



Neutral genetic diversity follows a latitudinal gradient in the endangered plant *Arnica montana* L.: a range-wide study

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Abstract

Arnica montana is a clonal, self-incompatible herb of economic and intrinsic ecological value which is declining in large parts of its range. With the employment of microsatellite markers, we characterized the population structure and distribution of genetic diversity of 40 populations of *A. montana* sampled throughout Europe. We detected a clear geographical pattern of isolation and strong population structure, indicating limited gene flow. We also observed a negative latitudinal gradient in genetic diversity. Such patterns can be explained by paleo-historical colonization routes following the last glacial maximum, with regions characterized by higher genetic diversity corresponding to former glacial refugia. We recommend the implementation of conservation measures such as assisted gene flow in the populations characterized by low genetic diversity under consideration of the observed population structure. For the populations where high levels of genetic diversity are still retained, we recommend the maintenance of suitable habitat conditions. Our study emphasises the importance of conducting large-scale population genetic studies that consider postglacial recolonization history when planning active conservation measures such as assisted gene flow.

Keywords Microsatellites · Distribution range · Postglacial colonization · Self-incompatible · Conservation

Introduction

In Europe, grasslands belong to the most endangered habitat types, with more than half of them threatened to some extent (European Commission 2017) because of human activities resulting in fragmentation, deterioration or even destruction of these habitats. Semi-natural nutrient-poor grasslands, in particular, have been facing a steady decline in the last decades (Peter et al. 2009). These habitats are characterized by high biodiversity levels including several biotic communities and numerous endangered species (Bailly-Maître et al. 2015) and therefore are of high ecological value.

Furthermore, they provide important ecosystem services such as water supply and flow regulation, carbon storage, and erosion control (Bengtsson et al. 2019). Land-use change, and in particular abandonment or farming intensification, are threatening these habitats thereby causing the decline of associated species.

One of these species is the perennial herb *Arnica montana* L., a characteristic species of species-rich *Nardus* grasslands (habitat type 6230 of the Habitats Directive, Council Directive 92/43/EEC, 21 May 1992), heathlands with moderate mowing or grazing levels (GalvANEK et al. 2008), and marginal coniferous forests (Radusiene and Labokas 2007). This plant is an irreplaceable component of the ecosystem, with specialist species depending on its presence for survival (Korneyev et al. 2015). Because of its sensitivity to soil nitrification and the consequent shift in community composition that follows eutrophication, it can be employed as an indicator species for habitat quality (Holmann et al. 2020). Besides, it is particularly valuable for its use in traditional and modern medicine due to the high content of secondary metabolites, such as sesquiterpenes, flavonoids, and phenolic acids (Petrova et al. 2012).

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The distribution range of *A. montana* is large, spanning from western Norway (up until 63.1°N) at its northern margin, to the Pyrenees at its South-Western margin, and the Carpathians at its eastern margin. It is also found at different altitudes from sea-level lowlands (e.g. Netherlands) to alpine grasslands (up to 3000 m a.s.l.). Although still relatively common in the Alps and in the Carpathians, *A. montana* is declining rapidly in Central and Northern Europe. For this reason, *A. montana* has been included in different national red lists (Zarzycki and Szelağ 2006; Rossi et al. 2013; Metzinger et al. 2018).

The task of detecting the “main” drivers for this decline is challenging because of the intrinsic complexity of anthropogenic activities and their impact on natural environments. Abandonment (Dullinger et al. 2003), eutrophication (Van der Berg et al. 2003), direct and indirect effects of climate change (Bruehlheide 2003; Vikane et al. 2019; Stanik et al. 2020), and invasive species (Bruehlheide and Scheidel 1999) are some of the proposed external factors causing population decline in this species. Also, due to its value for medicinal purposes, this species suffered from overexploitation from wild collection and for this reason has been included in the Habitats Directive (Annex V).

Also, genetic factors such as inbreeding, limited gene flow, and genetic erosion negatively affect *A. montana* populations (Duwe et al. 2017; Van Rossum and Raspé 2018) and are hence potential drivers for the species’ decline. Previous studies generated differing results in patterns of neutral genetic diversity. For example, Maurice et al. (2016) found lower levels of genetic diversity in montane populations compared to colline populations on a rather regional level, comparing populations from Western Germany, Luxembourg, Eastern Belgium as well as populations from the Vosges mountains in Northern France. Duwe et al. (2017), however, identified on a larger scale, covering the Alpine Arch as well as the whole of Germany, the opposite pattern with alpine populations showing slightly higher genetic diversity than central-European lowlands. Both studies described strong population differentiation between different regions and elevations finding also evidence of isolation by distance (IBD). On the contrary, the study from Van Rossum and Raspé (2018) found no evidence of IBD at the regional scale (Southern Belgium and French Ardennes).

Comparing different studies conducted with different genetic markers and at different spatial scales has its limitations and hence, for a better understanding of the population genetic structures of *A. montana* at regional scales, a wider, range-wide perspective is necessary. This is even more true when it comes to future conservation challenges (e.g. climate change). A clear understanding of the basic population genetic features of *A. montana* populations, such as distribution of genetic diversity, differentiation of populations,

and inbreeding levels, is fundamental for the development of appropriate conservation measures e.g., genetic restoration. Populations located in different regions of the species range can be expected to differ significantly in those population genetic parameters and, therefore, may require specific conservation strategies (Shay et al. 2021). Moreover, under current climate change, wild populations are predicted to shift northward and/or upward given the availability of suitable nearby habitat patches (Kelly and Goulden 2008).

Finally, it is important to ascertain whether genetic patterns arise due to human interference, such as fragmentation, or are the results of natural processes, such as range dynamics. This knowledge can be employed to determine if observed indications of genetic erosion necessitate proactive conservation interventions. It is worth noting that the footprints of natural processes are better evident at larger spatial scales (Hewitt 1999; Trense et al. 2021). Therefore, we argue that for a better view of the genetic features of *A. montana* populations a comprehensive sampling design is required.

Our study focuses on the range-wide population genetic features of the declining herbaceous plant *A. montana*. Using microsatellite markers, we aim to characterize (1) whether and how populations are differentiated at this large scale, and (2) how neutral genetic diversity is spatially distributed across the range of *A. montana* throughout Europe. Furthermore, we discuss the potential implications of our results for the future conservation of this valuable species.

