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Phylogeography of *Arabidopsis halleri* (Brassicaceae) in mountain regions of Central Europe inferred from cpDNA variation and ecological niche modelling

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The present study aimed to investigate phylogeographical patterns present within *A. halleri* in Central Europe, to propose hypotheses explaining the emergence of these patterns and to formulate hypotheses on the formation of the present day range of *A. halleri* in the region. 1281 accessions sampled from 52 populations within the investigated area were used in the study of genetic variation based on chloroplast DNA. Over 500 high quality species occurrence records were used in ecological niche modelling experiments. We evidenced the presence of a clear phylogeographic structure within *A. halleri* in Central Europe. Our results suggest that the species might have not survived the last glacial maximum in the Carpathians and Sudetes and that its range during the last glacial maximum might have consisted of at least two major parts: (1) a northern refugium consisting of vast refugial areas north and northeast of the Alps and (2) a southern refugium located in the Dinaric Alps and Balkan Mts. We postulate that the Sudetes and Western Carpathians were colonised mainly by plants originating from the northern refugium, whereas populations from the Eastern Carpathians originate from southern refugium. We also discuss our results in relation to the problematic taxonomy of the species.

Phylogeography of *Arabidopsis halleri* (Brassicaceae) in mountain regions of Central Europe inferred from cpDNA variation and ecological niche modelling.

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Abstract

The present study aimed to investigate phylogeographical patterns present within *A. halleri* in Central Europe, to propose hypotheses explaining the emergence of these patterns and to formulate hypotheses on the formation of the present day range of *A. halleri* in the region. 1281 accessions sampled from 52 populations within the investigated area were used in the study of genetic variation based on chloroplast DNA. Over 500 high quality species occurrence records were used in ecological niche modelling experiments. We evidenced the presence of a clear phylogeographic structure within *A. halleri* in Central Europe. Our results suggest that the species might have not survived the last glacial maximum in the Carpathians and Sudetes and that its range during the last glacial maximum might have consisted of at least two major parts: (1) a northern refugium consisting of vast refugial areas north and northeast of the Alps and (2) a southern refugium located in the Dinaric Alps and Balkan Mts. We postulate that the Sudetes and Western Carpathians were colonised mainly by plants originating from the northern refugium, whereas populations from the Eastern Carpathians originate from southern refugium. We also discuss our results in relation to the problematic taxonomy of the species.

Key words:

Arabidopsis halleri, phylogeography, Carpathians, Alps, Sudetes, Harz, Quaternary, taxonomy

Introduction

A phylogeographical approach has been used in numerous studies addressing the Quaternary history of the flora of Europe, shaped by repeated range contractions during cold periods and subsequent extension of available habitats during warmer periods [1,2]. These range oscillations, altering the patterns of gene flow, have been found to contribute to the genetic differentiation that can be detected between contemporary populations [3]. The presence of this

differentiation has allowed (with the advent of phylogeography) insights into processes responsible for range formation, reconstruction of (re)colonisation routes, detection of refugial areas and unravelling historical relationships among different parts of the contemporary species distribution.

Areas of relative ecological stability that provided suitable habitats for species survival during periods of glaciation are termed refugia [4]. Numerous studies carried out so far showed that major glacial refugia were locate in the southern part of Europe [5,6]. Recently, the possibility of full-glacial survival of temperate species at northern latitudes in so-called northern or cryptic refugia [7] was hypothesised and subsequently supported by fossil records from several species [8]. There is, however, still little molecular evidence for the existence of "northern refugia" in Central Europe [9].

In Europe the potential for phylogeographical research has been exploited intensively in the Alps, where the abundance and complexity of the available phylogeographic studies has already resulted in synthetic and comparative analyses [10,11]. The situation is quite different in other mountain ranges of Central Europe such as the Carpathians, Sudetes, Bohemian Forest (Sumava) and Harz Mts. A recent literature review pointed out clearly that the history of species range formation in the mountain plants of Central Europe is still only poorly known from phylogeographical studies [12].

Arabidopsis halleri with its pattern of occurrence covering nearly all mountain regions of Central Europe [13] seems to be an interesting model taxon to address all the problems raised above. Previous phylogeographical studies focused on the species, evidenced the presence of two major units within the species range and attributed the emergence of these units to vicariance associated with the isolation of two large populations groups during the Quaternary which were

located in Central and Southern Europe [14]. The sampling density adopted in the study was, however, too low to address the question of *A. halleri* range formation in the mountains of Central Europe.

In the light of these considerations we focused our study on the poorly investigated area covering the Carpathians, Sudetes, Bohemian Forest and Harz Mountains in order to reconstruct the phylogeographic history of this montane species *Arabidopsis halleri*. We decided to base our study on information obtained from chloroplast DNA (cpDNA) variation. This approach allowed us to overcome another limitation originating from the fact that the vast majority of phylogeographical analyses carried out hitherto on mountain species in Europe has been focused on alpine and arctic-alpine species, while our knowledge of the phylogeographic patterns present within herbaceous species having the centre of its occurrence in lower vegetation belts (subalpine and montane) still remains, with some notable exceptions [15,16], much poorer.

We aimed to achieve the following goals:

1. To reveal phylogeographical patterns present within *A. halleri* in Central Europe.
2. To propose hypotheses explaining the emergence of these patterns 3
3. To formulate hypotheses on the formation of the present day range of *A. halleri* in the region.

Material and methods

The study species

Arabidopsis halleri (L.) O'Kane & Al-Shehbaz is a perennial, self-incompatible and highly outcrossing [17], stoloniferous herb with a highly disjunctive distribution between Europe and Far East. It occurs in mountain and upland environments on slopes, forest margins, rocky crevices and river banks from 200 to 2200 m a.s.l. In Europe, it is widely distributed in the Alps,

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Carpathians and Sudetes. Its distribution covers also some upland regions north from the Alps (including the Harz Mountains) and the Western Carpathians [13]. The species is highly variable in leaf morphology, flower colour and traits connected with the development of stolons. At least three subspecies are quite distinct in terms of morphological variation [18]: *A. halleri* subsp. *halleri* and *A. halleri* subsp. *ovirensis* occur in Europe, whereas *A. halleri* subsp. *gemmifera* occurs in the Far East. According to other authors [19] the species can be alternatively divided into five distinct morphological subspecies, with four occurring in Europe. Only diploid (2n=2X=16) individuals have so far been reported from throughout the distribution range [18,19].

Sampling and DNA extraction

We sampled 1281 individuals from 52 populations (Table 1) scattered across the species range in seven geographic regions of Central and Eastern Europe (S1 Fig.). Twenty-five populations included in the present study have been already described in previous studies [14, 20]. In the present study, sampling was extended to improve sampling representativeness in the area of the Sudetes as well as the Carpathians. All the locations sampled belonged to the native species range [13]. In each locality individual samples were taken from plants separated by at least three meters in order to avoid sampling clones [21]. Sample size generally reflected population size and was almost exhaustive in small populations. Permits for plant sampling were obtained from the following national parks: Krkonošský národní park (CZ), Karkonoski Park Narodowy (PL), Tatrzański Park Narodowy (PL), Bieszczadzki Park Narodowy (PL) and Karpáts'kij biosférnij zapovidnik (UA). In other cases sampling was done outside protected areas.

