Tracing the evolutionary history of Dracocephalum austriacum: Insight from population genomics and species distribution modelling

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Abstract

Austrian dragonhead (*Dracocephalum austriacum*), a rare species of mountain steppe/steppe habitats, has a highly fragmented distribution across its entire range. Due to its narrow ecological requirements, it inhabits relict habitats that are increasingly threatened by climate change, human disturbance and ongoing succession. Across Europe, the species persists in less than 90 small and spatially isolated populations. Although it is legally protected, without targeted conservation efforts and tailored rescue programmes, its future remains uncertain. Therefore, we genotyped almost all of the known populations across the species' range using the DArTseq method to apply these data to support ongoing direct conservation efforts. We analysed key indices of genetic diversity, their spatial patterns, and phylogenetic relationships. To reconstruct the species' phylogeographical history, we also conducted MaxEnt modelling of habitat suitability. Our results reveal five geographically restricted genetic lineages considered as evolutionary significant units. The most distinct lineage in the eastern Pyrenees exhibits signs of long-term isolation and retention of ancestral polymorphism. In contrast, the remaining four lineages are of more recent origin and reflect a clear phylogeographic pattern of westward expansion from the Caucasus. The divergence between the two most distinct genetic lineages likely began during the Last Interglacial period, while a subsequent wave of east-to-west colonisation during the Last Glacial Period introduced newer lineages. Overall, genomic diversity is low and relatively uniform across the species' range. Effective population size estimates suggest that most populations are only viable in the short term.

Introduction

The study of genomic structure in endangered species is essential for conservation biology, evolutionary research, and ecosystem management (e.g., Breed et al. 2019, Luikart et al. 2019, Hohenlohe et al. 2021, Ma et al. 2023, Schneider 2023, Theissinger et al. 2023). In light of the ongoing threats to biodiversity posed by climate change (e.g. Wudu et al. 2023, Bose et al. 2024, Price et al. 2024, Rai et al. 2025), habitat destruction, and human activities (Laurance 2010, Beyer and Manica 2021, Hogue and Breon 2022), there is an increasing emphasis on understanding the genetic diversity and population structure of rare and declining species. This has become a priority for conservation efforts, as highlighted, e.g., by Allendorf et al. (2010), Frankham (2010), Laikre (2010), Hoban et al. (2013) or Heuertz et al. (2023). Reflecting these needs, new and courageous projects are emerging, such as the Darwin Tree of Life (Darwin Tree of Life Project Consortium 2022), the European Reference Genome Atlas (McCartney et al. 2024), or the Earth BioGenome Project (Lewin et al. 2018), which aim to facilitate genetic research.

Genetic diversity is key to species survival, allowing populations to adapt to changing environments, resist pathogens, and maintain reproductive viability. However, endangered species may suffer from reduced genetic variation due to small population sizes, habitat fragmentation, and historical bottlenecks, increasing their vulnerability to extinction (Ellstrand and Elam 1993, Kitner et al. 2012, Plenk et al. 2016, Szczecińska et al. 2016, Vaculná et al. 2021). Genomic studies provide important insights into the processes shaping genetic structure in endangered plants (Kitner et al. 2012, Teixeira and Nazareno 2021, Vaculná et al. 2021). Genome-wide analyses enable us to assess diversity levels, detect past demographic events, and reconstruct patterns of gene flow (Lee et al. 2020, Seidl et al. 2022, Gao et al. 2024, Li et al. 2024, Wariss et al. 2025), often at lower cost and higher resolution (Andrews et al. 2016). These data help identify distinct evolutionary lineages (Evolutionary Significant Units, ESUs – e.g. Funk et al. 2012, Hoelzel 2023), detect genetic bottlenecks, and determine whether populations are experiencing inbreeding or hybridisation (Frankham 2015, Jennigs et al. 2016, Gargano et al. 2022, Starkey and Gray 2022). Genomic approaches also facilitate the identification of adaptive traits that may be essential for the long-term survival of species facing environmental challenges (Meger et al. 2024, Jiang et al. 2025, Van Deurs et al. 2025).

Phylogeographic analyses, which integrate genetic data with historical biogeography, further enhance our understanding of species distributions over time. By reconstructing past range shifts and refugial dynamics, we can infer how past climatic oscillations and geological events have influenced population connectivity and divergence (Hewitt 2000, 2004, Hampe and Petit 2005, Kajtoch et al. 2016). Such knowledge is particularly valuable for endangered plants with fragmented distributions, as it helps pinpoint regions of high genetic uniqueness and conservation priority (Petit et al. 2003, Kutnjak et al. 2014, Vaculná et al. 2021, Seidl et al. 2022).

From a conservation perspective, genomic studies provide crucial information for relevant conservation authorities to maintain or restore the genetic variability of a given population (Whiteley et al. 2015, Hoban et al. 2020, 2023). For instance, conservation programs can use genetic data to guide seed banking efforts (e.g. Hamrick and Trapnell 2011, Peres 2016, Wambugu et al. 2023), design effective landscape/habitat corridors (e.g., Razgour et al. 2017) and develop strategies that maximise genetic resilience (Frankham 2015, Whiteley et al. 2015, Frankham et al. 2017). Without such knowledge, conservation interventions may overlook critical genetic factors, leading to ineffective or detrimental outcomes (e.g., Ralls et al. 2020). Given the increasing threats to global biodiversity, integrating genomic research into conservation planning has never been more important. By studying the genetic structure of populations of endangered plants, we not only uncover their evolutionary history but also equip conservationists with the necessary tools to justify the selection of management measures to safeguard these species for future generations.

The genus *Dracocephalum* L. comprises around 60 species distributed across temperate Eurasia, with one species native to North America (Chen et al. 2022). Most species are adapted to temperate continental climates, including alpine environments, and thrive under strong seasonal temperature and precipitation variations (Liu et al. 2024, Chen et al. 2022, Abdullaeva et al. 2019). Phylogenetic and biogeographic analyses suggest that the genus originated in West and Central Asia, extending into southern Siberia, where it initially diversified before spreading westward across Eurasia (Chen et al. 2022). *Dracocephalum austriacum* L. (Austrian dragonhead), the focus of this study, belongs to the basal "Ruyschiana" clade, along with *D. grandiflorum* L., *D. ruyschiana* L., *D. argunense* Fisch. ex Rchb., and the North American *D. parviflorum* Nutt. (Chen et al. 2022, Liu et al. 2024). Despite morphological diversity, members of this clade are diploid and share a basal chromosome number of $x = 7$ and consistently occupy basal positions in genus-level phylogenies (Chen et al. 2022).

Today, *D. austriacum* occurs in scattered, relic populations. Its distribution ranges from the Caucasus (Armenia, Georgia, and Russia: Dagestan and the Pyatigorsk region) to Eastern and Central Europe (Ukraine – 6 populations, Romania – 4, Hungary – 4, Slovakia – 5, Austria – 2, Czech Republic – 8). It also occurs in the Alps (Italy – 6, Switzerland – 31, France – 18) and extends westward to the Eastern Pyrenees in Spain (3 populations) (Meusel et al. 1978, Käsermann 1999, Hrouda 2000, Aymerich & Sáez 2001, Dostálek et al. 2010, Bonnet et al. 2020, Infoflora 2025). *Dracocephalum austriacum* is a perennial herbaceous to subshrub species characteristic of steppe/mountain steppe habitats. It is a heliophilous species with narrow habitat requirements, thriving on open sunny, well-drained, shallow calcareous soils in exposed areas, windy, rocky outcrops. Specifically, the species frequently inhabits the western edges of rocky outcrops (like upper parts of deep gorges, rims of cliffs), where it benefits from cold ascending winds from deep valleys and gorges (Fig. 1, our observations, see also Nicolè et al. 2011, Dostálek and Münzbergová 2013). Its ecological niche closely resembles that of its relative *D. ruyschiana* (Kyrkjeeide et al. 2020). The species occupies a wide altitudinal range, from ~ 230 m a.s.l. (e.g., Vanovice, Bohemian Karst) to over 2000 m (2008 m in Sevan National Park, Armenia; 2131 m in Vanoise National Park, France). Despite its broad geographic range, *D. austriacum* is a rare relict species with significant geographic isolation among populations (Hrouda 2000, Dostálek et al. 2010). It is listed in Appendix I of the Bern Convention (Council of Europe 1979), in Appendix II and IV of the EU Habitats Directive (Council of the European Communities 1992) and on national Red Lists in nearly all countries of occurrence (Witkowski et al. 2003, Bañares et al. 2010, Király et al. 2007, Kagalo et al. 2009, Tamanyan et al. 2010, Bilz et al. 2011, Eliáš et al. 2015, Grulich and Chobot 2017, Schratt-Ehrendorfer et al. 2022).

The species is threatened by habitat loss due to natural succession (e.g., shrub and tree encroachment), increased shading and degradation of its open rocky habitats. Climate change further compromises populations by altering rainfall patterns, resulting in severe droughts during the growing season (May–July), especially affecting individuals on shallow soils on steep slopes (Nicolè et al. 2011, Dostálek and Münzbergová 2013, our observations). Due to its limited distribution and strict habitat specificity, *D. austriacum* is particularly vulnerable to environmental change and anthropogenic disturbance. Despite its conservation importance, the species' evolutionary history and genetic diversity remain insufficiently understood. Previous studies indicate that the level of genetic diversity has an important effect on seed production and thus influences the fitness and vitality of the populations (Dostálek et al. 2010). It has also been shown that habitat quality and climatic variation significantly influence the population dynamics (Nicolè et al. 2011), and that habitat quality, genetic diversity, and climatic variation influence key steps that are essential for population growth rate (Dostálek and Münzbergová 2013). However, there are significant gaps in our knowledge of intraspecific genetic variation and historical biogeography of the species. Investigating the population structure and phylogeographic patterns of the species can provide insights into past dispersal events, genetic connectivity, and potential refugia during past climatic fluctuations, especially when done across the whole species range.

Recent advances in population genomics, particularly high-throughput sequencing and genome-wide markers (e.g. Satam et al. 2023), have enabled more comprehensive assessments of genetic diversity, gene flow, and historical biogeographic events in rare species. In this study, we aim to (1) characterise the genetic structure and genomic diversity of *D. austriacum* populations across its distribution range, (2) infer historical processes that shaped its current distribution, and (3) evaluate the future of the species using ecological niche modelling. Our results will contribute to a deeper understanding of the evolutionary processes that have shaped the distribution of *D. austriacum* and provide important information for its conservation management.