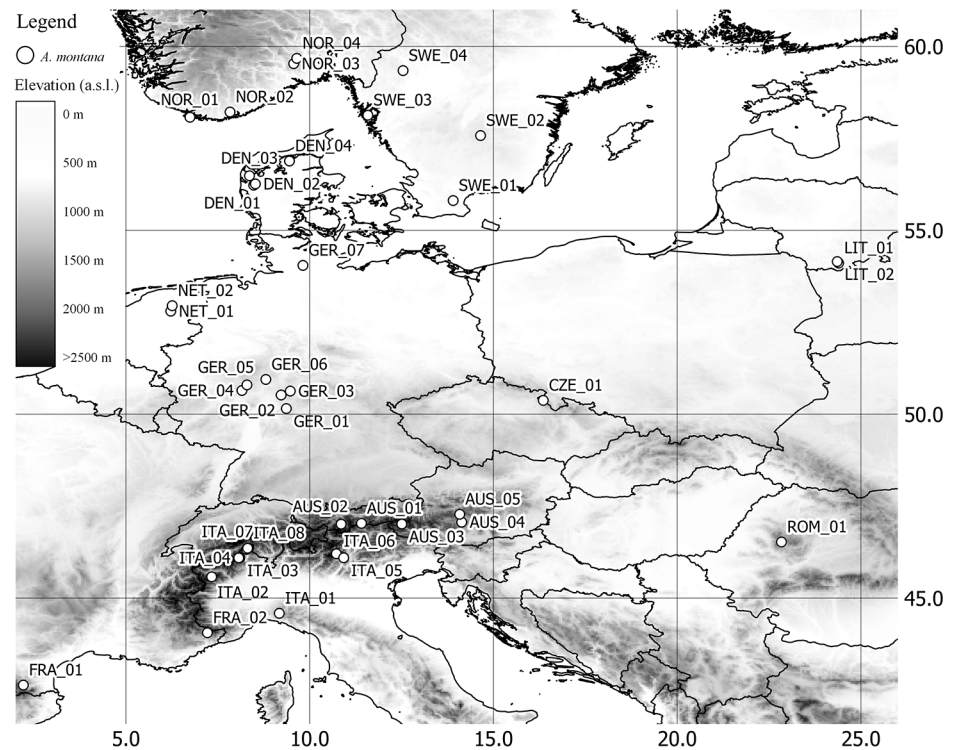
Materials and methods

Sampling

Overall, 43 populations with a total amount of 807 individual plants were randomly sampled throughout Europe (Fig. 1), spanning along the altitudinal, longitudinal and latitudinal gradients. To cover areas with lower geographic coverage, additional data from 90 samples from an earlier study (unpublished data) were included here. The sampling and genotyping methods for these samples are differing from our methods and are therefore described in detail in Appendix S1.

Coordinates of *A. montana* stands were obtained from publicly available datasets (via gbif.org), local nature agencies and collaborators. Locations were visited in 2018 and 2019 during the flowering/fruiting season, from June to August depending on the locality. Within each population, 20 plants were randomly sampled at a minimum distance of 1 m from each other in order to avoid the collection of ramets of the same clone. For each plant, two leaves were collected and stored in silica gel. Upon arrival in the laboratory, leaves

Fig. 1 Map of the sampling locations. Each of the 40 populations sampled across the *A. montana* range is represented by a white dot. Greyscale represents the altitudinal gradient



were dried for 24 h in a drying cabinet at 40 °C and stored at room temperature. The graphical output for the sampling map was produced with QGIS V3.18.3 (QGIS Development Team 2022). DEM profiles were obtained from the WorldClim dataset (Fick and Hijmans 2017).

DNA isolation and genotyping

DNA was extracted from dry leaves ground to a fine powder using a bead mill homogenizer (Omni Bead Ruptor 24, Biovendis, Mannheim, Germany). A CTAB protocol (Appendix, S2) modified from the original developed by Barbier et al. (2019) for extraction-recalcitrant tissues was employed to extract DNA. The working solution DNA yield for each sample was estimated with a spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, Massachusetts) and diluted to 1 ng/μl to minimize interference with PCR reaction observed at higher sample concentrations. PCR was conducted by multiplexing ten nuclear microsatellite loci previously developed by Duwe et al. (2015) (Appendix, S3), using the Type-it Microsatellite PCR Kit DNA (Qiagen, Hilden, Germany). Multiplexed PCR reactions (Appendix, S4) were developed taking into account possible dimer formation between primers by using AmplifX (Jullien 2019). Different parameter settings were used in the multiplexed set of markers during PCR amplification (Appendix, S4). Amplification products were analysed by capillary electrophoresis with a 3500 Genetic Analyzer using GeneScan™

600 LIZ™ as internal size standard (Applied Biosystems, Waltham, Massachusetts). Genemarker V2.6.7 (SoftGenetics, Hulce et al. 2011) was used for allele scoring.

Population genetic analyses

First, the dataset was scanned for identical multi-locus genotypes (MLGs) with Genalex V6.51b2 plugin (Peakall and Smouse 2012) and manually checked for allele-scoring errors and missing data by searching for outliers (by applying column filters in Excel). In case of multiple ramets per genotype, only one ramet was randomly chosen and kept for further analyses. Furthermore, only populations with at least ten different MLGs were retained for the following analyses. Departure from Hardy Weinberg Equilibrium (HWE) was tested with the “exact HW test” (Haldane 1954) with the online software Genepop On the Web (Rousset 2008). For populations showing deviation from HWE, heterozygote deficiency accounting for null allele presence was tested with a complete enumeration method (Dempster et al. 1977) with Genepop. Bonferroni sequential p-value correction was applied for accounting for multiple testing (Holm 1979).