Collected leaf tissue was immediately dried in silica gel prior to molecular analysis. DNA was extracted from 10 to 15 mg of dry material using NucleoSpin®96 Plant (Macherey-Nagel) and PCR amplifications were performed on 1:20 dilutions.

Genetic analysis

Genotyping procedure. During the genotyping process we screened 12 polymorphic sites previously detected by PCR-RFLP in three cpDNA regions: trnK intron and two intergenic regions (trnC-trnD, psbC-trnS). The observed polymorphisms are briefly characterized in Table 2. In contrast to Pauwels et al. [20], however, three different genotyping methods were employed.

SNaPshot assay

After PCR amplification of the three cpDNA regions of interest, we used SNaPshot assay for simultaneous detection of seven single nucleotide polymorphisms (SNP, cf. Table 2). In the analysis we employed ABI Prism® SNaPshot® Multiplex Kit (Applied Biosystems) and followed the protocol given by the manufacturer.

The following PCR conditions were utilized: a total volume of 15 µl consisting of 3 µl of template DNA (20-100 ng), 6.225 µl of water, 1.5 µl of 10X PCR Buffer, 2.1 µl of 25 mM solution of MgCl2, 1.2 µl of 2.5 mM solution of dNTP, 0.3 µl of BSA solution (10 mg/ml), 0.3 µl of 10 mM solution of each primer and 0.075 µl of AmpliTaq®DNA Polymerase (5U/µl). The reaction was carried out in Mastercycler® ep gradient S thermal cycles using one cycle of 5 min at 95^{o}C, and 36 cycles of 45 s at 92^{o}C, 45s at 58^{o}C - 62^{o}C (depending of the primers sequences, precise protocol upon request); and 2 min 30 s at 72^{o}C, followed by one cycle of 10 min at 72^{o}C. The primer sequences for specific PCR amplifications are given in S2 Table. Amplicons were used for genotyping following the manufacturer's instruction (ABI Prism® SNaPshot®

Multiplex Kit). The primer sequences used in the SNaPshot reaction are given in S3 Table. Sample electrophoresis was carried out in an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems) using a capillary of 36 cm, POP 4 Migration Buffer and Dye Set E5. The data were collected using Foundation Data Collection 4.0 software and analyzed with GeneMapper 4.0.

CAPS assay

The CAPS assay was used for genotyping three SNP polymorphisms in the trnC-trnD region by specifically amplifying the genomic region containing the SNP polymorphisms mentioned in Table 2 and digesting the amplification product using the *Acs*I restriction enzyme. The PCR primer sequences were given in S3 Table. The PCR mixture and the conditions followed the protocol given above for SNaPshot assay. Restriction enzyme reaction was performed on a total volume of 20 µl consisting of 10 µl of PCR product and 10 µl of restriction mixture containing 5.9 µl of water, 2 µl of SuRe/Cut Buffer B 10X, 2 µl of 2mM solution of spermidine and 0.1 µl of *Acs*I solution (10U/µl). The mixture was then incubated at 50°C for 1 hour, followed by enzyme deactivation at 70°C for 15 min. Both procedures were carried out in Mastercycler® ep gradient S (Eppendorf) thermal cyclers. Fragments were separated by electrophoresis on 2% agarose gels stained by ethidium bromide and photographed with BioImage system (Bioprobe) under UV light.

PCR length difference assay

Two indel polymorphisms in the trnK and trnC-trnD regions (cf. Table 2) were genotyped from a PCR product length difference assay following the method described by Oetting et al. [30]. The PCR mixture followed the protocol given above for SNaPshot assay. The primer sequences used in this reaction are given in table S2. PCRs were carried out in Mastercycler® ep gradient S (Eppendorf) thermal cyclers using one cycle of 5 min at 95°C, and

36 cycles of 30 s at 94°C, 30 s at 51°C or 56°C (depending of the primers sequences); and 30 s at 72°C, followed by one cycle of 10 min at 72°C. Fragments were separated on 8% polyacrylamide gels using Li-Cor 4200 Global IR2 DNA Sequencer.

Data analysis

Since cpDNA is a non-recombining molecule, alleles observed at all twelve loci were combined into cpDNA haplotypes (chlorotypes, Table 3). Chlorotype nomenclature is fully consistent with our previous study [20].

Phylogenetic relationships between chlorotypes

A minimum spanning tree (MST) was constructed on the basis of a distance matrix reflecting molecular differences between each pair of chlorotypes using a modification of the algorithm described by Rohlf [23]. Computations were made using the software Arlequin 3.11 [24]. The MST algorithm assumes that each chlorotype is linked to all the other chlorotypes by one or a series of mutations and constructs a tree with minimal number of required mutational steps between haplotypes [25]. The position of a chlorotype in the MST also gives information regarding relative age, since older haplotypes are expected to locate in internal nodes of the tree [26].

Molecular diversity indices

Allelic richness (A_{Sc}) was calculated for each population according to the rarefaction method [27,28] using Fstat software [29]. Estimates of A_{Sc} were standardized to the smallest sample size (n=7). Chlorotypic diversity (H_{Sd}) and its sampling variance were calculated according to the methodology given by Nei (1987) for each population separately and over the whole sample using Arlequin 3.11 [24]. To test for differences in allelic richness between the

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investigated geographical regions we employed a permutation test implemented in Fstat [29] using 10 000 permutations of populations between geographical regions.

Population genetic structure

To reveal structure in our dataset we used Spherikm [30], software designed to analyse multivariate datasets by means of spherical k-means clustering (SKMC). The computations were based on a matrix of chlorotype frequencies in analysed populations. The statistically optimal number of clusters was assessed using the quasi-Akaike information criterion [30]. The partitioning of genetic variation within and between groups of populations identified by SKMC as well as between geographical regions was tested by analysis of molecular variance (AMOVA) using Arlequin 3.11 [24]. AMOVA computations were based on a distance matrix among the identified chlorotypes. We also carried out separate AMOVA analysis for populations within each geographical region in order to test partitioning of genetic variance among and within investigated populations.

Distribution modelling

To reconstruct the potential distribution of *A. halleri* we used two palaeoclimate scenarios: mid Holocene (ca. 6 kyr BP) and Last Glacial Maximum (LGM, ca. 22 kyr BP). Paleoclimatic data were obtained from simulations in the following Global Climate Models: CCSM4, MIROC-ESM and MPI-ESM-P. Bioclimatic variables calculated on the basis of these models and downscaled to 5 arc-minute resolution were downloaded from the WorldClim dataset [31] (http://www.worldclim.org), together with present-day climate data at the same resolution. We tested all the variables for multi-collinearity by examining the cross-correlations among them (Pearson's r) based on the 544 species occurrence records. Highly correlated variables ($r > 0.7$) were excluded from the models [32], resulting in 8 variables representing temperature and

precipitation: annual mean temperature (bio_1), mean diurnal temperature range (bio_2), isothermality (bio_3), temperature seasonality (bio_4), mean temperature of the wettest quarter (bio_8), mean temperature of the driest quarter (bio_9), annual precipitation (bio_12) and precipitation seasonality (bio_15). Areas covered by ice sheet [33] were excluded from the climatic layers of the LGM paleoclimate scenarios.