Material and methods

Plant material

We aimed to sample the entire range of *D. austriacum*, targeting at least 10 individuals per population. All known populations in Austria, the Czech Republic, Hungary, Italy, Romania, Slovakia, and Ukraine were sampled. In Armenia, one of the two known populations was sampled. The number of populations in the Russian Federation remains uncertain, though the species is more widespread there. Sampling in Georgia was not possible due to the inaccessibility of the regions where the species occurs. In Spain, 2 of the 3, in France, 8 of the 18 and in Switzerland, 8 of the 31 known populations were sampled. In total, 62 populations from 12 countries were sampled, yielding 772 individuals (Fig. 1F, Table 1; SM 1). The outgroup consisted of 48 samples from: *D. ruyschiana* (42 samples/5 populations), *D. multicaule* Montbret & Aucher ex Benth. (2 samples/1 population), *D. grandiflorum* (2 samples/1 population) and *D. argunense* (2 samples/1 population) (SM 2). The closest relative (*D. ruyschiana*) was sampled from the western (Switzerland) and central (Dagestan) regions of its range. Due to the conservation status of *D. austriacum*, exact coordinates are not provided. In all countries, the sampling was carried out in accordance with the legal requirements (see Acknowledgement).

Table 1
Summary table showing the number of populations sampled (N_P), the number of samples collected (N_{SAM}), the number of samples analysed by DArTseq (N_{DART}), and mean number of samples analysed per population (N_M). Countries where all recent populations have been sampled are indicated by '*' (see Materials and Methods section for details).

Country	Label	N_P	N_{SAM}	N_{DART}	N_M
Armenia	AM	1	10	10	10.00
Austria*	AT	2	20	20	10.00
Czechia*	CZ	8	158	103	12.90
France	FR	8	136	80	10.00
Hungary*	HU	4	28	26	6.50
Italy*	IT	6	70	57	9.50
Romania*	RO	4	60	40	10.00
Russia	RU	8	27	27	3.40
Slovakia*	SK	5	68	48	9.60
Spain*	ES	2	25	20	10.00
Switzerland	CH	8	98	57	7.10
Ukraine*	UA	6	72	52	8.70
Total	**12**	**62**	**772**	**540**	**8.98**

DNA extraction and DArTseq genotyping

Genomic DNA was extracted from dried leaves using the protocol of Inglis et al. (2018) with minor modifications. DNA quality was verified via agarose gel electrophoresis, and concentration was measured by Quantus Fluorometer (Promega). DArTseq genotyping (Kilian et al. 2012) was conducted on 540 samples of *D. austriacum* (selected based on the quality of extracted DNA) and 21 outgroup samples by Diversity Arrays Technology Pty Ltd (DAT Pty Ltd., Bruce, Australia) using Medium density sequencing (1.2 million reads/sample). The DAT integrated analytical pipeline processed the raw data and delivered it to our laboratory for further statistical treatment.

Sanger sequencing of ITS locus and phylogenetic analysis

To check for taxonomic misidentification and compare the resolution of DArTseq with Sanger sequencing, 46 ingroup and 6 outgroup samples (SM 1 and 2) were sequenced for the ITS region (ITS1-5.8SrDNA-ITS2; primers ITS1 and ITS4 – White et al. 1990). PCR was performed in 15 µl reactions with 50 ng of template DNA, 0.1 mM primers, 1.45 mM dNTPs, 1× buffer (containing 3 mM $MgCl_2$), and 1 U of GoTaq® G2 DNA polymerase (Promega). Thermocycling conditions: 95°C for 5 minutes; 35 cycles at 95°C for 30 s, 52°C for 45 s, and 72°C for 45 s; final extension at 72°C for 5 min. Products were purified using the enzymatic purification protocol (Kim and Blackshaw 2001), GeneElute PCR Clean-Up Kit (Sigma-Aldrich®), and sequenced bidirectionally by Macrogen Europe. Sequences were edited and aligned in Geneious® 7.1.7 (Kearse et al. 2012). Data were submitted to GenBank (accession numbers available in SM 3) and compared to ITS sequences from GenBank for quality control (see SM 4 for the list of compared records). Phylogenetic analysis was performed in MrBayes v. 2.2.4 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) implemented in Geneious 7.1.7 using the GTR + G + I nucleotide substitution model, with two simultaneous runs of four parallel MCMCMC chains, each for 3,000,000 generations, with a random starting tree, subsampling frequency set to 1,000 and 30% of the trees discarded as burn-in.

DArTseq data analysis

For further genotypic analyses, we used SNP data, which are more informative than SilicoDArTs data type generated with the DArTseq genotyping platform. To achieve accuracy and reliability of analyses, the raw data (561 individuals; 88,658 loci) were filtered using the R (R Core Team 2020) package dartR v2.9.7 (Gruber et al. 2018, Mijangos et al. 2022). Filtering steps included: removal of "secondaries": reduction of linkage and redundancy; "read depth": default range of 5–50; "reproducibility": 1; "locus callrate": ≥ 0.97; "individual callrate" ≥ 0.90; minor allele count (MAC): ≥ 3; removal of monomorphic loci. These settings represent a compromise between the necessity of maintaining high data quality and reproducibility while ensuring an adequate number of individuals. These filtering steps were applied to three datasets: 1) full dataset including outgroup (561 samples), 2) ingroup-only dataset (540 samples), and 3) ingroup excluding Spanish Pyrenean populations (520 samples).

Clustering analyses

Phylogenetic relationships were examined using a split network (SplitsTree v.4.18.2; Huson and Bryant 2006) based on unbiased Nei's genetic distances (uNei; GenAlex; Peakall and Smouse 2012), and a tree built via the SVDQuartets method (Chifman and Kubatko 2014, 2015) implemented in the

PAUP*4.0a program (Swoford 2003) using 100,000 quartets and 1,000 bootstrap replicates, summarised as a 50% majority-rule tree. STRUCTURE (Pritchard et al. 2000) was used to detect Evolutionary Significant Units (ESUs) and admixture patterns. *K* values ranged from 1 to 15, with 10 replicates per *K*, each run for 500,000 MCMC iterations (burn-in: 50,000). Results were summarised using STRUCTURE Harvester (Earl and von Holdt 2012) and CLUMPP (Jakobsson and Rosenberg 2007). For the identification of the most likely number of the genetic clusters (*K*) within the analysed data, Evanno's Delta*K* method was used (Evanno et al. 2005). Maps were visualised in QGIS 3.28 (QGIS 2024). PCoA was performed in the dartR.

Genetic diversity, isolation by distance, fixed differences and effective population size

Genetic diversity metrics were calculated in GenAlEx, including observed (Ho) and expected (uHe) heterozygosity, allelic richness (Na, Ne), private alleles (Np), polymorphism (P%), Shannon's index (I), inbreeding coefficient (F_{IS}), and Wright's F_{ST}. For the calculation of these statistics, due to a low number of samples and the close proximity of Russian populations (RU_A, RU_E–H), they were pooled (RU_A*), and neighbouring Czech populations were merged (CZ_AB*; CZ_CD*). Differences between defined groups were analysed via one-way ANOVA and Tukey's HSD test (STATISTICA; TIBCO). Analysis of Molecular Variance (AMOVA) was conducted to assess the distribution of genetic variability, and the statistical significance of this distribution was evaluated using 1,000 replicates. Pairwise uNei genetic distances were visualised using the R package pheatmap (Kolde 2018).

Isolation by Distance (IBD) was analysed by correlating genetic and geographic distance matrices. Fixed differences (loci fixed for alternate alleles between populations, i.e. populations are reciprocally fixed for a different allele) were identified using the function "gl.fixed.diff" (threshold: tloc = 0). The generated matrix of pairwise differences was then used by the function "gl.collapse," which further amalgamated populations with fixed differences less than a specified threshold (tloc = 0). Both analyses were performed using the dartR package.

Effective population size (*Ne*) was estimated at the species level, lineage level and country level using the linkage disequilibrium (LD) method implemented in NeEstimator v.2 (Do et al. 2014). *Ne* was not estimated per population due to the small sample size. The rationale behind this is that 1) despite a strong population structure, populations from each country always belonged to the same genetic lineage and estimation was not skewed by the Wahlund effect; 2) diversity indices were similar between the populations; 3) the aim was to estimate *Ne* at a broader scale. Due to the strong difference between the two Spanish populations, *Ne* at the species level and for all lineages/countries except Spain were calculated with and without the two genetically distinct Spanish populations.

Estimation of divergence time using DIYABC-RF

To estimate the time frame of the divergence among genetic lineages and infer the demographic history, we used the software DIYABC Random Forest v1.2.1 (Collin et al. 2021). Three competing divergence scenarios were defined based on five genetic lineages identified using PCoA and STRUCTURE analyses: SC1: strictly hierarchical (tree-like) divergence; SC2: hierarchical divergence with later gene flow from a basal lineage; SC3: parallel divergence from two ancestral sources followed by a secondary merger (SM 5a). Because no prior estimates for demographic parameters were available, we used log-uniform priors spanning broad time and population size ranges. The training dataset was generated by running 20.000 simulations, and the full set of available summary statistics was used. The Random Forest (RF) algorithm was used for model selection, using 1.000 trees per forest.

To test the potential influence of strong genetic structure on model selection, an additional analysis was performed, excluding the most divergent lineage (populations from the Spanish Pyrenees, lineage N1), which showed markedly high genetic differentiation (SM 5b). This reduced analysis included only four lineages and three adjusted divergence scenarios. Again, log-uniform priors were used, and 20.000 simulations were performed using all summary statistics derived from the reduced SNP dataset. Model selection was repeated with the same RF settings.

The time parameters (t_1–t_4) were estimated using the full dataset. An additional set of 20,000 simulations was generated for the selected scenario for these estimations. Since it takes the Austrian dragonhead five years to reach generation time (our observations), this value was used to calculate the divergence times from the estimated number of generations by the DIYABC-RF analysis.