In detail, unbiased expected heterozygosity (uH_e), observed heterozygosity (H_o), genetic distance (F_{ST}) averaged across all loci were calculated with Genalex plugin. Inbreeding coefficient (F_{IS}) with corresponding 95% confidence interval (bootstrapped) for each population was

Table 1 Population names, geographic coordinates and genetic diversity indices for the 40 *A. montana* populations. N = sample size, Ho = observed heterozygosity, uHe = unbiased expected heterozygosity, Ar = allelic richness (for N=10), F_{IS} inbreeding coefficient with corresponding 95% confidence intervals (C.I.). * Data obtained from additional dataset (see S1)

Population	Latitude	Longitude	Elevation (m)	N	Ho	uHe	Ar	F_{IS} (95% C.I.)
FRA_01	42.63571	2.209420	2094	20	0.48	0.468	3.9	-0.054 (-0.176, 0.047)
FRA_02	44.05773	7.217400	1468	20	0.366	0.423	3.3	0.1093 (-0.009, 0.225)
ITA_01	44.58704	9.172926	1222	17	0.682	0.603	3.7	-0.165 (-0.254, -0.074)
ITA_02	45.57952	7.334350	2654	16	0.514	0.563	3.7	0.0562 (-0.083, 0.195)
ITA_03	46.09408	8.096917	2032	12	0.511	0.618	4.7	0.134 (-0.03, 0.241)
ITA_04	46.09439	8.069250	2373	20	0.591	0.632	5.2	0.0405 (-0.062, 0.138)
ITA_05	46.09739	10.92444	1122	19	0.481	0.507	3.5	0.0246 (-0.096, 0.145)
ITA_06	46.20688	10.72512	1755	12	0.463	0.496	3.1	0.0247 (-0.163, 0.235)
ITA_07	46.32706	8.281167	1222	18	0.581	0.612	4.1	0.0206 (-0.109, 0.132)
ITA_08	46.35628	8.311028	2334	20	0.622	0.647	4.9	0.0145 (-0.046, 0.063)
ROM_01	46.52600	22.83500	1343	20	0.349	0.336	2.7	-0.0658 (-0.294, 0.167)
AUS_01	46.84454	12.40865	2478	18	0.6	0.587	4.8	-0.0517 (-0.145, 0.029)
AUS_02	47.01123	10.85565	2033	19	0.424	0.454	2.8	0.0359 (-0.131, 0.176)
AUS_03	47.01454	12.50592	1422	19	0.657	0.639	5.4	-0.0568 (-0.143, 0.009)
AUS_04	47.06556	14.13583	1594	18	0.655	0.619	5.7	-0.0856 (-0.188, 0.013)
AUS_05	47.27928	14.08144	1807	19	0.57	0.63	5.8	0.0712 (-0.038, 0.2)
GER_01*	50.15390	9.364451	373	16	0.584	0.623	4	0.0363 (-0.048, 0.106)
CZE_01	50.37576	16.35439	766	19	0.289	0.34	2.6	0.1189 (-0.062, 0.285)
GER_02	50.52222	9.223056	700	19	0.601	0.586	4.1	-0.054 (-0.159, 0.039)
GER_03*	50.62905	9.471056	352	19	0.516	0.508	3.2	-0.0423 (-0.171, 0.074)
GER_04*	50.64504	8.156486	576	18	0.611	0.58	3.4	-0.0834 (-0.149, -0.035)
GER_05*	50.79687	8.293406	427	19	0.516	0.487	3.4	-0.087 (-0.188, 0.005)
GER_06*	50.94500	8.807000	342	15	0.427	0.372	2.2	-0.1874 (-0.374, -0.017)
NET_01	52.80181	6.220000	8	15	0.349	0.375	2.4	0.0369 (-0.156, 0.227)
NET_02	52.95011	6.259944	9	18	0.298	0.336	3.1	0.0865 (-0.069, 0.247)
GER_07	54.04580	9.816100	10	15	0.367	0.429	3.3	0.1158 (0.02, 0.181)
LIT_01	54.11025	24.38897	125	20	0.427	0.512	3.6	0.1448 (0.002, 0.274)
LIT_02	54.14945	24.35278	113	18	0.223	0.315	2.6	0.2696 (0.116, 0.428)
SWE_01	55.80800	13.90300	181	20	0.345	0.321	2.7	-0.1036 (-0.242, 0.033)
DEN_01	56.22656	8.477306	44	19	0.293	0.312	2.8	0.0332 (-0.196, 0.269)
DEN_02	56.26067	8.521556	44	18	0.35	0.368	2.4	0.022 (-0.094, 0.137)
DEN_03	56.48200	8.361139	24	19	0.316	0.373	2.5	0.1301 (-0.043, 0.276)
DEN_04	56.88325	9.449444	32	19	0.383	0.416	3	0.0817 (-0.117, 0.267)
SWE_02	57.57600	14.64900	321	19	0.37	0.363	3	-0.0471 (-0.185, 0.078)
NOR_01	58.07380	6.742692	11	14	0.284	0.282	2.2	-0.0454 (-0.266, 0.158)
SWE_03	58.12800	11.58200	37	20	0.279	0.282	2	-0.0162 (-0.19, 0.161)
NOR_02	58.21520	7.832570	181	16	0.138	0.23	1.8	0.3835 (0.109, 0.644)
SWE_04	59.34100	12.54000	221	15	0.149	0.194	1.9	0.2063 (-0.048, 0.434)
NOR_03	59.53100	9.565000	543	19	0.32	0.345	2.7	0.048 (-0.115, 0.197)
NOR_04	59.67402	9.648897	244	16	0.369	0.38	2.5	-0.0021 (-0.094, 0.082)

calculated with R (“*diveRsity*” package) (Keenan et al. 2013). Adjusted allelic richness was assessed with the rarefaction method implemented in HP-Rare v1.0 (Kalinowski 2005) with a sample size of $n=10$. In order to test how genetic parameters are distributed across space, the influence of geographical variables (elevation, latitude, longitude) on adjusted allelic richness (Ar), unbiased heterozygosity corrected for sample size (uH_e) and inbreeding coefficient (F_{IS}), were tested using linear regression analysis in R (R Core Team 2022) with a backward stepwise

selection of explanatory variables (LAT, LON, ALT). The collinearity between altitude and latitude, which is an intrinsic characteristic of European geography, was taken into account by deleting the variable with the lowest R^2 value between collinear variables. Results were plotted with the R package “*ggplot2*” (Wickham 2016).

