in Fig. 3A) and also higher speciation rates in the Alps and the Iberian mountains than in any other European mountain region (Supplementary figure SF1). Unlike speciation, the inferred extinction rates did not vary between bedrock types, elevation belts or regions (AICdiff<0 in all the cases). Interestingly, the differences in diversification rates were not the major force affecting proportions of species across bedrock types or elevation belts. In other words, higher speciation rate in certain habitats did not necessarily result in higher species richness in these habitats (Fig. 3E and 3F). Rather, the difference in contemporary species richness seems to originate from directional shifts along ecological gradients, as our analyses inferred strong net directional migration between bedrock types and elevation belts. The rate of migration from siliceous to calcareous bedrock habitats was higher than in the opposite direction (AICdiff=5.59 $_{[df=1]}$, Fig. 3C), which better explains comparable present-day proportions of species growing on silicate and calcareous bedrocks (Fig. 3E, Supp. fig. SF2) despite differing speciation rates. We found even more important asymmetric migration rates between elevation belts, as we inferred strong directional migration from high to mid-elevation habitats (AICdiff=19.79 $_{[df=1]}$, Fig. 3D). This result captures events of secondary migration of high altitude ancestors toward lower elevations, leading to relatively high present-day and equilibrium proportions of species inhabiting either mid-elevations or occurring in both elevation belts (Fig. 3F, Supp. fig. SF2).

Models of evolutionary assembly slightly differed among the six study lineages (AICdiff=7.39 $_{[df=30]}$ for bedrock, AICdiff=21.07 $_{[df=20]}$ for elevation). The most notable outliers concerning the assembly across bedrock types were *Androsace* and *Phyteuma*, which exhibit higher proportions of species occurring on siliceous than on calcareous bedrock (Fig. 3E). In *Androsace*, this was linked to marginally faster diversification rates on siliceous than on calcareous bedrock and symmetrical migration between the two bedrock types. *Phyteuma* showed marginally faster diversification on calcareous bedrock, but strong directional migration towards siliceous bedrock, thus explaining the higher species diversity of this genus on siliceous bedrock (Fig. 3A). A notable exception concerning assembly across elevation belts was *Androsace*, which has a higher proportion of species occurring at high elevations (Fig. 3F) due to directional migration from mid- to high elevations (Fig. 3B). Based on ancestral state reconstructions (Supp. fig. SF3), *Androsace* also contains two exceptional lineages (containing five species each) which most likely spent their evolutionary history exclusively in high elevation habitats. *Gentiana* also has a relatively high proportion of species in high elevation habitats, but here the very broad credibility intervals of model parameters did not allow us to further distinguish scenarios of evolutionary assembly.

Sister species overlap in geographical and ecological space. Due to computational limitations, the ClaSSE models used above could only be run with a maximum of five geographic regions. Hence, we performed a complementary analysis to infer how sister species vary in the dimension of overlap in terms of elevation belt, bedrock type and geographic range, the latter being estimated both with coarse-scale and fine-scale geographic regions (five and 87 mountain regions, see Fig. 1 and Supplementary methods SM5). Most sister pairs diverged over the fine geographic scale (Fig. 4), with 37% sister species pairs showing no geographic overlap at this scale. Similarly as in ClaSSE models, sister species differed less across bedrock types (27% of sister pairs with no overlap) and over the coarse geographic scale (22% of sister pairs with no overlap). Only 9% of sister species pairs showed no overlap in the occupied elevation belts.

Although the order of speciation modes remained the same among all six study lineages, the degrees of overlap slightly differed between lineages (Fig. 4). Notably, the lineages with lowest geographic overlap at the fine scale were *Primula* (63% of pairs with no overlap) and *Androsace* (43% of pairs with no overlap), suggesting that allopatric speciation at finer geographic scale was particularly important for these two lineages. The lineage *Campanula* exhibited high variance in sister species overlap distribution at fine geographic scale, on the one hand having a high proportion of sister pairs with no overlap (43%), but on the other hand having the highest proportion of sister species pairs showing complete geographic overlap (36%).

Discussion

Here we explored the tempo and drivers of species diversification across a representative sample of six diverse plant lineages whose evolutionary history is tied to the European mountain system. As we purposely narrowed our study down to clades that previously received a systematic revision, it is clear that many of our studied species had already been sequenced, although always for few genes only and using variable methodologies. Instead we employed a uniform next-generation sequencing approach to all study clades and produced over two hundred whole plastid genomes, a cytoplasmic genomic compartment that has proven to be highly valuable for plant phylogenetic reconstructions and dating while avoiding some pitfalls from nuclear phylogenomics (e.g. paralogy, [48]). Our study therefore constitutes a significant step forward towards documenting the evolutionary origins of alpine plant diversity in European mountains, because it is the first to harness next generation sequencing on multiple plant clades in parallel and to apply a multi-clade comparative framework to explicitly model the tempo and drivers of mountain plant diversification. Our results may thus allow certain generalizations across our study lineages, confirming previous knowledge or botanists intuitions regarding plant diversification in European mountains, documenting some quite surprising, and perhaps counter-intuitive patterns, and finally identifying some interesting variations between the evolutionary histories of different study clades.