MaxEnt niche modelling

MaxEnt (version 3.3.3; Phillips et al. 2006) was used for ecological niche modelling, utilising environmental data from 132 verified occurrence points (GBIF 2022; personal observations). To reduce spatial sampling bias, occurrence records were spatially filtered using a regular grid (0.01° resolution), retaining only one point per grid cell. Paleoclimate projections incorporated data from General Circulation Models (GCMs) representing the Last Glacial Maximum (LGM; ~ 22.000 BP), Mid-Holocene (~ 6.000 BP), and downscaled data for the Last Interglacial (LIG; ~ 130.000 BP; Otto-Bliesner et al. 2006) combined with WorldClim version 1.4 climate layers (Hijmans et al. 2005). Future projections (2021–2040 and 2081–2100) were based on CMIP6 data and WorldClim version 2.1 (baseline: 1970–2000; Fick and Hijmans 2017). The SSP3 (Shared Socioeconomic Pathway 3) scenario was adopted, as we considered it the most realistic under current global trends (Riahi et al. 2017). To approximate present-day conditions (~ 2025), we used the 2021–2040 SSP2-based projection, since 2025 falls within this interval and the original baseline (1970–2000; WorldClim v2.1) is now outdated. Spatial resolution was 30 arc-seconds for most data, except for LGM projections, which used 2.5 arc-minutes. GCMs used in ensemble modelling: CCSM4 and MPI-ESM-P for LGM; CCSM4, CNRM-CM5, HadGEM2-CC, HadGEM2-ES, and MPI-ESM-P for the Mid-Holocene, and EC-Earth3-Veg for future projections (SM 6a). These models were selected for their accuracy in reconstructing historical European climate (McSweeney et al. 2015, Palmer et al. 2023) and are widely used in species distribution modelling. Soil data from ISRIC (Hengl et al. 2017) and GlobalChange (Shangguan et al. 2014) were included to refine predictions for this calciphilous species.

Model performance was assessed using threshold-independent receiver operating characteristic (ROC) analysis. MaxEnt was initially run with default settings using all 19 bioclimatic variables along with selected soil variables. To reduce multicollinearity, we calculated pairwise Pearson correlation coefficients and removed one variable from each pair with |r| >0.8, resulting in a final set of nine relevant predictors (SM 6b). Climatic data from WorldClim version 2.1 were used for model training and evaluation under current conditions. To optimise model settings, we used the ENMeval R package (Kass et al. 2021), testing various combinations of feature classes and regularisation multipliers. Based on Akaike Information Criterion corrected for small sample sizes (AICc), the best combination was Linear + Quadratic features with a regularisation multiplier (RM) of 0.25; sequential model selection favoured Hinge feature with an RM of 3. However, neither configuration outperformed the default settings, which were therefore retained. The final model was run with 10-fold cross-validation, all other parameters left at default. Ensemble models for multi-GCM projections (LGM, Mid-Holocene) were generated using the dismo R package (Hijmans et al. 2024), and final predictions were visualised using QGIS.

Results

ITS phylogeny

ITS amplification was successful for all selected samples. The total length of the final alignment for the complete data set was 605 bp. Except for one transversion (position 5: C → A), no other intraspecific variability was detected within the investigated *D. austriacum* samples. This SNP was detected in one sample from the Czech Republic (pop. CZ_G), one sample from Italy (pop. IT_A) and two samples from Slovakia (pop. SK_H). The comparison with GenBank records did not reveal any further intraspecific diversity. The populations of *D. austriacum* appear to be highly homogeneous for the ITS locus. The two morphologically most similar species, *D. austriacum* and *D. ruyschiana*, differed by only one transition (19: T → C) within the sequenced ITS locus. Bayesian phylogenetic analysis revealed a dendrogram in which *D. austriacum* formed a terminal cluster with *D. ruyschiana* as a sister species. *Dracocephalum grandiflora* formed a sister branch to the *D. austriacum-D. ruyschiana* cluster (SM 7).

DArTseq data

Phylogenetic and clustering analyses

After applying a series of qualitative filtering steps to the SNP data, three final datasets were retained: 1) with outgroup – 5357 polymorphic loci, 561 samples; 2) only ingroup – 5026 polymorphic loci, 530 samples; 3) ingroup without Pyrenean populations – 4808 polymorphic loci, 511 samples.

Phylogenetic analysis of the complete dataset (with outgroup) produced a well-supported phylogram showing *D. austriacum* as a monophyletic group comprising five distinct genetic lineages (Fig. 2). The Pyrenean lineage formed the earliest diverging and most basal group. The next diverging lineage was the Caucasian lineage, consisting of populations from Armenia and Dagestan, and corresponds to the easternmost distribution of the species. This clade is followed by the Eastern European lineage, represented by populations from Ukraine and Romania. The remaining populations formed two geographically structured groups: the Alpine lineage (includes populations from the French, Italian and Swiss Alps) and the Pannonian lineage (includes populations from Hungary, Slovakia, Austria and the Czech Republic). The topology of the inferred tree, particularly the distinction between these five lineages, received strong statistical support. Split network provided a very similar view and showed *D. austriacum* as a well-defined group, including the Pyrenean lineage, which is clearly separated within this group, and forms a transition to phylogenetically more distant species (SM 8).

Principal coordinate analysis (PCoA, Fig. 3) yielded similar results. Populations from the Spanish Pyrenees were clearly separated along the first axis (Fig. 3A). When excluded, separation among the remaining four lineages (Caucasian, Eastern European, Alpine, and Pannonian) became more pronounced (Fig. 3B). A clear distinction was evident between the genetically similar Eastern European and Caucasian lineages. Notably, the Austrian population occupied an intermediate position between the Czech, Hungarian, and Slovak populations within the Pannonian lineage (Fig. 3B).

Bayesian clustering using STRUCTURE was conducted to assess the population genetic structure within the complete dataset. The analysis yielded clear results, identifying two distinct genetic clusters (K = 2) as the most supported differentiation for the examined data (Fig. 4). The first cluster comprised the Pyrenean populations, while all remaining populations formed a second genetic group.

To further resolve the genetic structure within this second genetic group, a subsequent STRUCTURE analysis was performed with the dataset without the Pyrenean populations. In this case, the highest statistical support (based on Delta$K$) was observed for a division into three genetic clusters (K = 3; Fig. 5A). This grouped the previously defined genetic lineages as follows: the Caucasian and Eastern European lineages formed a single cluster, the Pannonian lineage represented a second cluster, and the Alpine lineage formed the third. Some degree of admixture was evident among these genetic groups. An alternative grouping, which provided a more detailed resolution of the population genetic structure, suggested a division into five genetic clusters (K = 5; Fig. 5B). In this scenario, the Caucasian-Eastern European lineage remained a single genetic entity, while the Pannonian lineage split into Czech and Slovak/Hungarian populations, with Austrian populations in an intermediate position. Austrian populations represent a transition between these two subgroups and, simultaneously, between the Pannonian and Alpine lineages. The Alpine lineage was divided into two subgroups, separated geographically in Switzerland.

Genomic diversity

Genetic differentiation across the full dataset (including Pyrenean lineage) was high (${}^{+PYR}F_{ST}$ = 0.322), indicating strong population structure (presence of five major lineages) and limited gene flow among subpopulations. This implies that subpopulations are largely isolated and undergoing gradual genetic divergence due to restricted gene exchange and genetic drift. When Pyrenean populations were excluded, the differentiation remained very high (${}^{-PYR}F_{ST}$ =

0.225). The slightly negative inbreeding coefficient ($^{+PYR}F_{IS}$ = -0.011/$^{-PYR}F_{IS}$ = -0.020) suggests a slight excess of heterozygotes, with no evidence of inbreeding depression. Across the dataset, all statistical indices indicated low genetic diversity. The average polymorphism was 17.28% (SE = 0.45), while the mean number of distinct alleles per locus (Na = 1.157, SE = 0.001) and the number of effective alleles (Ne = 1.084, SE = 0), along with Shannon's index (I = 0.077, SE = 0), suggested very limited genetic variation distributed evenly across the species' range. This low genetic diversity was further supported by low heterozygosity values (Ho = 0.051, SE = 0; uHe = 0.053, SE = 0).

The genetic variability indices among the identified lineages did not differ largely, with mean values across all five lineages being largely similar (Tab. 2, SM 9). When examining genetic diversity within individual lineages, the Pyrenean lineage exhibited the highest values of least common alleles (LCA, Tab. 2, SM 9), suggesting a unique genetic composition. This distinctiveness was further emphasised by the highest number of private alleles (Np, Tab. 2, SM 9). Statistically significant differences among lineages were identified for multiple indices (one-way ANOVA, Tukey's HSD test; $p < 0.05$): 1) Ne - for pairs of lineages: Alpine vs. Caucasian, Alpine vs. Eastern European, Pannonian vs. Eastern European; LCA (≤ 25%) - for pairs of lineages: Pyrenean vs. all other lineages, Alpine vs. all other lineages; 3) LCA (≤ 50%) and Np - Pyrenean vs. all other lineages; 4) Ho - Eastern European vs. Pannonian and Alpine; 5) uHe - Eastern European vs. Alpine.

The pronounced genetic distinctiveness of the Pyrenean lineage was also evident in the heat map illustrating the degree of genetic similarity and differentiation among populations (SM 10a), whereas differences among the other populations were less pronounced. When the dataset was analysed without the Pyrenean lineage, the pattern of genetic similarity and differentiation aligned with the structure of the remaining genetic lineages (Caucasian, Eastern European, Alpine, and Pannonian; SM 10b).

Analysis of molecular variance (AMOVA) indicated that most of the genetic variation resided within individuals (58%). A substantial proportion of genetic variation (30%) was distributed among populations, highlighting the presence of strong population structure. In contrast, genetic variation within populations was relatively low, accounting for only 12% of the total variance. Fixed Differences analysis (SM 11) again highlighted the distinctiveness of the Pyrenean lineage, but no statistically significant fixed differences were identified among the remaining populations. When populations representing the Pyrenean lineage were excluded, all the populations were collapsed, indicating the absence of fixed differences (data not shown). Isolation by distance (IBD) showed a weak but significant correlation between genetic and geographic distance (R^2 = 0.143; p = 0.001), increasing to R^2 = 0.288 (p = 0.001) when the Pyrenean lineage was excluded. This underscores the role of spatial separation in shaping genetic structure and divergence of investigated populations.