Genetic differentiation within and among populations was calculated from a pairwise genetic distance (F_{ST}) matrix corrected for null alleles with the software FreeNa (Chapuis and Estoup 2006) and its significance was tested

with a hierarchical analysis of molecular variance (*poppr*: *amova*, 1000 permutations, ~ Regions/Populations), as implemented in the “*poppr*” R package (Kamvar & Grünwald 2015). Regions were defined on the basis of the first hierarchical level of the Structure analysis, differentiating between the detected clusters at $K=2$. Furthermore, to assess limitations in gene flow, isolation by distance (IBD) was assessed with a Mantel test on R (“*vegan*” package, Oksanen et al. 2022) by testing the relation between genetic distances ($F_{ST}/(1 - F_{ST})$) corrected for null alleles and geographic distances of populations. Additional Mantel tests were performed separately for the two main genetic clusters detected in the populations structure analysis.

Population genetic structure

The presence of genetic clusters (k) in *A. montana* populations was tested by performing a model-based Bayesian cluster analysis as implemented in the software Structure V2.3.4 (Pritchard et al. 2000) coupled with the parallel computation tool StrAuto (Chhatre and Emerson 2017) for optimizing computing time. An admixture model with correlated allele frequencies and without prior information about the regional membership of the populations was considered (100,000 burn-in period; 100,000 Markov chain Monte Carlo (MCMC), 15 independent runs for each K , for $K=1-40$). Estimation of K was calculated using Structure Harvester online tool based on Evanno’s ΔK approach (Evanno et al. 2005) (Appendix S5 and S6). The same procedure was repeated another time with a second hierarchy analysis, by running separately the two main groups ($K=2$) detected with the first run. Populations were assigned to one of these main groups based on their proportion of membership with one of the two clusters, with 50% considered as a threshold. CLUMPAK online tool (Kopelman et al. 2015) was used for aligning results from Q-matrices obtained for each run (including DISTRUCT pipeline). Graphical output for each K was produced with QGIS V3.18.3 (QGIS Development Team 2022), CLUMPAK and R.

Furthermore, a principal coordinate analysis (PCoA) on pairwise genetic distances of individuals was conducted within Genalex and plotted with *ggplot2*. A discriminant analysis of principal components (DAPC) was conducted with R (“*Adegenet*” package) (Jombart 2008) as a complement to the Bayesian analysis and the PCoA. The analysis was conducted using populations as *a-priori* groups and the number of PCA axes and DA axes to be retained was judged using the explorative tools present in *Adegenet* (60 PC axes and 8 DA axes retained, automatic selection of the axes). In this multivariate method, differences between groups are maximised while minute differences within the groups are overlooked. PCoA and DAPC do not need to fit

the assumptions of Hardy-Weinberg equilibrium and linkage disequilibrium (Miller et al. 2020) and thus might be more suitable to describe the population structure in case some assumptions are not met.