Distribution data were obtained from GBIF database (http://www.gbif.org/) as well as from our own field research carried out in France, Germany, Poland, Austria, Czech Republic, Slovakia and Ukraine. After initial screening for duplicates and data aggregation into a 5 minute resolution raster we obtained 544 unique records that were used to calibrate and validate the models (S4 File). Data handling was done using GRASS GIS ver. 6.4 (http://grass.osgeo.org).

Seven different algorithms implemented in biomod2 ver. 3.1-48 [34,35] and MaxEnt ver. 3.3.3k [36] were used : two regression methods (GLM – generalized linear models; GAM – generalized additive models), two classification methods (FDA – flexible discriminant analysis; CTA – classification tree analysis) and three machine-learning methods (GBM – generalized boosting model; RF – random forest for classification and regression and MAXENT – maximum entropy modelling). For each of the algorithms we ran 10 pseudo-absence replicates with 10000 of pseudo-absences, to meet the minimum requirements of the algorithms used [37]. The models were fitted with 10 different random presence sets for each pseudo-absence run. This gave us a total of 100 replicates for each of the algorithms. Occurrence records were randomly divided into two subsets containing data for calibration (70%) and evaluation (30%) of models.

We used the area under the receiver-operating characteristic (ROC) curve and true skill statistic (TSS) to evaluate model performance. These accuracy measures were calculated with reference

to the current potential distribution only, due to the lack of independent and reliable fossil records for *A. halleri*.

Permutation procedure was used to define contributions of the variables to the models. In order to identify areas classified as suitable for species survival by the majority of algorithms (final consensus models) we performed ensemble forecasting [34]. This procedure was used to eliminate the least reliable models (TSS<0.7) and provided 7 ensemble models: mean of probabilities, coefficient of variation of probabilities, two models of confidence interval around the mean of probabilities, median of probabilities, models committee averaging (average of binary predictions) and weighted mean of probabilities. Binary transformation was carried out using a threshold that maximized the true skill statistic (TSS) to generate the most accurate predictions [38].

Results

Chlorotype diversity

A total of 12 cpDNA haplotypes were found in the investigated populations (Table 4). All neighboring chlorotypes were linked by a single mutation (Fig. 1), except for G and H (separated by two mutations). Thus, the MST topology did not allow the division of chlorotypes into clearly demarcated groups separated by more than one mutation.

Chlorotypes did not show equal frequencies in overall sampling (Table 4). The most widely represented was chlorotype J, present in 26.4 % of the samples analyzed. Chlorotypes E, F and G had a share of 15.47 %, 12.19% and 12.11% respectively. The share of the remaining chlorotypes in the overall sampling was significantly lower than 10%.

Chlorotypes E and J were the most widespread geographically (Fig. 2), with their occurrence established respectively in 6 and 5 out of the 8 investigated geographical regions.

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Most of the haplotypes occupying tips and terminal branches of the MST were more localized geographically. Chlorotype B, C and H have been found only in the Bohemian Forest, chlorotype D in the Harz Mts., while chlorotype H only in the Eastern Carpathians (Fig. 2). Chlorotype G was found almost exclusively in the Eastern Carpathians, while chlorotype F showed a predominant occurrence in the Western Carpathians as well as in the geographically close region of the Northern Carpathian Foreland.

Genetic diversity indices: chlorotypic richness (A_{Sc}) and chlorotype diversity index (H_{Sd}) were calculated for each investigated population. They varied broadly from 1 to 4.983 for A_{Sc} and from 0 to 0.893 for H_{Sd} (Table 4). We examined also geographical pattern of variation in chlorotypic richness. As shown in Fig. 3, populations with a high level of genetic diversity were co-located in the Bavarian Forest, the Harz Mts. and in the Western Carpathians (Tatra Mts.).

Also we compared genetic diversity indices between different geographical regions (Table 5). As expected, geographical regions differed substantially in terms of genetic diversity. Three regions: Western Carpathians, Harz Mts. and Bohemian Forest were found to be most diverse. The lowest genetic diversity was found in the Alps and in the Sudetes.

Genetic structure

The clustering approach employed in the present study (spherical k-means clustering - SKMC) enabled us to study the structure present in our dataset on several levels. We examined a broad spectrum of different k values from k=2 to k=25. The results of SKMC from k=2 to k=10 were plotted on the map (Fig. 4). Clearly, the populations from Eastern Carpathians formed one stable cluster (present in all the k levels), that differed from all the other populations. A subdivision of the populations studied into 6 clusters was statistically optimal (Fig. 5A) and had a high support in AMOVA (Fig. 5B). We carried out separate AMOVA analyses to test the

distribution of genetic variance among and within groups identified by SKMC. Results of SKMC evidenced the presence of some level of genetic admixture in almost all studied geographical regions except in the Harz Mts., the Alps, and the Eastern Carpathians.

AMOVA performed without grouping populations showed that 79.16 % of the total genetic variation was found between populations (Table 6). When assuming six groups of populations (according to k-means clustering), as much as 65.8% of the total variation was observed between groups of populations, whereas 15.82% was found among populations within groups (Table 6). These percentages of genetic variation were 42.88% and 37.90%, respectively, when assuming seven groups (characterised according to the geographical regions sampled; Table 6). Separate AMOVAs performed within each investigated geographical region revealed the highest percentage of among-population variation in the Sudetes and the Bohemian Forest: 85.66 % and 74.57%, respectively (Table 6). The lowest values of among-population variation was found in the Alps, where we recorded the presence of just one chlorotype (0%; Table 6), and in the Harz Mts. (18.55%; Table 6). All the remaining geographic regions showed intermediate level of among-population variation.

Distribution modelling

Model performance was assessed using two different statistics: True Skill Statistic (TSS) and Area under Receiver Operating Characteristic (ROC). All models performed well and had TSS > 0.7 and AUC > 0.88. The performance of two models CTA and FDA was weaker than the performance of the remaining methods, but still of acceptable quality.

Two (CCSM4, MPI ESM) out of the three climatic models suggest that the species survived the LGM (~ 22 kyr BP) only in the southernmost part of the Carpathians (Fig. 6). The

Dinaric Alps and Balkan Mts. were also among the potential areas for species survival in southern Europe during the LGM (Fig. 6). Vast areas north from the Alps can be also considered as suitable for the survival of *A. halleri* (Fig. 6).

We also analysed the potential distribution of *A. halleri* during the Holocene climatic optimum (ca. 6 kyr BP). Our models showed that recolonization must have advanced very slowly in the Carpathians, when compared with areas north of the Alps. All the models showed that conditions facilitating the spread of *A. halleri* occurred much earlier in between the Western Carpathians, the Sudetes and areas north from the Alps, while suitable areas in the Eastern Carpathians were at first much more restricted (Fig. 6).