Pleistocene glacial oscillations have caused massive losses of plant diversity in Europe[49] and were often thought to have induced a diversification slowdown in European mountains[11,15,29]. Surprisingly, we detected neither strong nor homogeneous effects of Pleistocene climates on plant diversification rates across our six study lineages. All study clades together showed no indication for slowdown of species diversification with Pleistocene cooling, and a sensitivity analysis precluded universal Pleistocene reduction of diversification rates stronger than 63% of pre-Pleistocene rates. When analyzed at the level of individual lineages, only the two Primulaceae clades showed certain support for diversification slowdown, as was also demonstrated in an earlier study[11]. Yet more interestingly, the other four lineages showed no individual sign of slowdown during the Pleistocene. Moreover, the high elevation habitats and also the Alps *sensu stricto*, which were most severely impacted by glacial dynamics, did not show lower rates of speciation or higher rates of extinction.

Instead, high elevation lineages exhibited high rates of directional migration to lower elevations. This may suggest that the habitats suitable for high mountain plants were not severely reduced during glacial periods but rather shifted downwards and up again in the interglacials. Such shifts instead of loss in habitats may thus have resulted in relatively weaker and more lineage-specific declines in diversification rates. Strictly speaking, our findings are based on lineages that significantly diversified in the European mountain system, and should not be considered a contradiction of the paleontological evidence of massive Pleistocene extinctions in the European lowland flora[50–52]. The high rates of endemism and genetic diversity in low elevation refugia at mountain peripheries[7,8,53], and the evidence of mountain plants in lowland glacial palynological record (*Androsace sp., Gentiana sp.*, *Saxifraga sp.*, but also *Dryas octopetala*, *Polygonum viviparum* or *Saussurea sp.*, [54]) however suggest that the above described migration, diversification and survival processes may represent a general model for other extant mountain plant lineages not included in our study.

Why did diversification of some lineage slow down while other remain steady? Given that we have six cases for comparison and we only observed a slowdown in the Primulaceae family (*Primula* and *Androsace*), we can only hypothesize the answer. A scenario of ecological opportunity driving species diversification following mountain uplift[18,27] could provoke a diversification slowdown after saturation of the available ecological space. But such an effect should be observed in the oldest and most diverse lineages of our study clades, rather than in in the youngest and relatively least diverse ones such as *Primula* and *Androsace*. The observed diversification slowdown could alternatively be explained by a decreasing rate of allopatric speciation, as the two lineages that slowed down towards the present (*Primula*, *Androsace*) also show the highest rates of sister species allopatry. The prevalence of allopatric speciation can theoretically lead to an intrinsic slowdown of speciation rates, particularly in lineages with low dispersion capacity, once the spatial setting of species' geographic ranges no longer enhances further genetic isolation[55]. Another explanation could be that Pleistocene climatic oscillations promoted migrations between populations in areas that would otherwise remain isolated, thus reconnecting populations and inhibiting allopatric diversification. Simulations of diversification across dynamically fragmented landscapes indeed showed that more connected landscapes or a faster pace of connection-disconnection events can impede species diversification under certain conditions[23,56].

Allopatric speciation has long been regarded as a prevalent mode of speciation in mountain environments[11,15,36,37,57], and the high importance of allopatric speciation demonstrated by our results is generally consistent with this view. An additional interesting finding of our study is the relatively small spatial scale of allopatric speciation, which did not take place between major mountain systems such as the Alps or Carpathians, but rather within these systems. Although the small-scale allopatric speciation appeared to be the most important mode of speciation (37% of speciation events) in sister species of our study lineages, our results further suggest that bedrock-driven divergence has been another important driver (27%) of sister-species speciations. This contrasts with a previous study[11] finding an almost exclusive mode of allopatric speciation and little bedrock divergence in three European alpine Primulaceae genera. These previous results[11] may however reflect a peculiar situation in Primulaceae - a family containing *Androsace* and *Primula* - the two lineages in our dataset for which we found allopatry to be by far the most important mode of speciation.

Our estimates that bedrock shifts account for about one fourth (27% in sister species analyses) or one fifth (19% in ClaSSE analyses) of all speciation events across our study clades constitutes an important novel finding for mountain flora. Bedrock or edaphic species differentiation is well known from mediterranean floras[58–61], while in European mountain species it has mostly been studied at the intraspecific level[39], or in studies using non-quantitative comparisons to other speciation drivers[40]. To our knowledge, our result thus provides the first quantitative evaluation of the importance of bedrock driven speciation in European mountain plants. Still, it remains unclear to what extent these speciation events were truly parapatric, that is with reduced gene flow between adjacent populations due to bedrock adaptation, or whether bedrock specialization originated as a consequence of allopatry, given that calcareous and siliceous bedrocks rarely co-occur at small spatial scales. Experimental evidence suggests that new species can indeed arise due to strong divergent selection of calcareous and siliceous ecotypes, even in the presence of gene flow[62]. Our findings have far reaching implications: it shows that contrary to long standing expectations, many speciation events may not or not only have been due to selectively neutral geographic divergence, but also due to divergent evolution across a complex geological landscape requiring specific physiological adaptations to the divergent proportions of important nutritional elements[63].