Results

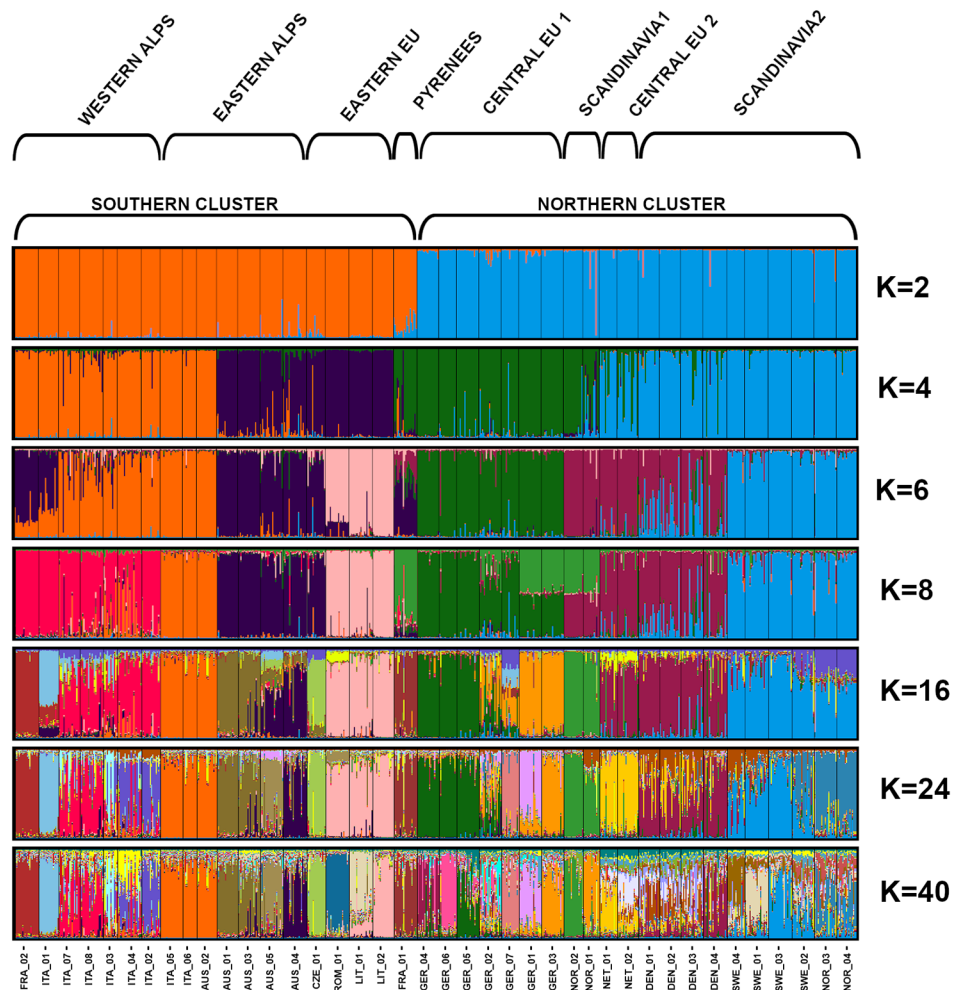
Genotyping

All the selected loci were polymorphic, spanning from 5 alleles in Armo3 to 26 alleles in Arm6. A few clones were present in the dataset despite the sampling design. Out of 807 samples, 76 individuals showed to share the same multilocus genotype (MLG) with at least another individual. In one specific case, a Norwegian population (not included in the present dataset) was composed of only 4 genets out of 20 sampled individuals. After discarding all but one ramet per MLG from the data set, overall 749 samples were left. Furthermore, three populations had less than ten different genets and were thus deleted from the dataset. The final filtered dataset contained 712 individuals and 40 populations (Table 1), with the smallest (ITA_03) containing 12 individuals. Out of the 10 SSR loci, two of them (Arm7 and Arm10) showed evidence of deviation from HWE after sequential Bonferroni correction. Subsequent analysis with Genepop using the EM algorithm (Rousset 2008), found that heterozygote deficiency explained the deviation, likely caused by null alleles. Moreover, in one case (ROM_01) we found evidence for a complete allelic dropout at Arm7 locus. Hence, the main analyses (Genalex genetic parameters, AMOVA, Structure, PCoA, DAPC) were conducted on two datasets. One including all markers and the other excluding Arm07 and Arm10. No significant differences were detected between the full dataset and the dataset without Arm7/Arm10. Due to the stability of the overall analysis despite the limited presence of null alleles, no markers were discarded for the final analysis, as the drawbacks of deleting them outbalanced the benefits of having more statistical power.

Population structure

Results of the Bayesian clustering analysis conducted with Structure showed that, by following the Evanno- ΔK approach, in the first hierarchy analysis, two main clusters were detected (Fig. 2, but see also Fig. S7, $H=1$). The two clusters are separated by the Alpine arc and Carpathian mountains, with all the populations north of the Alps belonging to one cluster, with the exception of Lithuanian and Czech populations, which are assigned to the southern cluster. This separation reflects the uppermost hierarchical

Fig. 2 Population genetic structure of 40 *A. montana* populations based on microsatellite data. The graphs show the results of the Bayesian cluster analysis (first hierarchy). Each bar chart specifies a different number of clusters assumed ($K=2, 4, 6, 8, 16, 24, 40$). Vertical bars display individuals grouped within populations. Different colours indicate different genetic clusters. On top of the graph geographical regions and assignment of populations to clusters (northern vs. southern) for $K=2$ are specified. Barcharts are produced with CLUMPAK online tool



level of genetic structure at a wider geographical scale. Targeting higher levels of K , populations as well as individuals were consistently assigned to distinct clusters pointing to further hierarchies, even down to the level of $K=40$ (Fig. 2). Analysis at the second hierarchy analysis (Fig. S7, $H=2$) identified two clusters in the northern cluster ($K=2$), the “continental” group composed of Central European populations and the “Scandinavian” group composed of the most northern populations. In the southern cluster, four main groups ($K=4$) were identified: Western Alps, Central Alps, Austrian Alps & White Carpathians and finally Eastern European populations (Lithuanian populations and Romanian Apuseni).

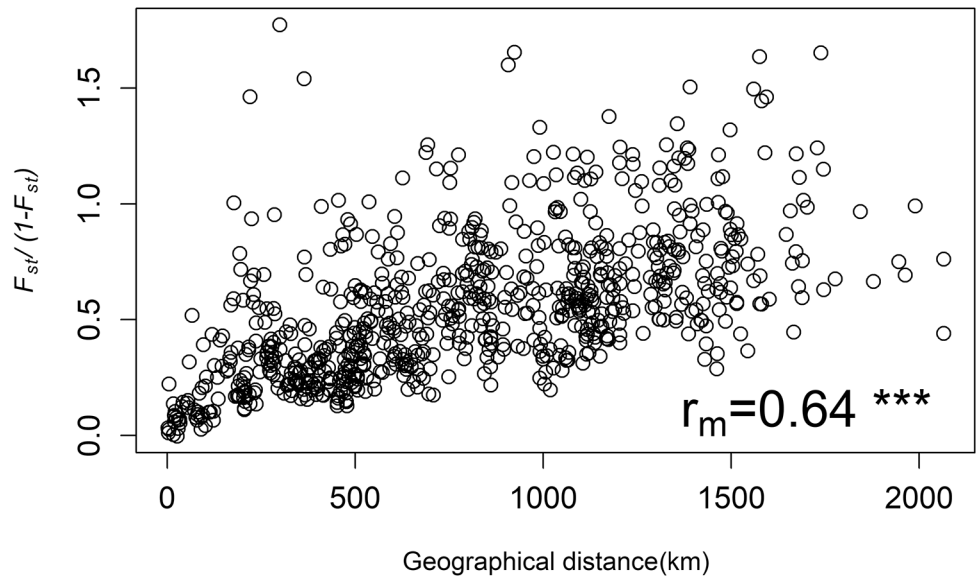
Similarly to the Bayesian clustering results, in DAPC (Appendix S8) and PCoA (Appendix S9) the separation of $K=2$ (North-South) of the first hierarchy level of the Structure analysis is described by the first PC axis (PC1) with the Lithuanian and the Pyrenean populations (LIT_01, LIT_02, FRA_01) taking an intermediate position between these groups. At the same time, the further separation described by higher values of k ($k>2$) are better described by the

second PC axis (PC2), especially in the PCoA. Hierarchical AMOVA (1000 permutations) showed high differentiation between populations, with 29.5% of the genetic variation found among populations: 13.55% between regions ($p<.001$), 15.97% between populations within regions ($p<.001$) and 70.5% within populations. Genetic differentiation (F_{ST}) across loci averaged 0.37. Furthermore, the Mantel test (Fig. 3) showed a strong ($r=0.64$) correlation between genetic distance and geographic distance. When Mantel tests were conducted separately on the “Northern” and Southern” clusters, similar results were obtained ($r=0.19$ and $r=0.20$, respectively, Appendix, S10 and S11).

Patterns of genetic diversity

H_o averaged 0.42 and was highest in ITA_01 (0.68) and lowest in NOR_02 (0.14). Unbiased genetic diversity (uH_e) averaged 0.44 and was generally higher in southern populations with the highest values found in ITA_08 (0.65) and lowest in SWE_04 (0.20). Most populations of *A. montana* showed inbreeding coefficient values (F_{IS}) close to zero

Fig. 3 Mantel correlation between genetic distances ($F_{ST}/(1-F_{ST})$) and geographical distances (in km). Black circles display pairwise differences of *A. montana* populations. *** $p \leq 0.001$



(averaging 0.03), with extremes represented by NOR_02 (0.38) and GER_06 (-0.19). Allelic richness (A_r) spanned between 1.80 (NOR_02) and 5.80 (AUS_05) (Table 1). Regression analysis displayed a statistically significant linear relationship between uH_e and latitude ($F_{3,36}=18.4$, $R^2=0.52$, $p < 0.001$; Fig. 4, upper). Additionally, between allelic richness (A_r) and latitude, there was a statistically significant linear relationship ($F_{1,38}=30.6$, $R^2=0.43$, $p < 0.001$; Fig. 4, middle). On the other hand, the inbreeding coefficient (F_{IS}) was not correlated with any of the geographical variables (Fig. 4, lower).