The calculation of the effective population size of *D. austriacum* gave very different estimates depending on the dataset used for the calculation. When *Ne* was estimated from the whole dataset (including the two distinct Spanish populations), LD*Ne* = 10.4, while when calculated from the dataset without the Spanish populations, it gave the more realistic value of LD*Ne* = 95.3 (Tab. 3 and SM 12). The difference in these estimates is most likely due to the distinction of the Pyrenean lineage and the rest of the populations studied, resulting in a strong population structure. Estimated *Ne* at the lineage level reached 50 or more only for the Caucasian, Pannonian and Alpine lineages (Tab. 4 and SM 13), country-level *Ne* (SM 14) were below 50 (considering the estimates without singletons), suggesting reduced long-term viability.

Table 2 Statistical indices of genetic variation presented as means for each recognised genetic lineage across the studied *D. austriacum* populations. The complete population statistics are presented in SM 9. N_P = number of populations, N = number of samples, N_M – mean number of samples analysed per population, P = polymorphism, Na = number of different alleles, Na (≥ 5%) = number of alleles with frequency ≥ 5%, Ne = number of effective alleles, LCA (≤ 50%/25%) = number of alleles with frequency ≥ 5% present in ≤ 50%/25% of populations, Np = number of private alleles, I = Shanon's information index, Ho = observed heterozygosity, uHe = expected unbiased heterozygosity, F_{IS} = inbreeding coefficient. Superscript letters indicate groups that are significantly different from each other, according to Tukey's HSD test.

Lineage	N_P	N	N_M	P (%)	Na	Na (≥ 5%)	Ne	LCA (≤ 25%)	LCA (≤ 50%)	Np	I	Ho	uHe	F_{IS}
Pyrenean^{A}	2	19	9.5	14.7	1.147	1.147	1.082	0.198^{BCDE}	0.215^{BCDE}	73^{BCDE}	0.073	0.048	0.051	0.006
Caucasian^{B}	4	35	8.8	18.2	1.182	1.182	1.088^{E}	0.069^{AE}	0.108^{A}	9^{A}	0.084	0.056	0.058	-0.034
EastEuropean^{C}	10	92	9.2	18.2	1.182	1.182	1.089^{ED}	0.062^{AE}	0.105^{A}	11^{A}	0.083	0.057^{DE}	0.058^{E}	-0.067
Pannonian^{D}	17	194	11.4	18.3	1.183	1.167	1.081^{C}	0.060^{AE}	0.100^{A}	5^{A}	0.079	0.052^{C}	0.053	-0.035
Alpine^{E}	22	189	9.0	16.2	1.162	1.160	1.077^{BC}	0.044^{ABCD}	0.083^{A}	6^{A}	0.074	0.050^{C}	0.051^{C}	-0.055

Table 3 The estimate of effective population size (*Ne*) of *Dracocephalum austriacum*. Estimates of *Ne* are in italics and underscored. DA^{+PYR} = estimate based on dataset including all the studied populations; DA^{-PYR} = estimate from dataset excluding two distinct Spanish Pyrenean populations; 0+ = estimate based on inclusion of all alleles; No S* = estimate excluding singletons.

Taxon/Whole range	DA^{+PYR}		DA^{-PYR}	
Lowest Allele Frequency Used	0+	No S*	0+	No S*
Harmonic Mean Sample Size	523.7	523.7	505.5	505.5
Estimated LD*Ne*	*10.4*	*10.4*	*95.4*	*95.3*
CI low JackKnife	10.4	10.4	99.3	99.0
CI high JackKnife	11.9	11.9	109.5	109.6

Table 4 Estimates of the effective population size (*Ne*) of defined *Dracocephalum austriacum* genetic lineages. Estimates of *Ne* are in italics and underscored. 0+ = estimate based on inclusion of all alleles; No S* = estimate excluding singletons.

Country	Pyrenean		Caucasian		Eastern European		Pannonian		Alpine	
Lowest Allele Frequency Used	0+	No S*	0+	No S*	0+	No S*	0+	No S*	0+	No S*
Harmonic Mean Sample Size	17.5	17.4	35.4	35.4	91.1	91.1	192.4	192.4	186.9	187.0
Estimated LD*Ne*	*10.5*	*6.7*	*79.8*	*55.2*	*61.0*	*37.9*	*75.9*	*55.5*	*98.5*	*72.6*
CI low JackKnife	7.6	4.2	59.9	39.6	45.7	28.0	67.2	50.4	86.0	66.7
CI high JackKnife	15.0	9.9	136.7	85.5	86.4	53.3	81.4	61.4	108.8	87.0

Divergence time estimation based on DIYABC-RF analysis

Analysing the full dataset, historical Scenario 3 (SC3) received the highest posterior probability in the Random Forest (RF) analysis (SM 5c, d). However, PCA projections (SM 5d) indicated a better fit of the observed data with Scenario 1 (SC1) – the observed dataset fell clearly within the simulated cloud of SC1. This discrepancy likely reflects a bias introduced by the strong genetic divergence of the Pyrenean lineage (N1), which dominates the variance structure and may drive scenario selection toward deeper divergence models. To test this hypothesis, we repeated the DIYABC-RF analysis using a reduced dataset excluding the Pyrenean lineage and evaluated three adjusted divergence scenarios (SM 5b). In this analysis, SC1 was strongly supported (SM 5e, f). It received the highest posterior probability, and the observed data clustered tightly within its simulated cloud in PCA space.

Based on these results, we estimated divergence times under SC1 using the full dataset (SM 5a). The divergence of the Pyrenean lineage (t_4) was estimated at 101–156 ka, broadly coinciding with the Last Interglacial Period. The remaining lineage splits were estimated as follows: Caucasian (t_3 = 5.3–15.6 ka), Eastern European (t_2 = 3.0–10.9 ka), and Pannonian–Alpine divergence (t_1 = 1.0–6.0 ka; Tab. 5). Estimates for t_1 and t_2 exhibited narrow credibility intervals, low out-of-bag (OOB) prediction errors, and were driven by meaningful summary statistics, indicating high reliability (SM 5g). The t_3 estimate was broader, with modest noise in the predictors, suggesting moderate reliability. In contrast, the estimate for t_4 was characterised by a broad, multi-modal posterior, high OOB error, and strong influence from noise variables, indicating low reliability, likely due to limited signal at deeper timescales.

MaxEnt niche modelling

The MaxEnt niche model was evaluated using a receiver operating characteristic (ROC) curve. The mean Area Under Curve (AUC) across replicate runs was 0.969 ± 0.021 (SM 15a), indicating excellent model performance. Among the environmental variables, 'Precipitation of the warmest quarter' (bio18)' contributed the most to the model (23.5%), followed by 'Mean temperature of the warmest quarter' (bio10; 22.2%; SM 15b). These results suggest that the modelled distribution of *D. austriacum* is mainly influenced by precipitation and temperature during the growing season. The species has an optimum at approximately 250–300 mm of rainfall during the warmest quarter (SM 15b), while its probability of occurrence decreases with increasing mean temperatures during this period (SM 15b). The third most influential variable was 'Calcium carbonate content' (CACO3; 16.2%), which aligns with the species' calciphilous nature. The response curve peaks for soils containing around 35% $CaCO_3$ (SM 15b). When considered in isolation, bio10 provided the highest gain, whereas the omission of bio18 caused the greatest decrease in model gain, further highlighting its importance.

For the oldest period representing the Last Interglacial (LIG; ~130,000 BP), the model predicted highly unfavourable conditions for the species, with a marked reduction in suitable habitats. Potential refugia during this period were restricted to isolated areas in the Alps, Pyrenees, western Caucasus, and possibly the Dinarides (Fig. 6A). The LIG climate was warmer than today, with mean temperatures during the warmest quarter (bio10) approximately 3°C higher than the 1970–2000 baseline, while minimum temperatures of the coldest month (bio6) could be up to 10°C lower, creating stressful extremes (data extracted, not shown). Under Last Glacial Maximum (LGM; ~ 22,000 BP) conditions (Fig. 6B), the model predicted a significant expansion of suitable habitats, particularly in southwestern Europe, including the Pyrenees, French Alps, and Massif Central, and the northwestern Caucasus. During the mid-Holocene (~ 6,000 BP) (Fig. 6C), a warm and humid period characterised by forest expansion, *D. austriacum* faced a contraction of suitable habitats. Suitable areas were largely confined to Alpine, Pyrenean, and Caucasian refugia—regions that still host the core populations of the species today.

The model for the current distribution (2021–2040) accurately reflects known centers of the species, particularly in the Caucasus and the Alps, and identifies potentially suitable areas in the Pyrenees and Carpathians (Fig. 6E). Interestingly, the model also predicts suitable habitats in the Dinaric Mountains, where the species has never been reported. Conversely, *D. austriacum* still persists in Austria and the Czech Republic, despite the model predicting low habitat suitability in these regions. Populations in these countries are declining, as they face worsening conditions during recent climatic

extremes (very warm and dry springs). The modelled distribution under historical climate conditions (1970–2000; WorldClim v2.1) suggests that conditions were more favorable in the end of the 20th century, with a wider range of suitable habitat (Fig. 6D). Future projections for the period 2081–2100 under SSP3 (Fig. 6F) indicate further habitat loss, especially in Central Europe (e.g., Czech Republic, Austria, Slovakia and Hungary) and the Pyrenees, where the species is likely to disappear from many of its current locations.

Discussion

Phylogeography of Dracocephalum austriacum

Our analyses of DArTseq data provide new insights into the phylogeny, phylogeography, and population-genetic structure of *D. austriacum*. The species forms a distinct, well-supported group, genetically clearly separated from other members of the Ruyschiana clade (Fig. 2, SM 7 and 8). Phylogenetic reconstruction reveals five major genetic lineages corresponding to geographic regions: Pyrenean, Caucasian, Eastern European, Pannonian, and Alpine (Fig. 2, SM 8). The Caucasian lineage is basal and strongly supported, indicating an origin or early divergence of the species in the Caucasus-Transcaucasus region. This finding supports the genus-level biogeographic model proposed by Chen et al. (2022) and extends it to the intraspecific level. The tree topology and clustering pattern suggest a gradual westward expansion. Notably, the Pyrenean lineage appears highly divergent and terminal, likely representing a relic lineage isolated since an early colonisation event. Its genetic distinctiveness suggests retention of ancestral polymorphism not preserved elsewhere in the species' range (Figs. 2, 3A, 4; Table 2; SM 9 and 8). This pattern is consistent with survival in long-term climatic microrefugia, where populations could persist through periods of unfavourable environmental conditions. The role of European glacial-interglacial refugia is well documented (Hewit 2000, Schmitt 2007, Provan and Bennett 2008) and is supported by both fossil and genetic evidence (Magri et al. 2006, Médail and Diadema 2009). There is substantial evidence that the Pyrenees, with their variety of habitats, likely served as important refuges for *D. austriacum* during the climatic oscillations of the Quaternary period. This notion is supported by several studies (e.g., Médail and Diadema 2009, González-Sampériz et al. 2010, García et al. 2022).