Discussion

Carpathian populations of *A. halleri*

Our data suggest a clear differentiation among populations from western and eastern part of the Carpathians. Those population groups differ in terms of chlorotype composition and frequencies. The same differentiation also appears clearly in the SKMC analysis since the western and eastern populations were grouped in different clusters from k=2. This pattern of genetic variation seems to follow the division between the eastern and western part of the Carpathians which was first recognized by Wołoszczak [39] and was established on the basis of floristic data. The nature of the barrier between the western and eastern part of Carpathians has been the subject of many studies employing different methodologies from floristic [40,41] to cytologic [42] and genetic [43,44]. It has been hypothesised that specific climatic and orographic conditions of the westernmost part of Bieszczady Mts. (also known as Bukovske Vrchy Mountains) are among the main factors influencing the genetic landscape of this part of Carpathians [45]. It seems that results of our study may suggest that differentiation between

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western and eastern part of Carpathians may be a historical phenomenon connected with recolonisation of the area by plants that survived in different refugia. In this case we do not need to postulate the presence of a specific barrier responsible for the existence of genetic discontinuity between western and eastern part of Carpathians since this phenomenon could be also explained by colonisation from two different directions together with the presence of gene flow between the two groups as was evidenced by our study (i.e. the presence of haplotype P in western and eastern Carpathians).

The Eastern Carpathian populations are characterized by the presence at very high frequencies of haplotype G. This haplotype was considered as ancestral by Pauwels [20]. The presence of the ancestral haplotype G at high frequencies has also been recorded in populations located south of the Alps as well as in the south-eastern part of this mountain range [14]. This suggests that populations from the Eastern Carpathians and southern part of the Alps share common ancestry. Our results indicate that both groups, despite the present geographical isolation, could be probably derived from the same refugium. It may be hypothesised that this refugium was located in the Dinaric Alps and/or the Balkan Mountains. Our genetic data showing relatively low genetic diversity in the Eastern Carpathians seems also to confirm hypotheses concerning relatively recent recolonisation of this area by *A. halleri* as suggested by our modelling experiments.

The Western Carpathian populations are characterized by high levels of genetic diversity and the presence of a private haplotype F at high frequencies. In SKMC analysis, populations from the Western Carpathians were clustered together with populations from upland regions of southern Poland. This fact supports the hypothesis of a Western Carpathian origin of populations located north of the Western Carpathians.

Survival of plant species in the Western Carpathians during the LGM has been a focal point of many studies employing different methodologies. These works have shown that the existence of a Western Carpathian refugium is quite probable for many plant species, including mountain plants. Macrofossil charcoal fragments found in Kraków, just about 30 km in a straight line from population PL8 and about 40 km from PL 7, indicate full glacial presence of *Pinus*, *Larix* and *Abies* from 26 to 28 kyr BP – during the coldest period of LGM that spanned between ~36 - 16 kyr BP [8,46,47]. Dates from humic soil further down in the sequence are even earlier, and indicate the presence of trees as early as 36 kyr BP [8]. There is also taxonomic evidence supporting the hypothesis of longstanding survival of different plant species in the Western Carpathians. *Saxifraga wahlenbergii* Ball. and *Delphinium oxysepalum* Borb. et Pax are good examples here. These two species are endemic to Tatra Mts. and occupy isolated systematic positions, what suggests that they are both of Tertiary age [48]. The survival of common yew (*Taxus baccata*), was also documented in charcoal for Moravany in Slovakia with a radiocarbon date of ca. 18 kyr BP [49]. There is also a large body of phylogeographic evidence that indicates the existence of a major northern refugium for a variety of animal taxa in the area around the Carpathians, with some lineages predating LGM [50].

Our results suggest, however, that in case of *A. halleri* the area of Western Carpathians was successfully re-colonised by plants originating from a refugium located north of the Alps. High genetic diversity observed in this area could be explained by relatively recent gene flow (occurring later than 6 kyr BP, according to our modelling experiments) from the Eastern Carpathians. The presence of a genetic admixture in Western Carpathian populations of *A. halleri* was also revealed by Pauwels et al. [14].

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We have shown that populations of *A. halleri* in western and eastern part of Carpathians form two different genetic groups that could be derived from two different refugia. Populations in the Eastern Carpathians clearly belong to the group that survived in a southern refugium (areas south of the Alps, Dinaric Alps and Balkan Mts.), while western Carpathian populations are formed by plants that survived in refugial areas north of the Alps. There are also traces of relatively recent gene flow (probably of late Holocene age) from the Eastern Carpathians.

Genetic differentiation between the Harz, the Bavarian Forest and the Alps

The situation in the areas north of the Alps is more complex than in the Carpathians. SKMC analysis showed that at least two groups of populations can be recognised regardless of the level of k: populations from the Harz Mountains and from the Alps. Populations from the Bohemian Forest were usually assigned to different clusters, forming very heterogeneous group. This heterogeneity was also evidenced in AMOVA.

It is not easy to explain this pattern of genetic differentiation. Some ideas might be provided by the results published by Tollefsrud et al. [51]. Investigating the genetic variation of Norway Spruce together with pollen data they established that one possible glacial refugium of the species might have extended from the northern slopes of the Alps up to the Šumava (the Bohemian Massif). This finding could be regarded as a conceptual framework for the process of building hypotheses on the Pleistocene history of *A.halleri* north of the Alps. It seems that *A. halleri* might have survived in a vast area, and that its Pleistocene distribution covered not only the region mentioned above, but also extended northwards and westwards up to the Ardennes and Hautes Fagnes (High Fens) in Belgium. So far one natural population from this area has been tested by Pauwels et al. [52]. This, nowadays isolated, population from Hautes Fagnes, harbouring a chlorotype with an extremely restricted geographical range [52], might be the trace

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of a past *A. halleri* distribution in Western Europe. The location of a glacial refugium in Ardennes has been hypothesized by Stewart and Lister [7] on the basis of observations made by Otte [53]. The vast extent of possible refugial areas north of the Alps was also clearly evidenced by our modelling experiments. Other studies based on molecular methods also suggest the presence of a glacial refugium in the Central Europe [54-56].

Differentiation is also apparent between the regions of the Harz Mts. and the Bayerischer Wald, with the first harbouring haplotype D, which is not present in the Bayerischer Wald. In the latter region the occurrence of haplotype C can be observed, which, in turn, is present neither in the Harz, nor in the Alps or the Šumava. A relatively recent (postglacial) origin of this differentiation could be hypothesized as both haplotypes occupy external nodes of the MST tree.

Genetic variation in the closely related species *Arabidopsis lyrata* from the Harz, southern Germany and the Alps [57], can give us some insights into possible explanation of this pattern. Studies on genetic variation of *A. lyrata* (carried out on the basis of nuclear microsatellite loci) showed high within-population diversity throughout central Europe, accompanied by low regional differentiation and geographically widespread polymorphism. The authors hypothesized that (given the unlikeliness of gene flow) a common gene pool must have existed for central European populations [57]. It should be noted that "central European" in this case is not a precise term and describes sites located approximately between the 10^{th} and 16^{th} eastern meridian. This area corresponds to the locations of the populations sampled by us north of the Alps in the Bayerischer Wald, the Harz and the Šumava. The same scenario is also probable for *A. halleri*, where haplotype E, present in all regions north of the Alps, can be interpreted as a testimony of a common gene pool in the past. Other haplotypes, with restricted

geographical range, would be, under this model, local derivatives that evolved after climatic warming and fragmentation of a previously vast range.