Discussion

Geographical patterns of genetic variation

Based on AMOVA and Mantel test results, very high differentiation exists between *A. montana* populations and strong isolation by distance (IBD) patterns are present throughout the species range. The present study displays at a larger scale what was found by Duwe et al. (2017) with the same microsatellite loci. We obtained comparable results in terms of differentiation between populations from the AMOVA (27% in Duwe et al. 2017 vs. 29.5% in our study, respectively), average F_{ST} values (0.27 vs. 0.37) and Mantel test correlation coefficient (0.43 vs. 0.64).

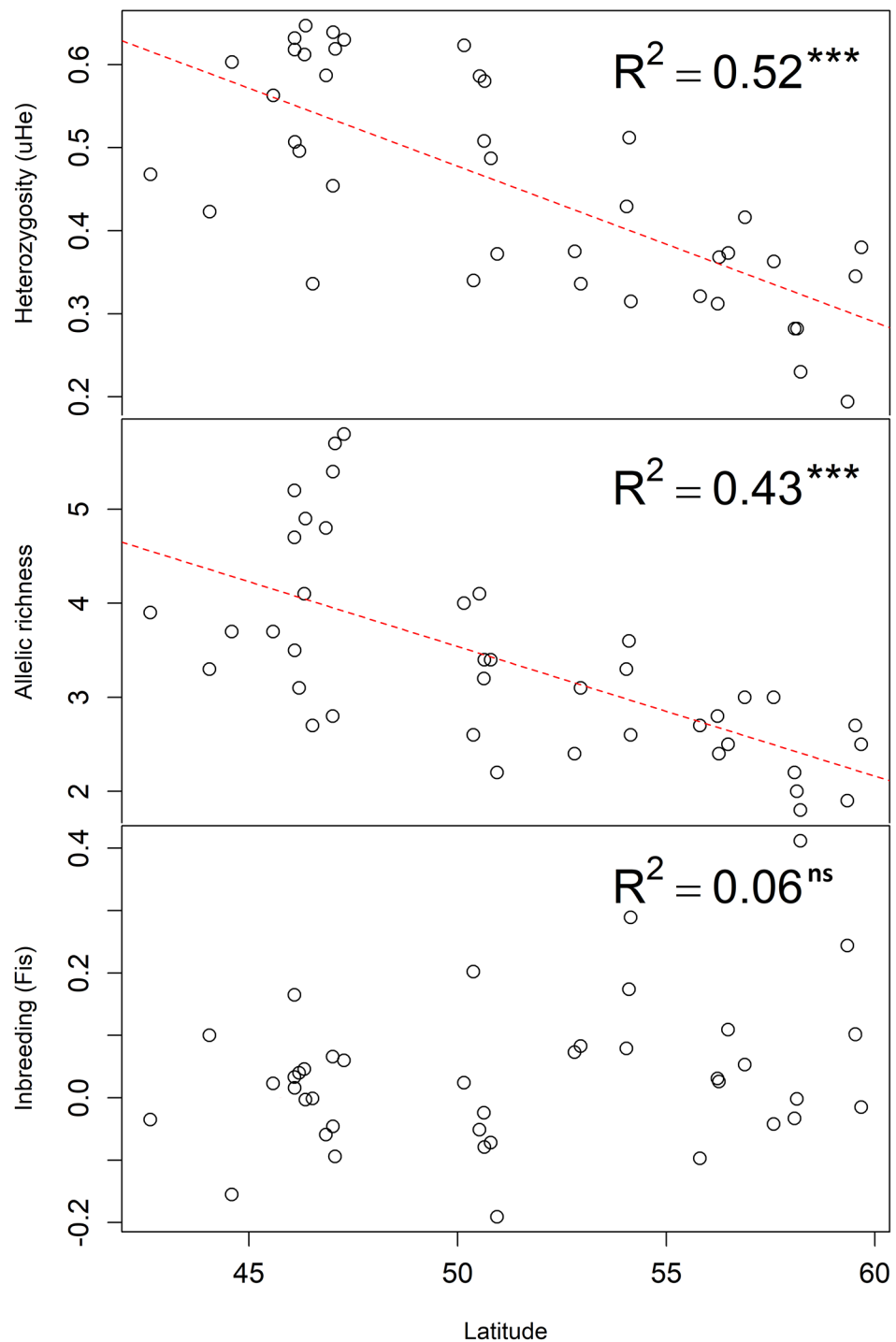
Overall, we conclude that gene flow across long distances is very limited in *A. montana* populations, explained most likely by poor dispersal ability. Evidence of limited seed

dispersal in *A. montana* at the local scale is present in the literature (Luijten et al. 1996). In particular, Strykstra et al. (1998) found that seeds disperse for a few meters only and fine-scale genetic structure detected by Luijten et al. (1996) with rosettes (genets) in the vicinity of each other sharing similar genotypes is indicative, too. Furthermore, the main pollinators of this species are solitary hymenopterans, syrphids and lepidopterans (Van Rossum and Raspé 2018) and hence restricted pollen dispersal can be expected, too.

In addition, the integrity of many natural habitats is affected by human activities, causing their fragmentation, with long-lasting consequences e.g. biodiversity loss (Haddad et al. 2015; Rogan and Lacher 2018). In a meta-analysis of 50 plant species, a large negative effect of fragmentation on pollination and reproductive success was found (Aguilar et al. 2006), which was stronger in self-incompatible species. Self-incompatible species are obligate outbreeders and therefore are more dependent on pollination that occurs between genetically different (and thus more distant) individuals. Consequently, *A. montana* as a self-incompatible species with very limited seed and pollen dispersal is particularly susceptible to habitat fragmentation. We underline therefore the importance of considering the aforementioned aspects when planning conservation measures.