Ecological niche modelling and current habitat preferences suggest that *D. austriacum* favours cooler and more continental climates typical of steppe or montane steppe environments. These conditions prevailed during glacial periods, which supported wider and more continuous distributions of steppes across large parts of Central Europe (Frenzel and Troll 1952, Janska et al. 2017, Lang 1994, Willis and van Andel 2004). During this period, *D. austriacum* might flourish, similar to other steppe-adapted species (see Kajtoch et al. 2016 and references therein, Meindl et al. 2016, Seidl et al. 2022). In contrast, warmer interglacial phases, such as the Last Interglacial (~ 130 ka BP), with temperatures exceeding present levels (e.g. Otto-Bliesner et al. 2013, 2021, Wilcox et al. 2020), likely resulted in range contraction, fragmentation and isolation in refugia (Fig. 6) (see Kajtoch et al. 2016 for other examples). The divergence of the Pyrenean lineage likely began during this period, when a previously continuous range started to fragment into isolated refugia (Fig. 7, Table 5). These refugial populations, including those in the Caucasus and Pyrenees, likely underwent genetic divergence driven by climatic and environmental pressures. Similar rocky habitats to those occupied by the species today might serve as a refuge for *D. austriacum* in the Pyrenees but also elsewhere, as they provide a variety of microniches and support local relicts (e.g., González-Sampériz et al. 2010, García et al. 2019, 2022, Birks and Willis 2008). With the onset of the Last Glacial Period (~ 110 ka BP), colder conditions allowed recolonisation from eastern refugia (Fig. 7). During the Last Glacial Maximum (~ 22 ka BP), the new lineage of *D. austriacum* was likely already present in Europe and persisted in multiple areas and the species continued to expand into the central European and Alpine regions until the end of the glacial period (Table 5). The transition into the Holocene brought warmer, more humid conditions that became less favourable for *D. austriacum*. The rapid expansion of forests across the open steppe and park-like landscape in Central Europe (e.g. Huntley and Birks 1983, Magri et al. 2006, Abraham et al. 2016, Giesecke et al. 2017) caused the gradual retreat of the species to relict habitats. In Alpine regions, it meant movement to higher altitudes; in the Pannonian region (and also in eastern Europe), it meant movement to relict stands with heliophilous, xeric steppe-like vegetation of calcareous soils. This led to fragmentation and the present-day patchy distribution of genetically structured populations (Figs. 6 and 7, Table 5). Thus, all extant populations outside the Iberian Peninsula appear to descend from an evolutionarily younger lineage (Fig. 2, Table 5), better adapted to cold, wind-exposed open habitats of the Last Glacial Period.

Our data support the proposed scenario through multiple lines of evidence. The marked genetic distinctiveness of the Pyrenean lineage suggests ancient isolation, as their genetic distance from other lineages precludes recent origin (e.g. SM 8 and 9). In contrast, the remaining *D. austriacum* populations share a more recent evolutionary history, as indicated by both phylogenetic (Fig. 2, SM 8) and STRUCTURE analyses (Figs. 4, 5). In the eastern range, populations form a cohesive lineage. The Caucasian group occupies a basal position, and Eastern European populations (Ukraine, Romania) show genetic continuity with it. STRUCTURE analysis indicates a single genetic cluster encompassing both groups, consistent with a common origin and possible migration from the Caucasus into eastern Europe. In the west, two distinct lineages are present: the Pannonian lineage (Hungary, Slovakia, Austria, and the Czech Republic) and the Alpine lineage (Italian, Swiss, and French Alps). Along with the Pyrenean and Caucasian–Eastern European lineages, these may be recognised as evolutionarily significant units (ESUs – as discussed by Funk et al. 2012, Hoelzel 2023, etc). Results of the DIYABC-RF also strongly support the above-presented diversification time frame, although the predictive accuracy for the older divergence time (t_4) has lower reliability (Table 5, SM 5).

MaxEnt niche modelling further supports this interpretation (Fig. 6), suggesting very restricted habitat availability during the LIG and more extensive ones during the LGM. Suitable areas in western and southern Europe may have once supported the now Pyrenean-restricted lineage. However, there are no records of *D. austriacum* from the central Apennines or the Dinaric Mountains. The inability of the model to predict suitable areas in Central and Eastern Europe during the LGM can be explained by the limited resolution of the data layers, which fail to predict microclimatic and microhabitat conditions on a finer scale in these regions.

The distribution pattern of *D. austriacum* mirrors that of its sister species *D. ruyschiana* on its European range, which has a more continuous range in northern Europe and Russia extending to Mongolia but is highly fragmented in southern and central Europe. There it occurs in relic habitats in the Pyrenees, Alps, Carpathians, Dinaric Mountains, and Caucasus (Lazarević et al. 2009, Kyrkjeeide et al. 2020, Guadiola 2022). The Serbian population, for example, grows in a habitat with a cold continental climate supporting many steppe species, likely remnants of Pleistocene steppe expansions (Lazarević et al. 2009). These two sister species thus not only share an evolutionary origin but also exhibit similar phylogeographic histories shaped by Quaternary climatic oscillations.

Genomic diversity and effective population size of Dracocephalum austriacum

The observed genetic diversity pattern, characterised by similar diversity indices across geographically and genetically distinct groups, is likely shaped by a combination of factors, including demographic bottlenecks, niche conservatism, uniform selection pressures, and life history traits (e.g., longevity, limited dispersal capacity). This pattern supports a scenario of gradual lineage spreading rather than rapid, leading-edge colonisation. If rapid colonisation were the cause, we would expect the typical genetic signature of colonisation: a gradual decrease in genetic diversity with increasing distance from the source population (Templeton 1980, Austerlitz et al. 1997). Although the IBD analysis showed increasing genetic differentiation with geographic distance, the correlation was weaker than expected in this scenario. Nevertheless, the low and homogeneous levels of genetic diversity across lineages (e.g., Na ≥ 5%, Ne, Ho, uHe; Table 2, SM 9) suggest demographic bottlenecks, likely associated with postglacial population dynamics of the species during the current interglacial period, the Holocene. This is further supported by species distribution modelling (MaxEnt), which indicates repeated restrictions in habitat suitability over time (Fig. 6).

The narrow ecological preferences of *D. austriacum* may also contribute to low genetic diversity, as adaptation to a specific ecological niche with relatively uniform selective pressures could have led to allelic fixation and reduced heterozygosity (e.g. Ellstrand and Elam 1993). Furthermore, the geographic isolation of subpopulations limits gene flow, reinforcing the population structure, which is expected to become more pronounced over time due to genetic drift (Ellstrand and Elam 1993). The distribution of genetic variation, largely found among individuals rather than populations, means that *D. austriacum* shares much of its genetic diversity across its range. This is consistent with adaptation to narrow ecological conditions. Additionally, life history traits influence the genetic diversity (e.g., Hamrick and Godt 1996, Thiel-Egenter et al. 2009, Hernández-Espinosa et al. 2022). *Dracocephalum austriacum* is a long-lived perennial with low dispersal ability (primarily via barochory; Dostálek and Münzbergová 2013) and a small effective population size (Tables 3, 4; SM 12, 13 and 14). This results in slow mutation accumulation, limited increases in heterozygosity, and increased genetic drift (Kimura and Ohta 1969, Nei et al. 1975, Templeton 1980).

Dostálek et al. (2010) conducted a study on the same Czech and Slovak populations used in this study, using allozymes. Their findings show a similar level of genetic diversity, despite employing a different methodology. Detected differences (e.g., higher F_{IS} and expected heterozygosity He) can be attributed to methodological differences and the fact that their study focused on populations from the same Pannonian lineage. This further corroborates the robustness and credibility of our results.

When comparing the genetic diversity of *D. austriacum* with its closest relative, *D. ruyschiana*, the latter species shows higher genetic diversity (Kyrkjeeide et al. 2020), likely due to its higher population connectivity, different historical dynamics, and larger effective population size. The genetic diversity in *D. austriacum* is consistent with that observed in other rare species with small, fragmented populations, such as *Adenophora liliifolia* (Vaculná et al. 2021), *Artemisia pancicii* (Kitner et al. 2012), and *Pulsatilla patens* (Szczecińska et al. 2016).

Effective population size (*Ne*) is a theoretical but useful concept in population genetics (Wright 1931) and has significant implications for conservation, especially for rare species. It is linked to the long-term survival of a species because it influences genetic diversity and the rate of genetic drift, which can affect a population's ability to adapt to environmental changes and avoid inbreeding depression (Frankham 1995, Charlesworth 2009, Fedorca et al. 2024). Estimating *Ne* from genetic data requires careful consideration as it is influenced by factors like low genetic diversity, high inbreeding, strong population structure (Wang 2005, Fedorca et al. 2024, Gargiulo et al. 2024). In this study, strong population structure influenced species-level *Ne* estimates, with the Pyrenean lineage showing a much lower *Ne* (LD*Ne* = 10.4, Table 3, SM 12) compared to the estimate excluding this lineage (LD*Ne* = 95.4; Table 3, SM 12). For practical purposes, it is more appropriate to interpret *Ne* within the range of the confidence intervals and for estimations excluding singleton allelic variants. At the level of individual lineages, estimated *Ne* values are slightly above 50 (Table 4, SM 13), which, according to the 50/500 rule (Franklin 1980, Franklin and Frankham 1998), is sufficient only for short-term population viability. For countries with fewer than 20 samples (e.g., Armenia), our estimates may not be reliable. Across the species' range, *Ne* occasionally reaches the threshold of 50 at the country level (SM 14), which again suggests that populations can only ensure short-term persistence under current conditions.