Another important finding of our study is the inference of frequent migration events between bedrock types. Intriguingly, we found that these migrations were asymmetrical, with silicate habitats being more often the source of limestone-dwelling lineages than the other way around. This finding seemingly contrasts with previous literature suggesting that plant adaptation to calcareous soils is more restrictive than adaptation to silicate bedrocks[62,64–66]. The higher pleiotropic fitness costs of adaptation to calcareous bedrock may however prevent lineages from switchback to siliceous habitats, and calcareous habitats might thus function as the evolutionary trap[67,68]. Moreover, the stronger migration from silicates to limestones we detected here may also be linked to geographic contingencies: central high elevation parts of European mountain ranges (such as Central Alps, Central Pyrenees or High Tatras) typically consist of silicate bedrocks, while peripheral lower mountain systems are more often calcareous[44]. Elevation belts may therefore constitute a confounding hidden factor in the models of evolutionary assembly across bedrock types (*sensu* [69]), where the inferred migrations from high to low elevations and from siliceous to calcareous bedrocks may partly reflect the same process of historical migration out of the central ranges of the mountain systems, possibly due to Pleistocene glaciations.

The evolutionary assembly of our study clades across elevation belts was apparently marked by massive directional migration from high to mid-elevations, causing the mid-elevation habitats to host more species despite speciation rates being roughly equivalent in both elevation belts. This directional migration was likely fostered by Pleistocene glaciations causing downward shifts of communities during glacial periods, which then may have left relictual populations at lower elevations during interglacials. Such climate-induced range shifts may have had two-fold consequences on diversification dynamics. On the one hand, it may have prevented species extinctions due to habitat loss during glacial periods, as discussed earlier. On the other hand, intense altitudinal migration may have hampered speciation across elevation belts, as we did not find evidence for speciation along elevational gradients. This finding makes an important distinction with known mechanisms of diversification in the Andes or other tropical mountains, where speciation across elevation gradients seems to have been a relatively important driver of plant speciation[1,5,28,43]. More specifically, high and mid-elevation populations in the European mountain system may have differentiated at intra-specific levels[38,70], but the strong migration from high to lower elevations in response to the severe Pleistocene climate oscillations has likely prevented the emergence of reproductive barriers necessary for speciation events. Important historical migrations along elevation gradients may thus provide an explanation why species diversification has not been as explosive in the European mountain system as in tropical ones[15,29].

The lineage *Androsace* sect. A*retia* constitutes a notable exception regarding the general scenario of evolutionary assembly highlighted above. This lineage shows a greater species richness in high elevations due to directional migration from mid- to high elevation habitats, and according to the ancestral state reconstruction it contains two sub-lineages that likely evolved exclusively in high elevation habitats. In fact, several species of *Androsace* sect. *Aretia* stretch to the highest limits of vascular plant life in the European mountain system (above 4000 m a.s.l., [71]), and in Himalaya (6350 m a.s.l., [72]). Unlike other study lineages, *Androsace* have likely undergone specific adaptations facilitating the repeated entrance into the harsh adaptive zone of high elevation habitats and continued population persistence at high elevations throughout the Pleistocene. A clear manifestation of such adaptation is that *Androsace* repeatedly developed a dense cushion life form, an architecture seemingly perfectly adapted to plant life at high elevations not only in this genus[73] but also in many other angiosperm lineages[74]. Given the inferred long-term affinity of *Androsace* to high alpine environments, it can be reasonably hypothesized that high-elevation sub-lineages of *Androsace* have survived glacial periods *in situ* in so called nunatak refugia – i.e. rocky outcrops at high elevations protruding the glaciers[75] – rather than in peripheral refugia, which may have been the prevalent scenario for the majority of the other study lineages. The survival in the limited area of nunataks could also explain Pleistocene diversification slowdown observed in *Androsace,* although the consequences of nunatak survival for species diversification remains unclear. The high elevation sub-lineages of *Androsace* sect. *Aretia* identified in this study thus constitute a perfect system for further tests of the nunatak survival hypothesis at smaller spatial and phylogenetic scales.

Conclusions

Our study provides an unprecedented window to the history of diversification of the temperate mountain flora in Europe. It shows that plant diversification within European mountains was a complex evolutionary process, with a strong interplay between altitudinal migration, allopatric speciation and bedrock adaptation of different lineages. Importantly, the onset of Pleistocene climate did not cause a strong diversification slowdown, as was previously expected, but rather stimulated strong migrations of mountain biota across elevation gradients, particularly towards lower altitudes. We hypothesize that these massive altitudinal migration events on the one hand buffered extinctions due to habitat loss during glacial periods, but on the other hand impeded Pleistocene adaptive radiations across the elevation gradient, as is classically known from tropical mountains. We found speciation events to be mostly driven by geographic divergence but almost as frequently by bedrock shifts, which is a novel finding. Overall, the absence of obvious adaptive diversification across the elevation gradient and the prevalence of allopatric speciation likely contributed to the lower richness and slower diversification dynamics generally observed in temperate mountains compared to tropical ones.