The highly differentiated *A. montana* populations were hierarchically clustered into geographically concise groups, as shown by the Bayesian analysis and confirmed by the PCoA and DAPC. The observed pattern is likely to be a result of the climatic cycles of the quaternary, which have shaped the genetic structure of many species (Hewitt 1996).

Fig. 4 Relationship of latitude with unbiased heterozygosity (*uHe*) (upper), allelic richness (*Ar*) (middle) and inbreeding (*Fis*) (lower) in *A. montana*. Black circles represent *A. montana* populations. The red dotted line represents the regression line. *** ($p \leq 0.001$); ns (non-significant)



Thus, the geographically concise genetic clusters might either originate from distinct glacial refugia, which had been isolated during the glaciation or might result from barriers that have established after recolonization. Anyway, with our current coverage of the range, it is difficult to evaluate whether the “northern” cluster arose from a refugium outside of the Alpine region or directly from the “southern”

cluster, which would mean that the differentiation is due to isolation after migrating north. The separation within the southern cluster seems more likely to originate from separate refugia because no apparent barriers or distribution gaps between East and West are evident and a parallel colonisation of the Alps from several refugia seems plausible as was observed also for other species (e.g. Wagner et al. 2015).

When the population structure was analysed at even higher resolution, we observed that more, separated genetic groups could be defined. Individuals were consistently assigned to geographically concise genetic groups and, even at the highest possible K value ($K=40$, Fig. 2), well defined and almost “pure” genetic clusters can still be observed in some regions, further confirming the strong population differentiation detected by the AMOVA.

Besides a strong hierarchical genetic structure, we observed a strong geographical cline of genetic diversity, with both heterozygosity (uHe) and allelic richness (Ar) decreasing from the Alpine region towards Scandinavia. Based on our range-wide sampling we could show that the major driver of genetic diversity is latitude, which may coincide with altitude in large parts of the range. However, at smaller geographic scales other factors may modulate genetic diversity (Maurice et al. 2016). Due to our large-scale study, we can now re-assess the distribution of genetic diversity in *A. montana* and put into context the previous studies conducted on the population genetic features of this species. In detail, Duwe et al. (2017) found an altitudinal gradient in genetic diversity (He) with populations at lower altitudes showing slightly lower levels of genetic diversity compared to populations at higher altitudes. Altitude was interpreted as the major factor modulating genetic diversity. Additionally, increased anthropogenic activities associated with lower altitudes (either due to eutrophication or habitat destruction) were considered the main responsible factors for lower genetic diversity in Central Europe. The finding that decreased habitat quality and habitat loss can cause a reduction in genetic diversity due to genetic erosion has been described in the scientific literature as a main result of increased fragmentation and consequent reduction in population sizes (Pitra et al. 2011; Schlaepfer et al. 2018). Hence, the loss of genetic diversity caused by human activities is subject of concern and the conservation of adequate levels of genetic diversity is fundamental for the preservation of natural habitats (Salo and Gustafsson 2016). However, by following the hypothesis that anthropogenic activities explain the latitudinal gradient in genetic diversity, the distribution of *A. montana*'s genetic diversity should be associated with habitat quality and/or habitat loss. That is, the areas of lowest genetic diversity (i.e. Scandinavia) would match geographically the regions where anthropogenic pressure on natural habitats is the highest. This is not the case, since habitat quality is generally higher in Scandinavia than Central Europe (Haberl et al. 2014). As such, low levels of genetic diversity are not necessarily a consequence of human activities but can also be footprints of natural phenomena, such as past founder events or isolation caused by natural barriers. Although we did not test directly for correlations with habitat availability or quality, we claim

that, based on our results, the anthropogenic activities are unlikely to be driving the distribution of genetic diversity at this scale in *A. montana*.

Instead, the observed *gradual* decrease in genetic diversity is in line with the previously analysed patterns (differentiation, gene flow, population structure) explained by migration events. Such migratory processes are likely to have led to recurrent founder events and population bottlenecks. As such, our results are interpretable in the context of past colonization routes following the end of the last ice age. Postglacial migration events resulted in a progressive loss of neutral genetic diversity from south to north in various European species (Excoffier et al. 2009; Sebasky et al. 2016; Daco et al. 2022). The direct consequence of this gradual genetic diversity loss is that most of the genetic variation present in *A. montana* is stored in the former glacial refugia while the northern parts of the distribution range exhibit much less variation. The strong geographical pattern in genetic structure and differentiation we find, further support our interpretation of the genetic diversity gradient in *A. montana* being the product of postglacial colonization routes from south to north. Other studies reached similar conclusions. For instance, the study by Daco et al. (2022) on the herb *Anthyllis vulneraria* conducted on a comparable scale detected a latitudinal gradient in genetic diversity, which was best explained by past founder events during postglacial colonization (and not population size or habitat quality). Furthermore, in several pan-European species differing in life-history traits, a comparable S-N latitudinal gradient in genetic diversity was observed and interpreted as a footprint of postglacial colonization: *Silene vulgaris* (Sebasky et al. 2016), *Rhinanthus angustifolius* (Strykstra et al. 1996) and more (Pellissier et al. 2016).

Concluding, by conducting a large-scale investigation that included populations from potential former glacial refugia (e.g. Alps, Pyrenees, Carpathians) as well as populations from the range margins of the species' distribution, we were able to recognise the traces of postglacial colonization events. Moreover, the scale of the study enabled us to define how much differentiation exists between populations located in different biogeographical regions at the continental scale and thus to identify the presence of distinct genetic groups, which can be later acknowledged for conservation purposes (Moritz 1994; Picó and Van Groenendael 2007). Indeed, conservation studies greatly benefit from inclusive sampling designs, because understanding where genetic diversity sources are present in a certain species can help planning conservation actions such as ex-situ conservation, assisted gene flow and reintroductions (Picó and Van Groenendael 2007; Van Rossum et al. 2020).