Concluding Remarks and Conservation Implications

The Austrian dragonhead (*Dracocephalum austriacum*) is a threatened species now surviving in highly scattered, isolated populations. It is a relic of the Ice Ages, and its persistence depends strongly on the preservation of its specialised habitats. Therefore, conserving both the species and its habitats must be a joint priority. Previous studies have shown that genetic diversity in *D. austriacum* significantly affects seed production and overall population fitness (Dostálek et al. 2010). Additionally, habitat quality and climatic variability influence population dynamics and key demographic processes essential for population growth (Nicolè et al. 2011, Dostálek and Münzbergová 2013). Our study builds on these findings by providing a comprehensive overview of genetic diversity across nearly all extant populations.

The generally low genetic diversity observed likely reflects demographic bottleneck and long-term adaptation to narrow, specialised habitats under similar selective pressures. This pattern suggests that low diversity may not, in itself, constitute the primary threat, as it is relatively uniform across the species' range. Instead, the species' vulnerability lies in its habitat specificity, fragmented distribution, and limited competitiveness and adaptive potential in the face of environmental change. Species that depend on specialised habitats are especially prone to extinction due to stochastic events and anthropogenic pressures (Ellstrand and Elam 1993, Aguilar et al. 2008, Haddad et al. 2015, Wiens 2016). Future habitat suitability projections (Fig. 6F) suggest a gradual decline, mirroring patterns predicted for the LIG. History may repeat itself, with *D. austriacum* retreating to interglacial refugia, undergoing genetic divergence, and recolonising during a future glacial phase (Fig. 7). However, a key difference now is the unpredictable influence of human activity on ecosystems. According to Nicolè et al. (2011), ongoing climate change over the next 30 years is unlikely to drastically reduce habitat availability for Alpine populations in France. Our findings support this, indicating that Alpine populations may remain viable for longer. In contrast, populations from the Pannonian and Eastern European lineages appear more prone to the risk of extinction.

This study identifies five genetic lineages within *D. austriacum*, corresponding to Evolutionarily Significant Units (ESUs). These should form the basis of coordinated international conservation strategies. Protecting regional lineages and maintaining genetic diversity across the species' range is crucial. Most populations have small effective sizes, making them especially susceptible to genetic drift and inbreeding. Although the rocky habitats occupied by *D. austriacum* may be relatively buffered against direct impacts of climate change, shifts in temperature and precipitation patterns could still pose severe threats to remnant populations. Conservation planning, guided by genetic and ecological data, is essential to ensure the species' long-term survival.

Declarations

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Author Contributions Miloslav Kitner, Ľ. Majeský and L. Vaculná designed the study, performed statistical analyses and interpreted the results. The first draft of the manuscript was written by Ľ. Majeský, commented by M. Kitner and L. Vaculná. Wet laboratory work was done by L. Kobrlová, M. Kitner, L. Vaculná and G. Sramkó. All other co-authors participated in sample collection, processing of sampling permits and in interpretation of the results. All authors read and approved the final manuscript.

Data Availability The nucleotide sequences of ITS region were submitted to GenBank (see SM 3). The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

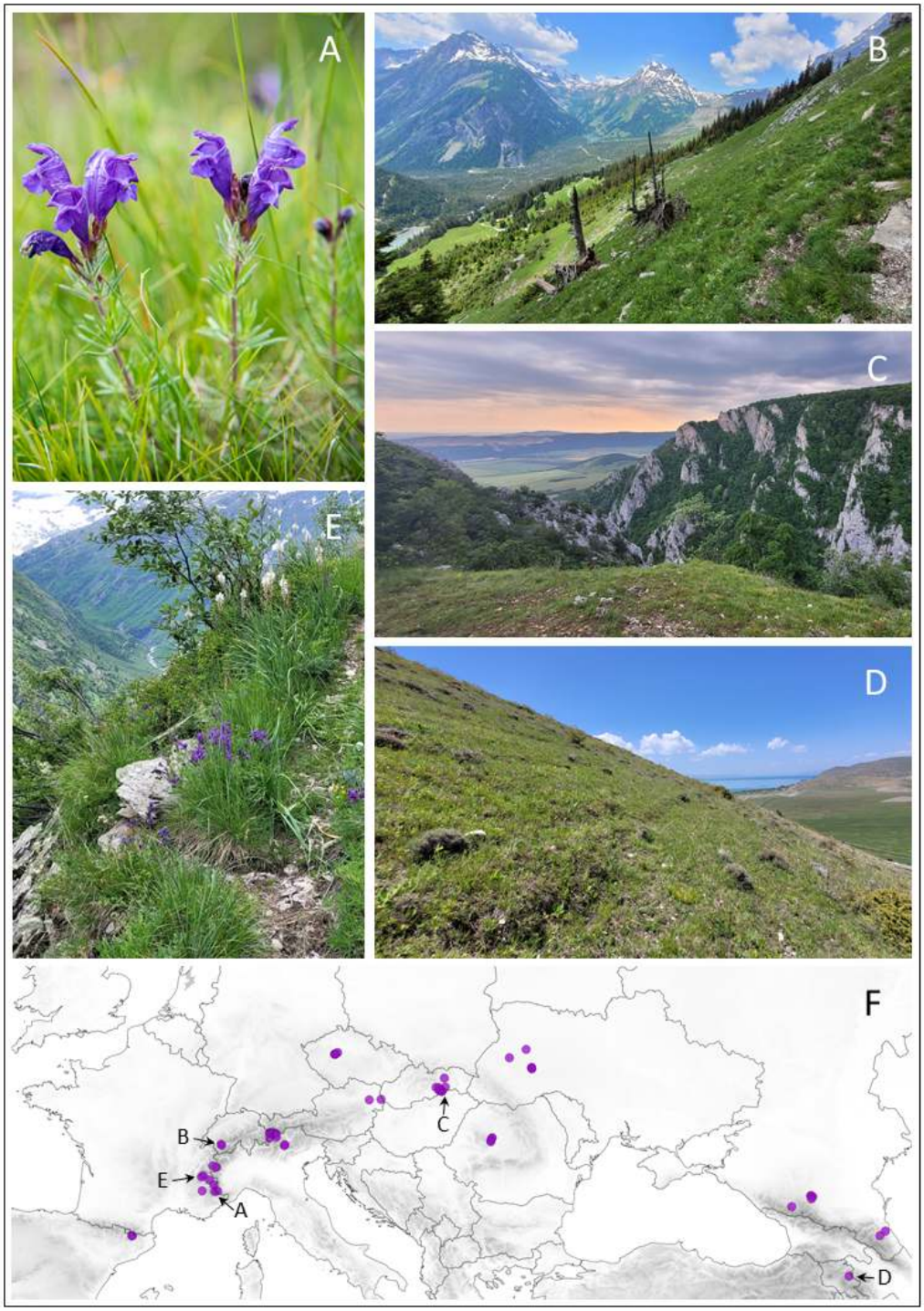

Figure 1

Dracocephalum austriacum (A) prefers open, sunny habitats exposed to cold winds (B – Godet, CH; C – NP Slovak Karst, SK; D – NP Sevan, AM; E - NP Écrins, FR). The map (F) is showing the localisation of sampling populations with arrows indicating the origin of the images above. This map actually represents the whole distribution range of the species.

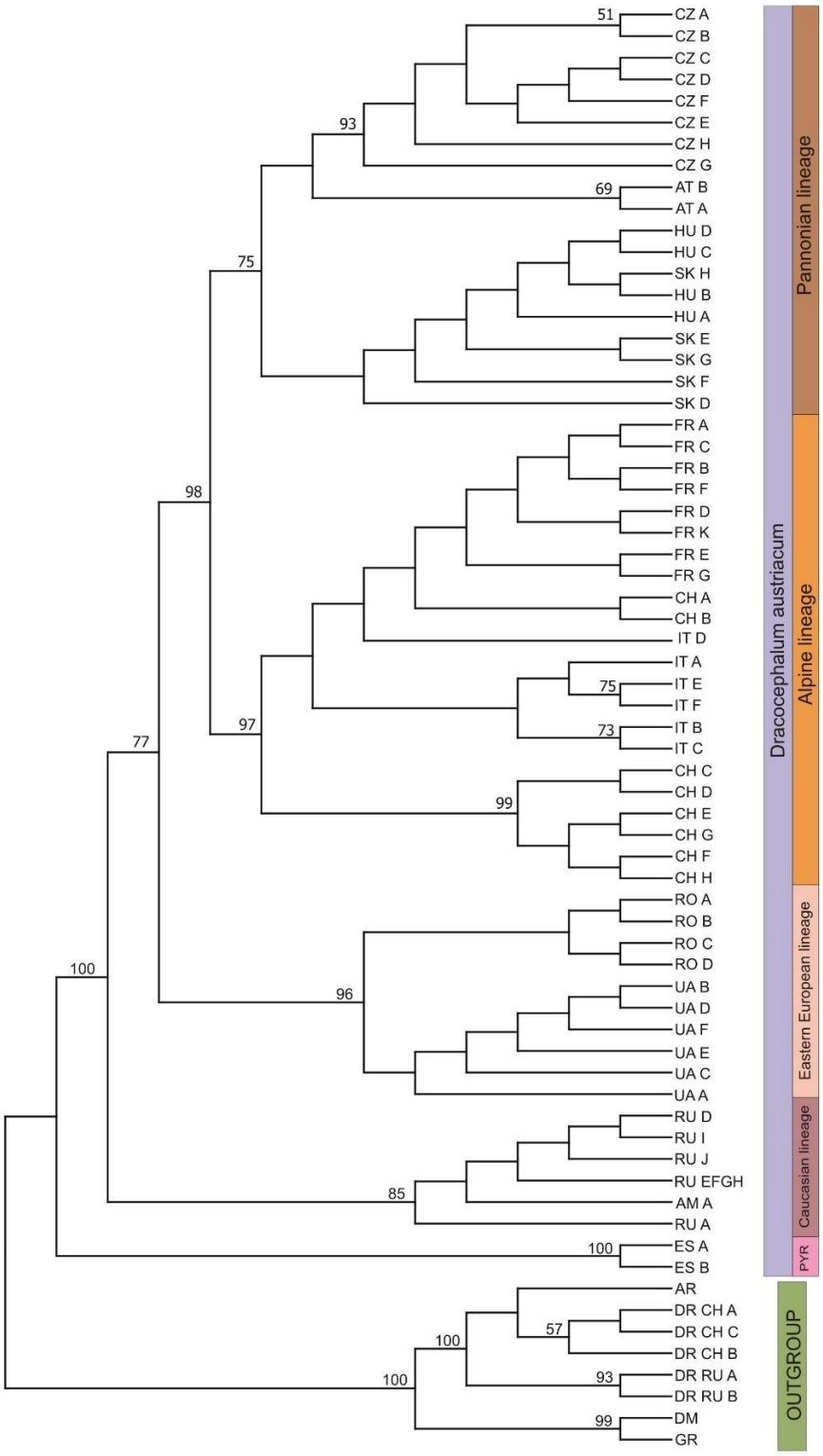

Figure 2

Phylogenetic tree, generated by the SVDQuartets method, showing relationships among the studied populations of *Dracocephalum austriacum.* The tree is rooted by the outgroup taxa *D. argunense* (AR), *D. ruyschiana*(DR), *D. multicaule* (DM) and *D. grandiflorum* (GR). Bootstrap support ≥ 50% is shown above the branches.