Methods

Clade selection, chloroplast genome reconstructions and phylogenetic inferences. The study lineages were selected as representative cases of plant species diversification in the European mountains by the following criteria: they contain more than 20 species in total; they contain at least 10 species inhabiting alpine and nival elevational belts in the European Alps (based on [76]); they were recently subject to taxonomic or phylogenetic revision suggesting that Europe is their center of diversity; they are dicots; and they do not follow derived life strategies as is myco-heterotrophy, parasitism or carnivory. Within the PhyloAlps consortium, which is an extensive network of collaborating institutions (see Supplementary note SN1), we sampled the majority of ingroup species and well established subspecies from these lineages (212, 84% of the total of 251 known ingroup species and subspecies). The well established subspecies were treated and referred to as species throughout the study. The majority of ingroup (sub)species were covered by one sample, but in several cases we included two or more samples for control purposes. For all lineages, we followed the most up-to-date taxonomic treatments and took into account phylogenetic studies providing group circumscription (Supplementary methods SM6). As outgroups for our analyses, we sampled an extensive collection of species from the families Campanulaceae, Primulaceae, Gentianaceae, Saxifragaceae and Grossulariaceae. The latter one was added to Saxifragaceae because of their close phylogenetic relationships[77] and to compensate the lack of fossil calibration points within Saxifragaceae. For details on sample counts and identities, please refer to Supplementary methods SM6 and accession table in Supplementary dataset SD1.

We extracted DNA from collected samples and prepared genomic shotgun libraries that were sequenced with Illumina HiSeq technology. The protocols of molecular biology for DNA extractions and library preparation are detailed in Supplementary methods SM7. The resulting paired-end reads were used to reconstruct the chloroplast genomes, using Org.Asm 1.0.3, a *de novo* organelle assembler based on De Bruijn graph[78]. We retrieved complete circular plastomes except for samples belonging to Campanulaceae (*Campanula* and *Phyteuma* lineages) where the assembly typically resulted in several discontinuous contigs that however covered the majority of chloroplast coding regions. We annotated all complete and fragmented plastomes, extracted focal coding and non-coding regions and aligned them region-by-region at the family level, resulting in high quality sequence matrices for each family ranging between 35471-47102 bp for up to 72 coding regions and 2435-5112 bp for up to 17 non-coding regions, with less than 3% missing data overall. See Supplementary dataset SD2 for a list of regions used in phylogenetic inference for each family, and Supplementary methods SM8 for more details on sequence processing.

The resulting coding and non-coding alignments for each of the four families were used for inferring dated phylogenies in BEAST 2[79], using different site models for the non-coding alignment and each codon position in coding alignment, Yule tree prior and lognormal clock with uniform fossil dating priors on at least two nodes of each family. Note that we applied uniform priors between minimum and maximum bounds because the sparse fossil record of our study clades does not allow applying more informative priors – this therefore constitutes a very conservative approach. In some cases, the fossil record allowed multiple intepretations of age and positioning in the phylogenetic tree, we therefore explored robustness of our dating to this factor. For further details on phylogenetic and dating analysis, see Supplementary methods SM1. Maximum credibility trees and reduced posterior trees were pruned to only include ingroup species and one individual per species, for details on species tree inference see Supplementary methods SM6. We obtained well resolved species-level phylogenies with 87% of nodes receiving >0.95 posterior probability (see Supplementary dataset SM3 for maximum credibility trees with node supports).

The phylogenies based on chloroplast genomes provide high resolution and accurate dating due to the large number of orthologous genomic regions with variable mutation rates, and the universality of the bioinformatic pipeline allowing to obtain homogeneous phylogenetic information across virtually any angiosperm group[48]. The limitation of this approach is that chloroplast-based phylogenies are only tracking the evolution of maternal lineages, which might be problematic in systems with frequent hybridization, and also at shallow phylogenetic scales due to incomplete lineage sorting. The absence of polyphyletic species and the low amount of identified paraphyletic species (see Supplementary methods SM6 for details) suggest that neither hybridization nor incomplete lineage sorting are causing important biases in our study groups.

Moreover, although hybridization and incomplete lineage sorting may influence the topology of specific tree, they are unlikely to systematically bias whole-region diversification patterns explored it this study.

Ecological and geographic characteristics of species. We attributed information about elevation range and bedrock affinity to each study species, using local floristic literature supplemented with expert knowledge of some groups (see Supplementary methods SM5 for details). This served to estimate each species' presence in habitats above treeline (referred to as high elevation habitats), below treeline (referred to as mid-elevation habitats), calcareous, dolomitic or ultrabasic bedrocks (referred to as calcareous bedrocks) and bedrocks with neutral or acidic reaction (referred to as siliceous bedrocks).

In order to compile information about species geographical distribution, we defined smallest operational geographical units across Europe for which we could get credible presence/absence information of every species in our dataset. We used this approach rather than grid-based processing of point occurrence data (as e.g. in [11]), because of imbalanced point data quality depending on country and broader European region. For the purpose of ClaSSE models, operational geographic units were merged to five major mountain regions of Europe and surrounding areas (Fig 1.). For details on focal regions, elevation belts, bedrock definitions and data sources, please refer to Supplementary methods SM5 and Supplementary dataset SD4.