Persistence of *A. montana* and conservation aspects

While genetic diversity is rather low in some parts of the distribution, there is no evidence of widespread inbreeding at the genetic level, despite the acknowledged population decline occurring in this species. This is not surprising considering that *A. montana* is self-incompatible and partially clonal. Self-incompatibility prevents mating between closely related individuals with the same S-allele, a strategy known as inbreeding avoidance (Furstenau and Cartwright 2017). Consequently, populations displaying inbreeding at the genetic level are probably rather rare in *A. montana* (Luijten et al. 2000). Moreover, in a partially clonal species, even small and isolated populations could maintain their genetic diversity temporarily by shifting to predominantly clonal reproduction. In our study, we did not compare populations for clonal diversity because our sampling design was not appropriate in this context. However, further studies aiming at evaluating the extent and features of clonality at range-wide level in this species might reveal important aspects about the consequences of reductions in population sizes and genetic diversity in a self-incompatible and clonal species like *A. montana*. In this context, we highlight the importance of monitoring shifts toward predominantly asexual reproduction, as observed in one case (Norwegian population excluded from the dataset for this reason). In fact, an increased level of clonality could make populations more susceptible to future threats, like climate change or diseases, because of lower genetic variation and potentially inbreeding following the breakdown of the self-incompatibility system (Navascués et al. 2010). In fact, in a study conducted by Luijten et al. (2000), hand-selfing resulted in the germination in up to 45% of the seeds (although in a limited number of individuals), which could indicate a partial breakdown of the self-incompatibility system. Therefore, further studies are necessary to unveil the mechanism behind sexual strategies in this species, considering the potential consequences from the conservation perspective.

Most of the genetic variation in *A. montana* is retained within in the Alps. This region is generally recognised as a hotspot of biodiversity (Petit et al. 2003). However, suitable habitats are disappearing also from the Alps (Dirnböck et al. 2003) where populations retain high levels of genetic diversity. The main reason is a shift in floristic associations caused by the abandonment of traditional pasture. Such deleterious changes can happen as fast as 30 years (Korzeniak 2016). Furthermore, it is likely that *A. montana* populations in these regions will undergo contractions because of unsuitable climate (La Sorte and Jetz 2010; Stanik et al. 2020) and limited habitat patches available as they migrate toward higher elevations (Hampe and Petit 2005). Hence, habitat-focused conservation becomes particularly important in

those areas where high levels of genetic diversity are still retained. In particular, the conservation of managed alpine meadows is necessary for enabling the survival of established individuals and the recruiting and development of new rosettes in available habitat patches at higher altitudes.

On the other hand, populations situated at the geographical centre of the distribution (Central-European lowlands and low-altitude montane areas) have lower neutral genetic variation and are likely experiencing non-optimal habitat quality due to higher human population density (Picó and Van Groenendael 2007) and climate change (Stanik et al. 2020). In addition, such populations have limited possibility of shifts toward higher latitudes/elevations and less available habitats for colonization, as they become more fragmented within patches of non-suitable habitats. Finally, while some northern European species are predicted to expand further north as the climate shifts (van der Meer et al. 2016), some are constrained by climate shifts in unsuitable directions (Vikane et al. 2019), limited genetic diversity and small population sizes (Vucetich and Waite 2003). Populations of *A. montana* situated at the northern edges are predicted to suffer from increased extinction risk and susceptibility to increasingly unsuitable climate (e.g. increased temperatures and rainfalls) (Vikane et al. 2019). Moreover, as we can see from our data, genetic variability in northern populations is heavily reduced, likely due to serial founder effects. Although the link between adaptive and neutral genetic variation is under discussion in the scientific community (García-Dorado and Caballero 2021), we can assume that adaptive diversity was also severely reduced due to founder effects. Therefore, northern populations are likely more susceptible to diseases and environmental change due to a lack of adaptive variation. These populations with increased extinction risk due to low genetic diversity, small population size might benefit from conservation practices such as genetic restoration and the following monitoring of genetic parameters (Van Rossum and Raspé 2018; Van Rossum et al. 2020). However, geographically distinct genetic groups were recognised in our study. Consequently, the indicated genetic clusters should be considered in the context of conservation practices such as relocations and assisted gene flow (Moritz 1994). Successful translocations of individuals for genetic restoration purposes have been reported by Van Rossum et al. (2020). It must be mentioned, though, that the source and target populations were all from the same region and had much lower differentiation levels than the ones recorded in our study.

Thus, we strongly suggest conducting further studies at larger scales on the adaptive genetic diversity of *A. montana* to indicate the best strategy (e.g. assisted gene flow) for supporting the survival and expansion toward new habitat patches of those northern low-diversity populations.

Conclusions

Our results show strong geographical patterns in differentiation, genetic diversity and genetic clustering in the endangered plant *Arnica montana*. Spatially-limited gene flow is present among populations, as shown by the isolation by distance (IBD) pattern. We interpret such patterns as the genetic signature of postglacial colonization events combined with limited dispersal ability, which are still visible in the extant genetic structure and distribution of genetic diversity. These patterns have important implications for the future conservation of this valuable species. Since climate change is expected to force *A. montana* populations to shift toward higher altitudes and latitudes, studies focusing on the potential responses to climate change in relation to genetic variation could help conservation programmes at maximising survival to climate change. Where neutral genetic diversity is low, and especially in Scandinavia, populations must be closely monitored for signs of reduced recruitment and the consequent population decline. Where population decline is caused (or followed) by a limited S-allele set and the consequent interruption of sexual reproduction, the introduction of new individuals from more diverse populations should be considered. Here, the genetic clusters we identified might help as a first starting point to define distinct evolutionary units for conservation practices such as relocations and assisted gene flow. Also, studies focusing on adaptive genetic variation could help conservation programmes at maximising survival to environmental change (e.g. global warming) in translocated and resident populations. At the same time, where genetic diversity is higher (e.g. Alps), habitat-focused conservation will be fundamental for the survival of this species in the medium and long term. In the meanwhile, ex-situ conservation of *A. montana* genetic heritage, such as activities aimed at sharing genetic material between botanical gardens and research facilities, could be the last resort for this species in some regions where assisted gene flow is impractical or economically unsustainable.

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Authors' contributions L.C. wrote the manuscript text and conducted the fieldwork and lab work. E.M. and S.L. supervised the writing process and contributed to the interpretation of the results. E.M., S.L. and I.L. helped with the experimental design. K.E. supervised the DNA extraction process. S.L. supervised the genotyping process. E.M. supervised the research project. All the authors reviewed the manuscript.

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Declarations

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