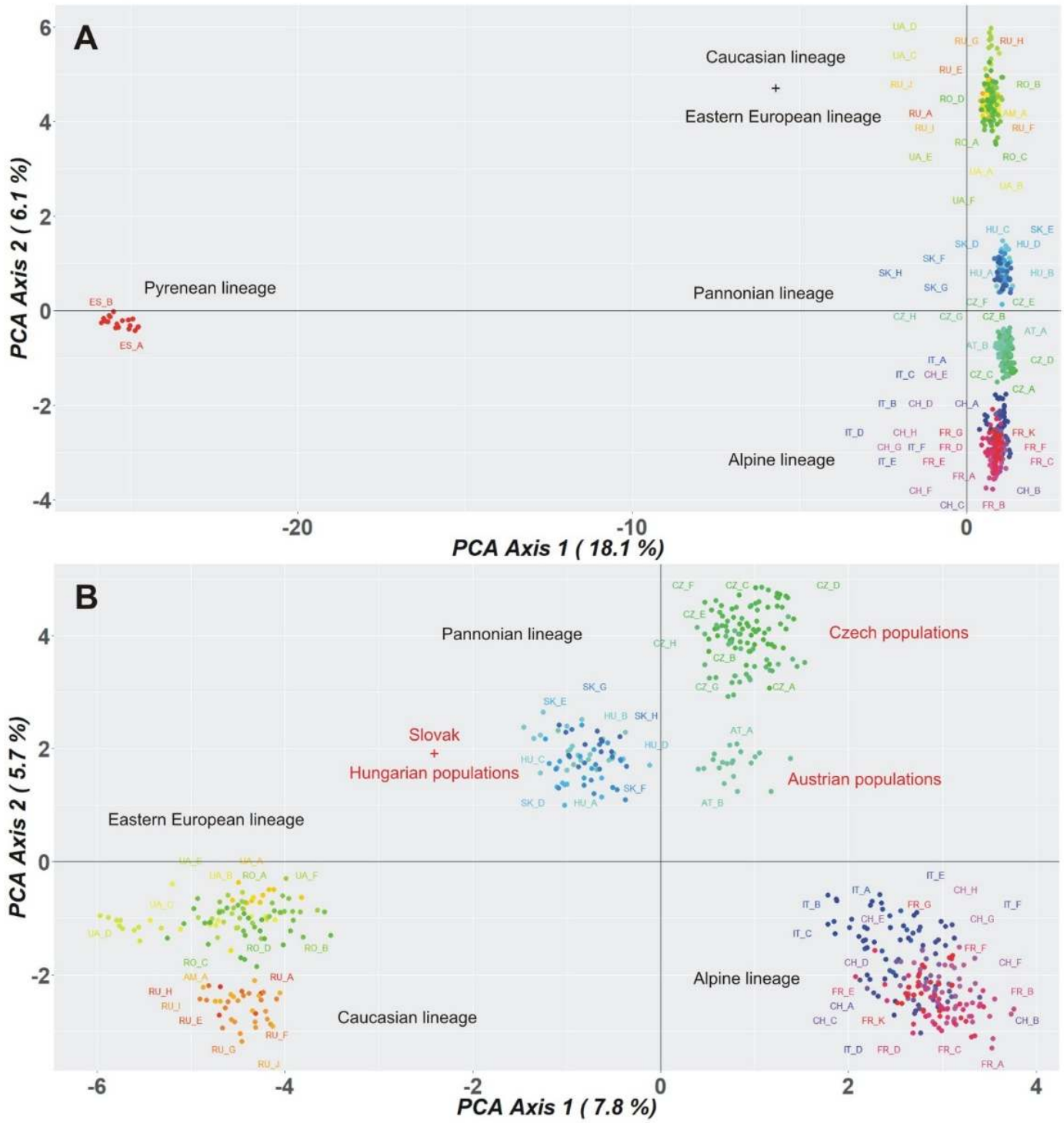

Figure 3

Results of the Principal Coordinate Analysis (PCoA) of: A) the whole dataset including the Pyrenean populations (total variability explained by the first two axes account for 24.2%), and B) the dataset excluding the Pyrenean populations (total variability explained by the first two axes account for 13.5%).

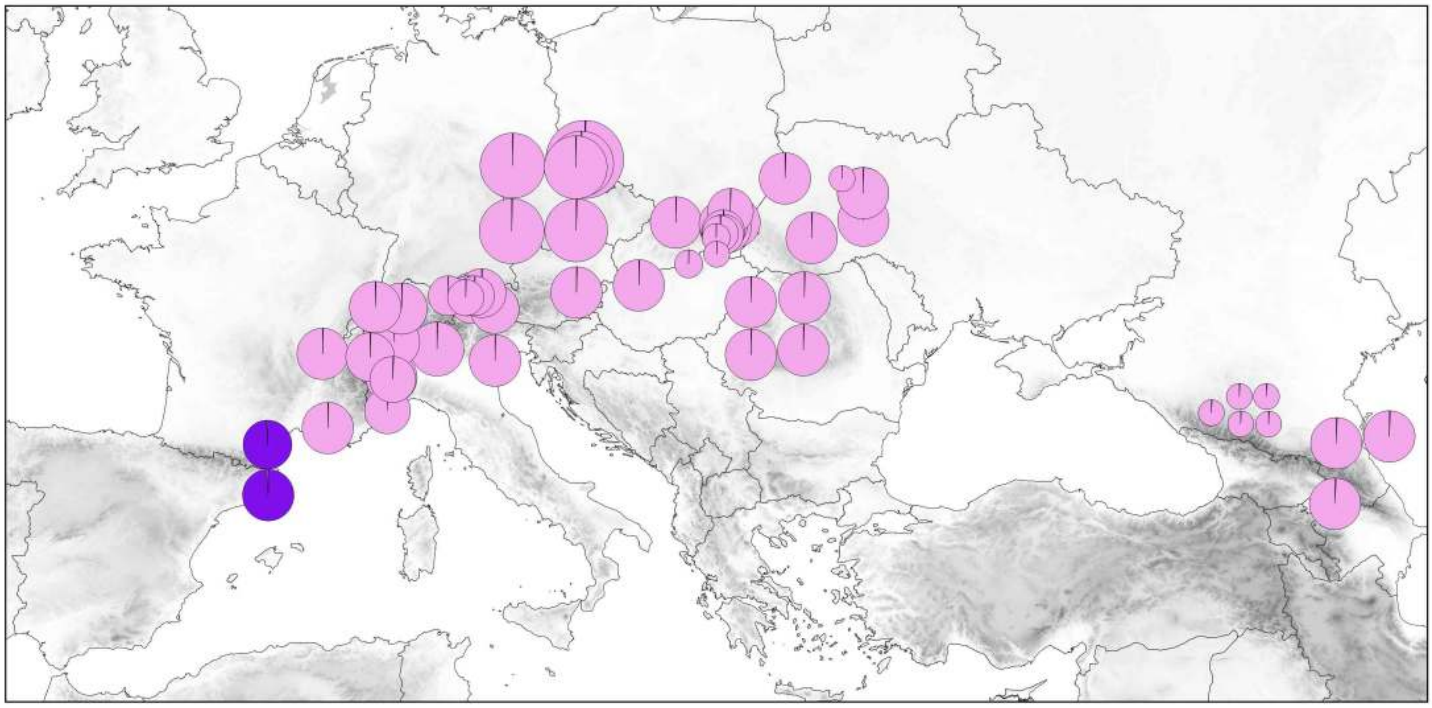

Figure 4

The STRUCTURE analysis of the whole dataset differentiates the studied populations into two distinct genetic clusters.