Tempo of species diversification. Based on the inferred phylogenies of each lineage, we fitted five models depicting different temporal dynamics of species diversification (see Table 1). For the models with temperature-dependent speciation or extinction, we used exponential dependence on ^{18}O isotope ratio time-series from Greenland ice cores[80]. To accommodate for phylogenetic uncertainty, we fitted every model on 100 trees randomly selected from the Bayesian posterior distribution of phylogenetic trees, and report either the whole distribution (parameter estimates) or median of values (AIC comparisons).

To explore the overall diversification dynamics across all study lineages, we developed a multi-clade framework to fit the above described diversification models across multiple evolutionary lineages simultaneously. We assumed that each of our lineages is an independent realization of shared diversification dynamics, which allowed us to construct the joint likelihood functions of temporal diversification models as a product of likelihood functions of each of the six lineages, and harnessing the lineage-specific parameters into shared values. A similar approach with joint likelihood was previously used for state-dependent diversification models[46,81]. We optimized model parameters using the joint likelihood function with a simplex routine, equivalent to default implementation of single-lineage models in the R package RPANDA 1.5 [82] (see Supplementary methods SM2 for implementation details).

To test for time- and temperature-dependence of diversification in both multi-clade and single-lineage analyses, we calculated AIC difference (AICdiff) between constant speciation and constant extinction model vs. the other respective models (Table 1). The constant speciation and constant extinction model is nested in the other respective models, and has one less degree of freedom. AICdiff=-2 thus suggests that the focal model parameter does not improve likelihood at all, AICdiff=0 suggests that both models are equally valuable from information-theoretic point of view, whereas AICdiff>2 suggests substantial support for the focal model[83], i.e. that the focal model would be outperforming the null model even if there was one completely non-informative parameter added on top of the focal one. We used equivalent interpretation of AICdiff values also in other AIC comparisons throughout the paper.

To test whether the dynamics of diversification are indeed shared across the six lineages as assumed by the multi-clade models, or whether they quantitatively differ among them, we compared the AIC of the multi-clade model with the respective sum of AIC values across the six lineage-specific models. Such a comparison is meaningful, because the sum of AIC values of the six lineage-specific models is equal to the AIC calculated from a model with joint likelihood function, but with each model parameter kept lineage-specific.

The time dependent diversification models were recently criticized due to identifiability problems[45]. In our analyses we address this issue in several ways: First, we use diversification models corresponding to explicit hypotheses of past diversification dynamics, rather than hypothesis free approaches that were the main subject of criticism. Second, following the recommendations in [45], we use parametrization where only speciation or only extinction is variable in time, and we interpret their results acknowledging that speciation or extinction variability may fall in the same congruence classes. Finally, we perform validation tests and a sensitivity analysis with values realistic for our dataset to show that both single-lineage and multi-clade models correctly identify parameter values from the simulated data (see Supplementary methods SM2 and SM3).

Evolutionary assembly across bedrock types, elevation belts and geographic regions. We used cladogenetic state-dependent speciation-extinction models[46,47] (ClaSSE), to study separately how diversification and migration occurred across bedrock types, elevation belts and regions. In the models for bedrock types and elevational belts we used parametrization equivalent to GeoSSE[46] that attributes one of 3 states to each species – exclusive for one bedrock type or elevation belt; exclusive for another bedrock type or elevation belt; present in both bedrock types or elevation belts. For the model for geographic regions we newly developed a generalization of GeoSSE for more than two regions (see Supplementary methods SM4 for script). In this generalization, the number of model states is growing exponentially with number of regions, which prevented us from using a more detailed division of European mountain system than 5 regions (and thus 31 states). All analyses were run with the maximum credibility phylogenies for each of the six focal lineages. Similarly to time-dependent diversification models, we ran all models for each lineage separately, and also a multi-clade model with parameters shared for all the lineages, using the same procedure of likelihood multiplication as described above and equivalent to one in [46] or [81].

For each evolutionary assembly model (on bedrock types, elevation belts, regions), we performed a series of AICdiff comparisons of maximum likelihood model fits to test the importance of different model features: (i) presence state-change speciation (following the terminology of ClaSSE models[47], that is, speciation associated with splits of ancestor species occupying both bedrock or elevation types, or multiple regions), (ii) difference of constant-state speciation rates for each bedrock type, elevation belt or region, (iii) difference of extinction rates for each bedrock type, elevation belt or region, (iv) directionality of migration between bedrock types, elevation belts and regions. The inferred best model was subsequently rerun in a Bayesian setup in order to obtain probability envelopes of parameter estimates, using slice MCMC sampler run for

10000 iterations and a burn-in of 5000 iterations. The proportions of state-change to all speciation events were obtained by multiplying the present-day numbers of species in different model states (e.g. calcareous specialist, siliceous specialist, bedrock generalist) by their respective speciation rates. The analyses were performed using the R package diversitree 0.9-11[84].