Results published by Koch and Matschinger [58] may suggest that the area of possible glacial survival of *A. halleri* discussed above, could be extended even further eastwards to cover the region of "unglaciated east" comprising the non-glaciated part of the eastern Alps, as well as the non-glaciated area between the eastern Alps and the western Carpathians [58]. According to Koch and Matschinger [58] the region of "non-glaciated east" (the eastern Alps) is the most diverse in cpDNA haplotypes. A similar pattern of high genetic diversity has been also observed by Pauwels et al. [52] within the Southeastern Alpine Foreland. The reason for these observations could be as discussed above, but another scenario is also possible. The phylogeography of *Rosa pendulina* [59] suggests that the area corresponding to the "non-glaciated east" could be a contact, or suture zone between northern and southern lineages, that have spread from two different refugia. It seems that these results largely agree with our findings.

Origin of *A. halleri* populations in the Sudetes

We have shown that *A. halleri* populations in Sudetes are characterized by a very low genetic variation, with most of the populations harbouring only one cpDNA haplotype. This finding is not surprising, given the evidence from geological research showing that this region was severely impacted during the glacial period. Is seems that at least one glacial maximum (48-43 kyr BP) had a devastating effect on the regional flora, mainly due to the close proximity of the continental ice sheet [60]. Geological evidence show that even during LGM this region was affected by the presence of mountain glaciers [61]. Therefore, it has been postulated that in situ glacial refugia, supporting the remains of the autochthonous flora, did not exist in the Sudetes

[62]. Quite a different scenario involving survival on nunatak and in peripheral refugia has been suggested for the Alps [63]. It is noteworthy that even nowadays the climate of the highest parts of the Sudetes is cold enough to maintain the occurrence of tundra-like ecosystems [64]. It seems therefore, that *A. halleri* populations in the Sudetes could be of a recent (postglacial) origin. This is supported by very low genetic variation found in Sudetes as well as the fact that despite dense sampling, we have not found any chlorotypes private to this area. On the basis of the evidence from cpDNA variation, it could be hypothesized that populations from the Bohemian Forest could be a source of migrants that established new populations of the species in this region after LGM.

We have also found the presence of haplotype I in high-mountain populations of *A. halleri* in the Sudetes. The occurrence of this uncommon haplotype has also been recorded in high-mountain populations in the Western Carpathians as well as for one population in the Bohemian Forest. Similar pattern have been also recognized in *Pulsatilla vernalis* [65]. The most reasonable explanation for the observed pattern is the assumption that a contact between the Sudetic and Carpathian flora occurred in the past. Mitka et al. [62] suggested that this contact could have occurred especially for high-mountain taxa, which could easily disperse within the open landscapes that were present between the Sudetes and the Carpathians during glacial maxima. This supposition is also supported by the floristic evidence showing that several high-mountain taxa such as *Erigeron macrophyllus* Herbich, *Melampyrum herbichii* Woł., *Sesleria tatrae* (Degen) Deyl and *Thymus carpathicus* Čelak. that are present in the Carpathians, occur also in high-mountain environments of the Sudetes [66]. The connections between the Carpathian and Sudetic populations of *A.halleri* surely require further studies.

Taxonomic implications

Taxonomy of *A. halleri* is still much debated and to date three central European subspecies have been recognised: subsp. *halleri*, subsp. *tatrica* (Pawł.) Kolník, and subsp. *dacica* (Heuff.) Kolník [67]. This division is based on a morphological study published by Kolnik and Marhold [19]. Our results seem not to support this taxonomic division and/or the geographical distribution of the taxa within *A. halleri* published by Kolnik and Marhold [19] and subsequently cited by various authors [68,69]. On the basis of morphological analyses, Kolnik and Marhold [19] have recognized four European subspecies: the most widespread, *A. halleri* subsp. *halleri*, occurring in vast areas of Europe (from France and Belgium, through Germany, to Poland), *A. halleri* subsp. *tatrica* (endemic to Western Carpathians), *A. halleri* subsp. *dacica* (occurring in eastern and southern part of Carpathians) and *A.halleri* subsp. *ovirensis* (growing in only one locality in Eastern Austria). We have shown that main genetic groups identified on the basis of cpDNA variation are not fully consistent with the division proposed by Kolnik and Marhold [19], nor with the distribution of the hypothesized taxa. Our results suggest that Eastern Carpathian populations of *A. halleri* should not be classified within subsp. *halleri* as it was originally postulated by Kolnik and Marhold [19]. Our study showed that this group originates from a refugium located probably in the Balkan Peninsula and is therefore quite distant from accessions sampled north of the Alps that should probably constitute the core part of subsp. *halleri*. Our results clearly showed that populations from western Carpathians and from the Northern Carpathian Foreland share a common ancestry. The presence of this genetically quite distinct group may support the hypothesis on the existence of subsp. *tatrica* [67].

It seems to us rational to conclude that three major groups of populations defined on the basis of genetic data are present in the areas investigated by us. These groups may correspond to three subspecies: subsp. *halleri*, subsp. *tatrica* and another subspecies occurring in the eastern

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part of Carpathians. It is difficult to say whether the Eastern Carpathian populations should be classified as subsp. *dacica*, as this option was ruled out by Kolnik and Marhold in their original study (2006). It is also very unclear whether plants from these three groups differ morphologically and what morphological characters could be identified as responsible for this hypothetical differentiation. Clearly, taxonomic division of the species requires further studies as was also suggested by Hohmann et al. [67].

Conclusions

We have shown that clear phylogeographic structure is present within *A. halleri* in Central Europe. Our results suggest that the species might have not survived the last glacial maximum in Carpathians and Sudetes. It seems that the range of the species during the LGM consisted of at least two major parts: (1) a northern refugium consisting of vast refugial areas north and northeast of the Alps and (2) a southern refugium located probably in the Dinaric Alps and the Balkan Mts. From these two regions the species started to spread after LGM towards the north and east forming the current range. It seems that both the Sudetes and the Western Carpathians were colonised mainly by plants originating from the refugium located north of the Alps, though some traces of gene flow from the Eastern Carpathians are evident. Populations from the Eastern Carpathians originate from the southern refugium located in the Balkan Peninsula.

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Supporting Information

S1 Fig. Geographic origin of populations sampled.

S2 Table. Primer sequences used to obtain analysed cpDNA fragments.

S3 Table. Primer sequences used in SNaPshot assay.

S4 File. ESRI Shapefile with distribution data used in modelling experiments.