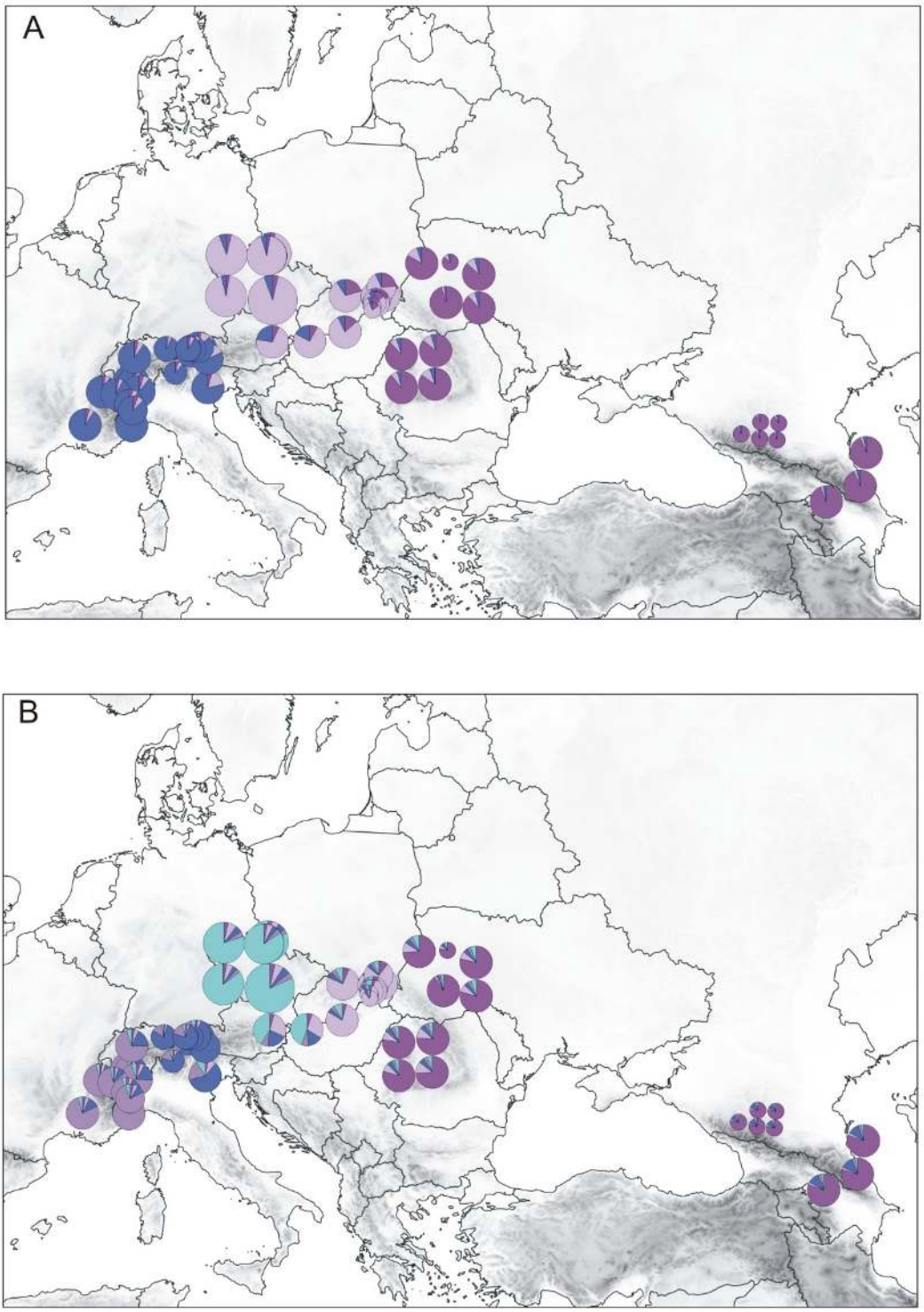

Figure 5

In the STRUCTURE analysis of the data set excluding the Pyrenean populations, the best results were obtained for: A) three different genetic clusters (*K* = 3); B) and five different genetic clusters (*K* = 5).

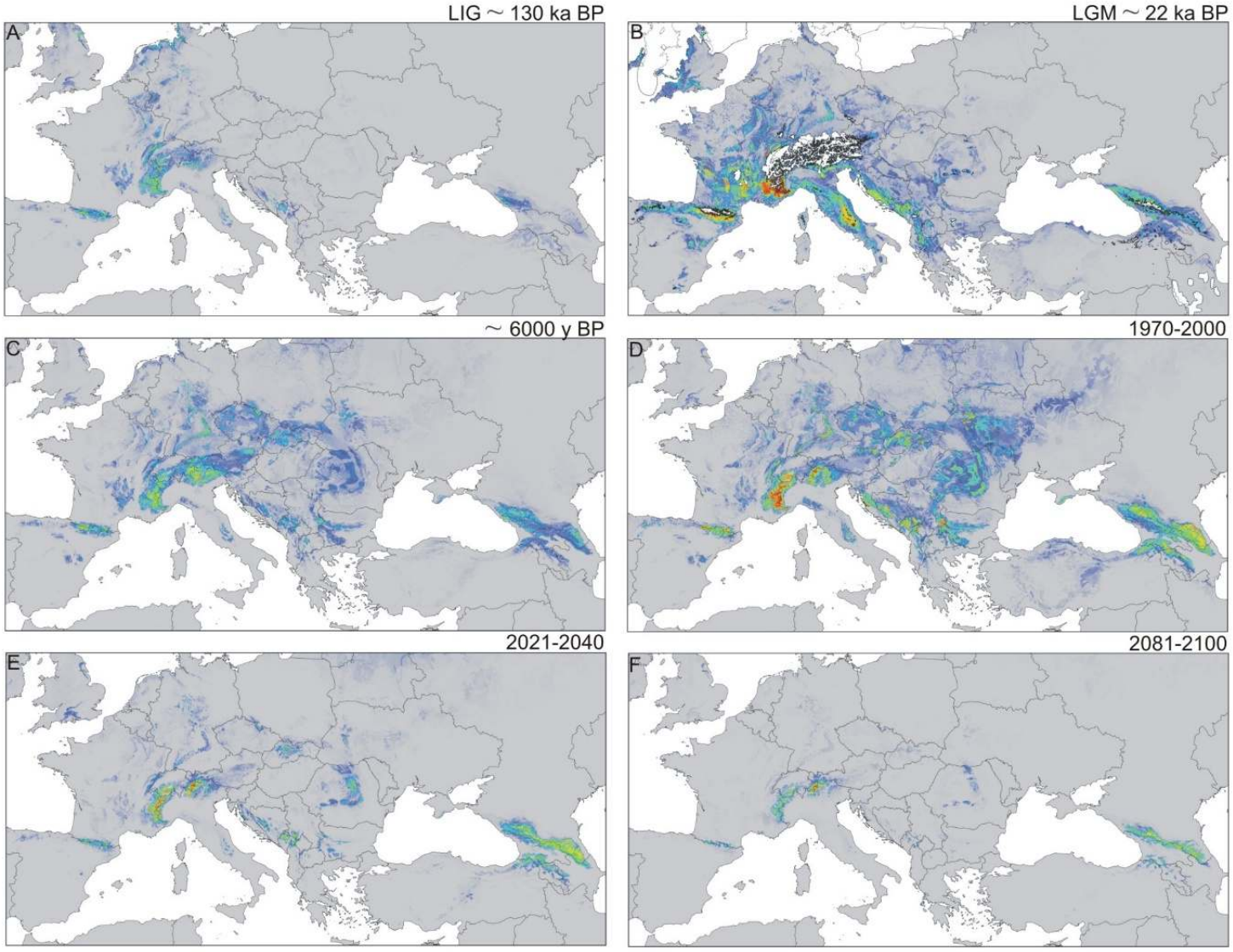

Figure 6

Predictions of past, present and future habitat suitability based on MaxEnt niche modelling. A) Last Interglacial (LIG, ~ 130 ka BP); B) Last Glacial Maximum (LGM, ~ 22 ka BP); C) Middle Holocene (~6,000 y BP); D) 1970-2000; E) prediction of habitat availability for the present time (2021-2040); F) prediction of habitat availability for the future (2081-2100).

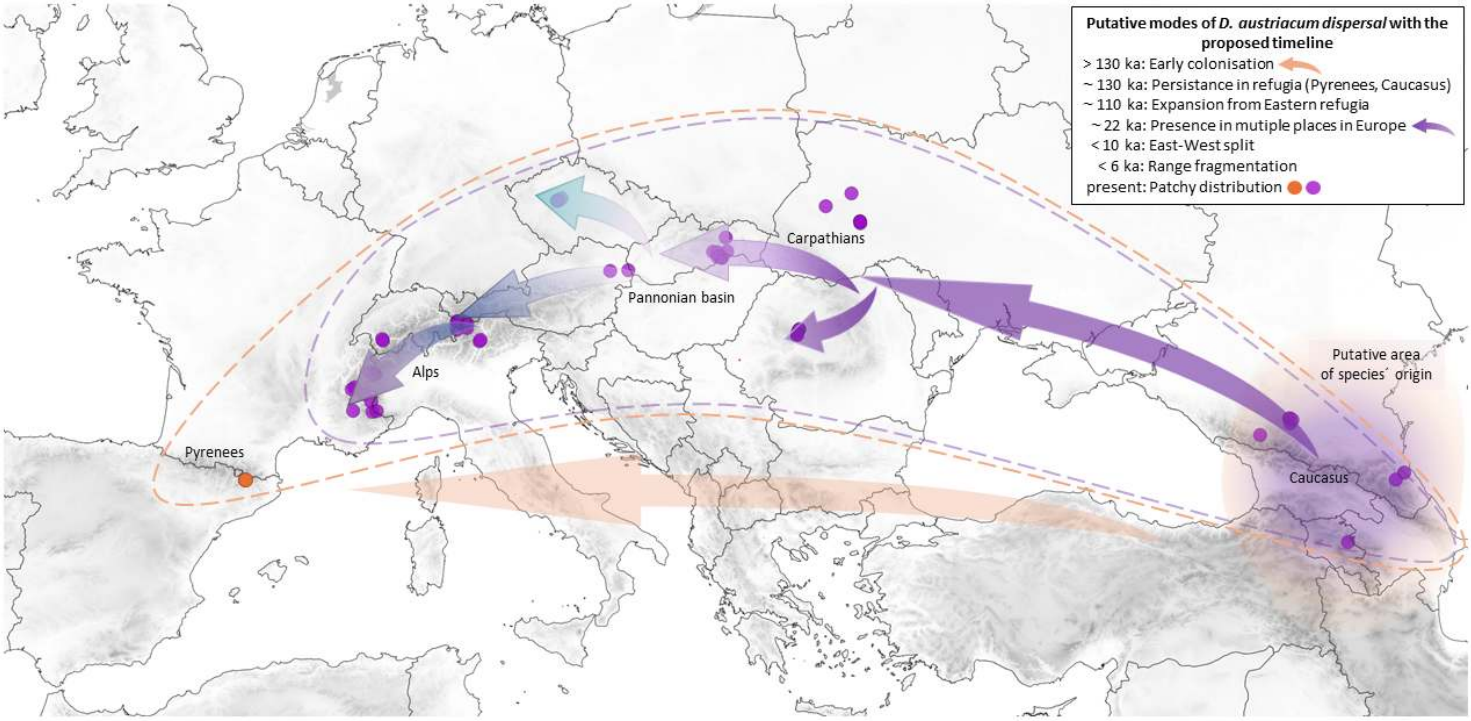

Figure 7

Proposed phylogeographic history of *D. austriacum* with timeline. The arrow colours use the same colour pattern as is used in the visualisation of the STRUCTURE analysis for *K* = 5 on Fig. 5B.

Supplementary Files

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- SM.zip