The SSE models are known to suffer from elevated Type I errors when testing constant-state diversification rates differences between states, and various correction strategies were proposed to address this issue[69,85–89]. However, this issue is relevant mostly when the evolutionary states are stable[69,85], which is clearly not the case of our study plant lineages with important role of migration and state change speciation across bedrocks, elevational belts and regions. Moreover, all constant-state speciation and extinction rate differences presented in this paper turned unsupported or only weakly supported even without these corrections, and are interpreted as such. Instead, we used the SSE framework for testing and interpreting rates of migration and state-change speciation, and subsequent ancestral state reconstructions, where the SSE methodology is adequate[90]. For estimating state-change dynamics and ancestral state reconstructions, the SSE methodology is known to statistically outperform the alternative approaches not explicitly accounting for diversification dynamics, as is BioGeoBEARS[91] or Mkn model[90]. As recommended[69,88], we also tested the ability of used single lineage and multi-clade SSE to correctly recover parameters, using simulated datasets resembling our data (Supplementary methods SM4).

Sister species overlap in geographical and ecological space. We performed an overlap analysis between sister species in order to address importance and frequency of allopatric speciation at two different spatial scales, along with speciation between bedrock types and elevation belts. To do this, we identified all sister species pairs in maximum credibility phylogenies. For each of the pairs we calculated the Schoener's D niche overlap index[92] for 87 operational geographic units (fine scale geography), five European mountain regions (coarse scale geography), elevational belts (high elevation vs. mid-elevation) and bedrocks (calcareous vs. siliceous). The overlap estimates were calculated using the R package spaa 0.2.2[93].

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Figures and tables

Figure 1: Map of the European mountain system depicting the five major geographic regions and the six mountain plant lineages used in this study. The colors used for the six different phylogenies are used accordingly throughout following figures. The timescale unit of phylogenetic branch lengths is Ma.

Figure 2: Tempo of species diversification for the six study lineages. (A) Lineage-through-time curves of each lineage. The thick lines represent maximum credibility phylogenetic reconstructions, while the semi-transparent lines represent 100 trees sampled from Bayesian posterior distributions. The dashed line marks the onset of the Pleistocene at 2.6 Ma before present. The number of lineages is plotted in logarithmic scale, i.e. the exponential growth expected under pure birth model would appear linear here. (B) Parameter estimates indicating the effect size of the temperature control on speciation rates in temperature-dependent models of species diversification. The black dots represent the estimates from the multi-clade model with shared parameters among lineages. Higher values above the dashed line indicate lower speciation in colder geological periods. Thick dots correspond to estimates based on the maximum credibility phylogenetic trees, and semi-transparent dots on the 100 trees sampled from the posterior distribution.