1 **Table 1. Location of sampled populations and sample sizes.**

2 n – sample size

		GPS coordinates		altitude	
Population	Locality; collectors	latitude	longitude	(m a.s.l.)	n
A05	Mutters, Alps, Northern Tyrol, Austria; MP, PSL	47°13'46,68''	11°22'46,72''	807	14
A08	W from Mehrn, Alps, Northern Tyrol, Austria; MP, PSL	47°25'10,56''	11°51'57,46''	522	45
A09	W from Mehrn, Alps, Northern Tyrol, Austria; MP, PSL	47°25'14,57''	11°51'53,71''	519	20
CZ04	SW from Vimperk, Bohemian Forest, Czech Rep.; MP, PSL	49°02'08,72''	13°45'08,41''	772	14
CZ05	N from Kubova Hut', Bohemian Forest, Czech Rep.; MP, PSL	48°59'15,54''	13°46'23,40''	998	24
CZ06	Kubova Hut', Bohemian Forest, Czech Rep.; MP, PSL	48°59'00,00''	13°46'00,00''	1060	12
CZ14	near Starý Heršťajn, Bohemian Forest, Czech Rep.; MP, PSL	49°28'37,19''	12°42'92,15''	842	20
CZ16	Horská Kvilda, Bohemian Forest, Czech Rep.; MP, PSL	49°03'21,05''	13°33'18,19''	1052	57
CZ18	NW from Zhuři, Bohemian Forest, Czech Rep.; MP, PSL	49°05'42,14''	13°32'10,31''	1039	9
CZ20	Labská, Sudetes, Czech Rep.; PW, EPW	50°42'55,9''	15°35'00,9''	698	33
CZ21	Herlikovice, Sudetes, Czech Rep.; PW, EPW	50°39'41,6''	15°35'44,5''	555	30
CZ22	Rýchorská Bouda, Sudetes, Czech Rep.; PW, EPW	50°39'29,4''	15°51'00,00''	995	32
D01	NE from Ramspau, Bohemian Forest, Germany; MP, PSL	49°10'06.40''	12°09'08.80''	345	7
D02	near Hirschling, Bohemian Forest, Germany; MP, PSL	49°11'31.00''	12°09'52.00''	452	8
D03	W from Cham, Bohemian Forest, Germany; MP, PSL	49°13'10.00''	12°39'66.00''	362	9
D04	S from Hochfeld, Bohemian Forest, Germany; MP, PSL	49°09'50.95''	12°47'45.43''	383	11
D08	S from Oker, Harz Mts., Germany; MP, PSL	51°53'47.45''	10°29'23.97''	279	12
D09	S from Glosar, Harz Mts., Germany; MP, PSL	51°53'27.55''	10°25'05.62''	325	18
D11	E from Hahnemklee, Harz Mts., Germany; MP, PSL	51°51'16.17''	10°21'56.68''	644	18
D12	SE from Lautenthal, Harz Mts., Germany; MP, PSL	51°51'54.09''	10°17'53.85''	415	18
D13	SW from Langelsheim, Harz Mts., Germany; MP, PSL	51°55'13.40''	10°18'29.68''	231	20
D14	SE from Heersum, Harz Mts., Germany; MP, PSL	52°06'08.66''	10°06'57.62''	89	11
PL02	Żyglinek, Western Carpathian Foreland, Poland; MP, PSL	50°29'40.81''	18°56'40.29''	298	21
PL03	W from Żyglinek, Western Carpathian Foreland, Poland; MP, PSL	50°29'30.34''	18°57'34.57''	302	15
PL07	Ujków Stary, Western Carpathian Foreland, MP, PSL	50°17'00.92''	19°29'03.10''	325	19
PL08	N from Chobot, Western Carpathian Foreland, Poland; MP, PSL	50°05'51.87''	20°22'32.89''	193	12
PL32	Kościelisko, Western Carpathians, Poland; AK	49°16'27.72''	19°52'45.58''	990	27
PL33	Zakopane, Western Carpathians, Poland; AK	49°17'34.02''	19°55'34.59''	879	21
PL37	Szklarska Poręba, Sudetes, Poland; PW, EPW	50°49'08.50''	15°31'24.08''	690	40
PL38	Orle, Sudetes, Poland; PW, EPW	50°49'00.59''	15°22'51.85''	828	29
PL39	E from Kowary, Sudetes, Poland; PW, EPW	50°47'23.66''	15°51'55.82''	583	39
PL40	Kowarska Pass, Sudetes, Poland; PW, EPW	50°45'37.77''	15°52'04.37''	729	39
PL41	Hala Izerska, Sudetes, Poland; PW, EPW	50°50'52.36''	15°21'45.62''	837	39
PL42	Łabski Szczyt, Sudetes, Poland; PW, EPW	50°47'14.47''	15°32'17.18''	1189	39
PL43	Mały Staw, Sudetes, Poland; PW, EPW	50°44'54.90''	15°42'09.56''	1199	39
PL44	W from Zieleniec, Sudetes, Poland; PW, EPW	50°22'54.28''	16°21'47.26''	755	41
PL45	Zawadzkie, Western Carpathian Foreland, Poland; PW, EPW	50°37'23.06''	18°26'39.06''	208	39
PL46	Sianki, Eastern Carpathians, Poland; PW, EPW	49°01'14.40''	22°53'08.40''	800	23
PL47	Roztoki Górne, Eastern Carpathians, Poland; PW, EPW	49°09'04.30''	22°19'16.90''	728	24
PL48	Krzywe, Eastern Carpathians, Poland; PW, EPW	49°11'64.00''	22°21'28.80''	647	20
PL49	Nasiczne, Eastern Carpathians, Poland; PW, EPW	49°10'22.90''	22°35'50.80''	643	22
PL50	Łężyce, Sudetes, Poland; PW, EPW	50°26'44.8''	16°20'58.3''	712	34
SK02	Vysný Klátov, Western Carpathians, Slovakia; MP, PSL	48°46'10.33''	21°07'48.49''	586	22
SK05	NE from Javorina, Western Carpathians, Slovakia; MP, PSL	49°16'59.11''	20°09'14.51''	994	46
SK10	Štrbskié Pleso, Western Carpathians, Slovakia; PW, EPW	49°07'07.26''	20°03'42.24''	1322	21
SK12	Plihov, Western Carpathians, Slovakia; PW, EPW	49°24'15.30''	20°42'08.52''	466	16
UA01	Rakhiv, Eastern Carpathians, Ukraine; PW, AR, PSL	48°01'31.60''	24°10'02.80''	434	31
UA02	Kvasy, Eastern Carpathians, Ukraine; PW, AR, PSL	48°07'59.00''	24°16'26.10''	530	32
UA04	Vil'shany, Eastern Carpathians, Ukraine; PW, AR, PSL	48°19'57.60''	23°36'22.80''	555	32
UA05	Synevyr, Eastern Carpathians, Ukraine; PW, AR, PSL	48°32'04.80''	23°38'53.50''	699	32
UA06	Synevyr Lake, Eastern Carpathians, Ukraine; PW, AR, PSL	48°37'01.20''	23°41'10.20''	1006	16
UA07	Synevyrska Poliana, Eastern Carpathians, Ukraine; PW, AR, PSL	48°36'00.60''	23°41'54.30''	829	8

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Table 2. cpDNA polymorphism observed in investigated material.