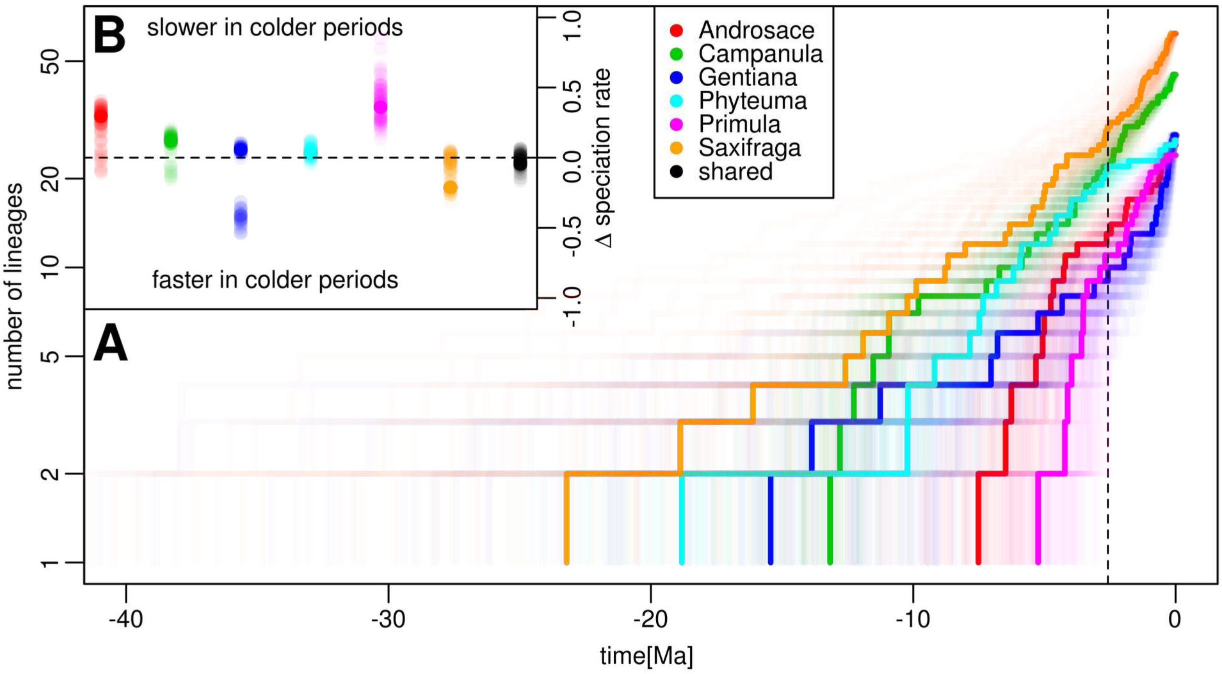

Figure 3: Results of ClaSSE models of evolutionary assembly across ecological gradients of bedrock (A, C, E) and elevation (B, D, F). The panels (A) and (C) show differences between siliceous and calcareous habitats in constant-state speciation and migration rates, respectively. The panels (B) and (D) show differences between high and mid-elevation habitats in constant-state speciation and migration rates, respectively. The dots represent mean Bayesian estimates and the bars indicate 95% credibility intervals. Black dots and bars represent the parameter estimates from the multi-clade model with shared parameters among lineages. The panel (E) represents proportions of species inhabiting siliceous, calcareous or both types of habitats (light, dark and middle gray, respectively), and the panel (F) represents proportion of species inhabiting high elevation, mid-elevation or both types of habitats (light, dark and middle gray, respectively).

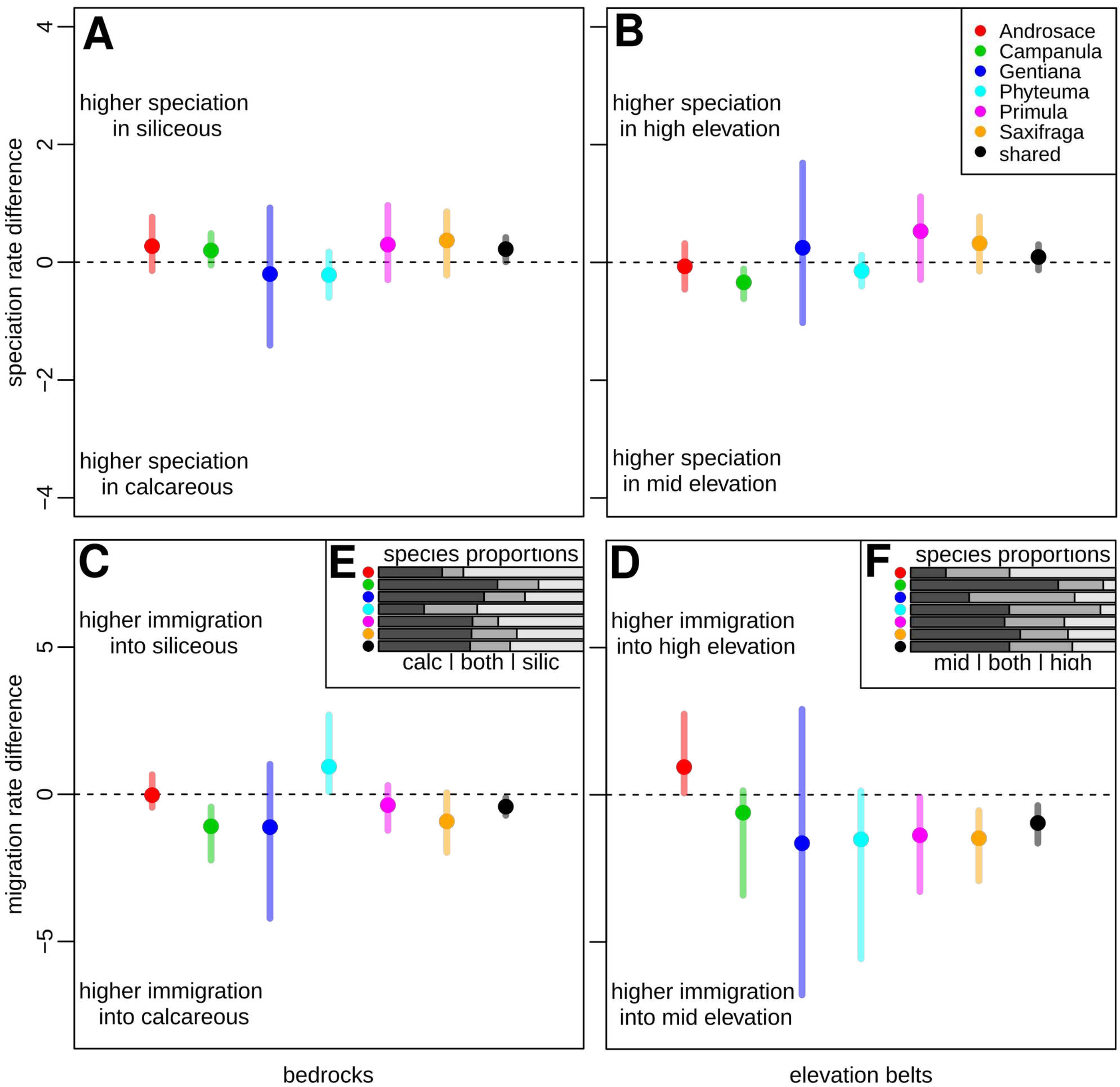

Figure 4: Sister species overlap in geographic and ecological space. Violin plots show distribution of Schoener's D index of overlap of sister species pairs across 87 operational geographic units (fine geo), five European mountain regions (coarse geo), bedrock types (calcareous vs. siliceous) and elevational belts (high vs. mid-elevations). The point within each plot represents median overlap value. Low overlap between sister pairs is considered as an indication of frequent speciation along the respective geographic or ecological dimension.

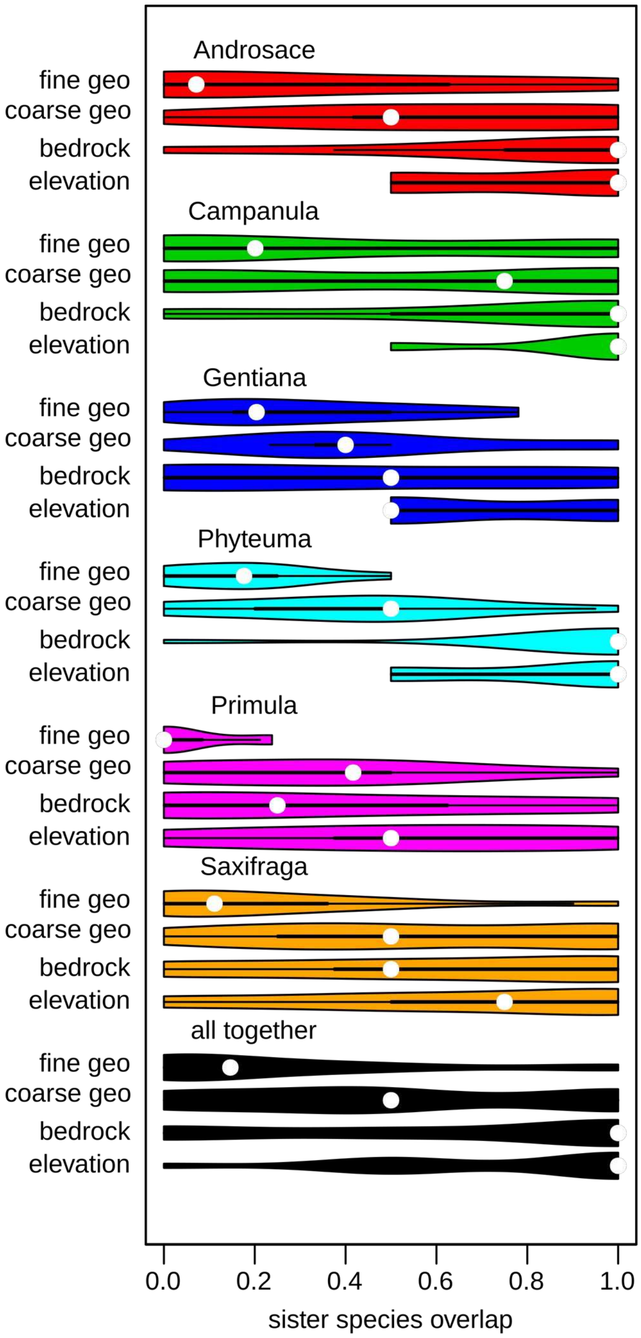

Table 1: Median AIC differences (AICdiff) between multiple time- or temperature-dependent diversification models and the nested null model with constant speciation and constant extinction. Values with median AICdiff>2 are depicted in bold.

	Androsace	*Campanula*	*Gentiana*	*Phyteuma*	*Primula*	*Saxifraga*	shared
speciation constant, extinction constant	0.00	0.00	0.00	0.00	0.00	0.00	0.00
speciation dependent on past temperature, extinction constant	0.40	-0.99	-1.42	-1.82	**2.09**	-1.70	-1.71
speciation constant, extinction dependent on past temperature	-2.00	-1.82	-0.25	-2.00	-2.00	-1.83	-1.16
speciation exponentially dependent on time, extinction constant	0.00	-0.95	-0.45	-1.89	**2.04**	-1.81	-1.16
speciation constant, extinction exponentially dependent on time	-0.08	-1.53	-0.31	-2.00	1.62	-1.80	-1.25

Table 2: Comparison of ClaSSE models of evolutionary assembly containing parameters of state-change speciation between bedrocks (siliceous vs. calcareous), elevation belts (high elevation vs. mid-elevation) or five coarse geographic regions, with the nested models lacking state-change speciation terms. Values with AICdiff>2 are depicted in bold.

	rate of state-change speciation (Ma^{-1})	proportion of state-change to all speciation events	AICdiff
bedrock types	**0.453**	**0.19**	**8.96**
elevation belts	<0.001	<0.001	-2.00
geographic regions (coarse scale)	0.013	0.063	-1.62

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DatasetS1Accessiontable.csv
- DatasetS2Genomicregions.csv
- DatasetS3Maximumcredibilityspeciestrees.txt
- DatasetS4Ecologicalandgeographicinformation.csv
- DatasetS5Alternativemaximumcredibilityspeciestrees.txt
- 6cladessupplementaryfinal.pdf