Observed mutations were numbered from 1 to 12 (numbers correspond with those given in fig. X and in tab. X)

mutation number	RFLP (PAUWELS I IN. 2005)	mutation type	methodology	Allelic variation		coding	fragment size (bp)
				base detected (SNaPshot)	polymorphism type		
1	K1K2 HpaII3	SNP	SNaPshot	A	*ATC***A**GG	1	-
				C	*ATC***T**GG	9	-
2	K1K2 AscI3	SNP	SNaPshot	T	*AGG***T**AT	1	-
				A	*AGG***A**AT	9	-
3	K1K2 Tru9I5	SNP	SNaPshot	G	*TT***G**AAT	1	-
				T	*TT***T**AAT	9	-
4	CS AluI1	SNP	SNaPshot	C	*XTAGC***C**ACTT	1	-
				T	*XTAGC***T**ACTT	9	-
5	CS HinfI4	SNP	SNaPshot	C	*XAATA***C**ACTC	1	-
				G	*XAATA***G**ACTC	9	-
6	CD AscIA	SNP	CAPS	-	*X***T**AATTT	1	908
				-	*X***A**AATTT	9	731+171
7	CD AscIB	SNP	CAPS	-	*XGAA***A**TTN	1	908
				-	*XGAA***T**TT	9	613+295
8	CD AscI4	SNP	CAPS	-	*XGAA***A**TT	1	497
				-	*XGAA***T**TT	9	351+146
9	K1K2HinfI6B	SNP	SNaPshot	T	*TTT***T**AT	1	-
				G	*TTT***G**AT	9	-
10	K1K2 HinfI5A	INDEL	SNaPshot	T	**ATATCT***TATTCTTATTG*	1	-
				A	**A---------** *TATTCTTATTC*	2	-
11	K1K2 Tru9IB	INDEL	length polymorphism	-	*X*AAA**TAACT**TTTTTGT	1	227
				-	*X*AAA --------- TTTTTGT	2	222
12	CD HinfI8A	INDEL	length polymorphism	-	*X*GGA**TTTTTTTTTTAGA**AAT	1	81
				-	*X*GGA----------AAT	2	69

Table 3. Description of cpDNA chlorotypes identified in investigated populations of A.halleri.

Characters used in coding correspond to coding column in Table 2. Correspondence to the mutations observed by Pauwels et al. (2005) in RFLP study was given. Mutation numbers corresponds with Figure 1.

Mutation number		1	2	3	4	5	6	7	8	9	10	11	12
Mutation name (cf. Pauwels et al. 2005)		K1K1 HpaII 3	K1K2 AcsI3	K1K1 Tru9I5	CS Alu I1	CS HinfI4	CD,AcsI 1A	CD AcsIB	CD AcsI4	K1K2 HinfI6B	K1K2, HinfI5A	K1K2 Tru9IB	CD HinfI8A
Chlorotype	A	9	9	9	1	1	1	1	9	1	1	1	1
	B	9	9	9	9	1	1	1	9	1	1	1	1
	C	9	9	9	1	1	1	9	9	1	1	1	1
	D	9	1	9	1	1	1	1	9	1	1	1	1
	E	1	9	9	1	1	1	1	9	1	1	1	1
	P	1	9	9	1	1	1	1	9	1	2	1	1
	F	1	9	9	1	1	1	1	9	1	1	2	1
	G	1	9	9	1	1	1	1	1	1	2	1	1
	H	1	9	1	1	9	1	1	1	1	2	1	1
	I	1	9	9	1	1	1	1	1	9	2	1	1
	J	1	9	9	1	1	9	1	1	1	2	1	2
	M	1	9	9	1	1	1	1	1	1	2	1	2

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Table 4. Chlorotype distribution among investigated populations of A.halleri and molecular diversity indices.

		Chlorotype																
Pop	n_i	A	B	C	D	Eα	E	F	G	H	I	J	K	L	M	O	a_7	H_{Sd}
A05	14	.	.	.	.	.	14	.	.	.	.	.	.	.	.	.	1.000	0.000
A08	45	.	.	.	.	.	45	.	.	.	.	.	.	.	.	.	1.000	0.000
A09	20	.	.	.	.	.	20	.	.	.	.	.	.	.	.	.	1.000	0.000
CZ04	14	.	13	.	.	.	.	.	.	.	1	.	.	.	.	.	1.759	0.143
CZ05	24	.	22	.	.	.	.	2	.	.	.	.	.	.	.	.	1.762	0.159
CZ06	12	.	12	.	.	.	.	.	.	.	.	.	.	.	.	.	1.000	0.000
CZ14	20	.	.	.	.	.	.	.	.	.	.	20	.	.	.	.	1.000	0.000
CZ16	57	.	.	.	.	.	56	.	.	.	.	1	.	.	.	.	1.231	0.035
CZ18	9	.	.	.	.	.	9	.	.	.	.	.	.	.	.	.	1.000	0.000
CZ20	33	.	.	.	.	.	.	.	.	.	11	22	.	.	.	.	1.999	0.458
CZ21	30	.	.	.	.	.	.	.	.	.	24	6	.	.	.	.	1.972	0.331
CZ22	32	.	.	.	.	.	.	.	.	.	.	32	.	.	.	.	1.000	0.000
D01	7	.	.	2	.	.	4	.	.	.	.	1	.	.	.	.	3.000	0.667
D02	8	2		2	.	.	1	.	.	1	.	2	.	.	.	.	4.983	0.893
D03	9	2	.	.	.	.	6	.	.	1	.	.	.	.	.	.	2.960	0.556
D04	11	5	.	.	.	.	2	.	.	4	.	.	.	.	.	.	2.990	0.691
D08	12	1	.	.	10	.	1	.	.	.	.	.	.	.	.	.	2.674	0.318
D09	18	3	.	.	15	.	.	.	.	.	.	.	.	.	.	.	1.962	0.294
D11	18	5	.	.	9	.	4	.	.	.	.	.	.	.	.	.	2.987	0.660
D12	18	4	.	.	6	.	8	.	.	.	.	.	.	.	.	.	2.989	0.680
D13	20	.	.	.	17	.	3	.	.	.	.	.	.	.	.	.	1.940	0.268
D14	11	1	.	.	10	.	.	.	.	.	.	.	.	.	.	.	1.879	0.182
PL02	21	.	.	.	.	.	.	14	.	.	.	7	.	.	.	.	1.999	0.467
PL03	15	.	.	.	.	.	.	13	.	.	.	2	.	.	.	.	1.934	0.248
PL07	19	.	.	.	.	.	4	15	.	.	.	.	.	.	.	.	1.985	0.351
PL08	12	.	.	.	.	.	.	5	.	.	.	7	.	.	.	.	2.000	0.530
PL32	27	.	.	.	.	.	11	9	.	.	7	.	.	.	.	.	2.992	0.681
PL33	21	.	.	.	.	.	.	7	.	.	14	.	.	.	.	.	1.999	0.467
PL37	40	.	.	.	.	.	.	.	.	.	.	40	.	.	.	.	1.000	0.000
PL38	29	.	.	.	.	.	.	.	.	.	.	29	.	.	.	.	1.000	0.000
PL39	39	.	.	.	.	.	.	.	.	.	.	39	.	.	.	.	1.000	0.000
PL40	39	.	.	.	.	.	.	.	.	.	.	39	.	.	.	.	1.000	0.000
PL41	39	.	.	.	.	.	.	.	.	.	1	38	.	.	.	.	1.329	0.051
PL42	39	.	.	.	.	.	.	.	.	.	28	11	.	.	.	.	1.994	0.416
PL43	39	.	.	.	.	.	.	.	.	.	.	39	.	.	.	.	1.000	0.000
PL44	41	14	.	27	.	.	.	.	.	.	.	.	.	.	.	.	1.999	0.461
PL45	39	.	.	.	.	.	2	36	.	.	.	1	.	.	.	.	1.883	0.148
PL46	23	.	.	.	.		.	.	23	.	.	.	.	.	.	.	1.000	0.000
PL47	24	.	.	.	.	4	.	.	20	.	.	.	.	.	.	.	1.952	0.290
PL48	20	.	.	.	.		.	.	20	.	.	.	.	.	.	.	1.000	0.000
PL49	22	.	.	.	.	2	.	.	20	.	.	.	.	.	.	.	1.798	0.173
PL50	34	.	.	34	.	.	.	.	.	.	.	.	.	.	.	.	1.000	0.000
SK02	22	.	.	.	.	.	4	18	.	.	.	.	.	.	.	.	1.967	0.312
SK05	46	.	.	.	.	.	.	19	1	.	25	1	.	.	.	.	2.565	0.545
SK10	21	.	.	.	.	7	2	11	.	.	.	1	.	.	.	.	3.377	0.633
SK12	16	.	.	.	.	7	2	7	.	.	.	.	.	.	.	.	2.915	0.642
UA01	31	.	.	.	.	21	.	.	10	.	.	.	.	.	.	.	1.998	0.452
UA02	32	.	.	.	.	30	.	.	2	.	.	.	.	.	.	.	1.638	0.121
UA04	32	.	.	.	.	5	.	.	27	.	.	.	.	.	.	.	1.932	0.272
UA05	32	.	.	.	.	6	.	.	14	.	.	.	.	.	12	.	2.962	0.653
UA06	16	.	.	.	.	1	.	.	12	.	.	.	.	.	3	.	2.671	0.425
UA07	8	.	.	.	.	.	.	.	6	.	.	.	.	.	2	.	2.000	0.429
Total	1280	37	47	65	67	83	198	156	155	6	111	338	0	0	17	0	6.927	-

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Table 5. Comparison of within-population diversity indices among different geographical regions.

n – number of populations in a region, ASc - chlorotypic richnes , HSd - chlorotype diversity index

geographical group	n	A_{Sc}	H_{Sd}
Western Carpathians	5	2.636	0.547
Eastern Carpathians	11	1.895	0.285
Sudetes	12	1.358	0.143
Harz	6	2.405	0.420
Alps	3	1.000	0.000
Bohemian Forest	10	2.169	0.181
N Carpathian Foreland	5	1.960	0.303

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Table 6. Results of AMOVA analysis.

All results were significant with $\alpha=0.05$. Significance test were carried out using permutaion test (10100 permutations).

	Source of variation	d.f.	Sum of squares	Variance components	Precentage of variation
All populations	Among populations	51	1329.890	1.09838	79.16
	Within populations	1233	356.479	0.28912	20.84
Western Carpathians	Among populations	5	33.292	0.23835	23.95
	Within populations	147	111.244	0.75676	76.05
Eastern Carpathians	Among populations	9	32.980	0.14726	46.44
	Within populations	230	39.066	0.16985	53.56
Sudetes	Among populations	11	408.052	1.01023	85.66
	Within populations	427	72.235	0.16917	14.34
Harz	Among populations	5	6.934	0.06793	18.55
	Within populations	91	27.148	0.29833	81.45
Alps	Among populations	2	0.000	0.00000	0.00
	Within populations	76	0.000	0.00000	0.00
Bohemian Forest	Among populations	9	133.130	0.91778	74.57
	Within populations	161	50.379	0.31291	25.43
N Carpathian Foreland	Among populations	4	18.696	0.20423	26.78
	Within populations	101	56.408	0.55849	73.22
k-means clustering (6)	Among groups	5	1102.418	1.03618	65.82
	Among populations	46	290.472	0.24899	15.82
	Within populations	1233	356.479	0.28912	18.36
Geographic location (7)	Among groups	6	759.806	0.64481	42.88
	Among populations	45	633.085	0.56992	37.90
	Within populations	1233	356.749	0.28912	19.23

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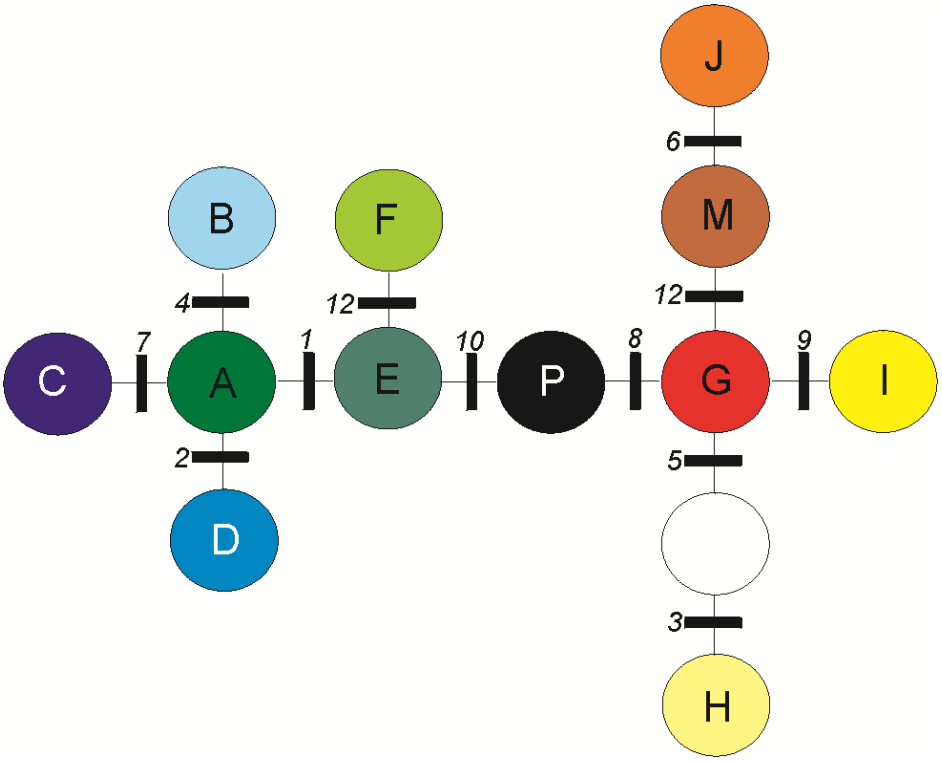

Fig 1. Minimum spanning tree (MST) presenting relationships between cpDNA haplotypes in A. halleri. Coloured circles represent haplotypes, white circle represent missing haplotype. Numbers indicate mutations as given in Table 1.

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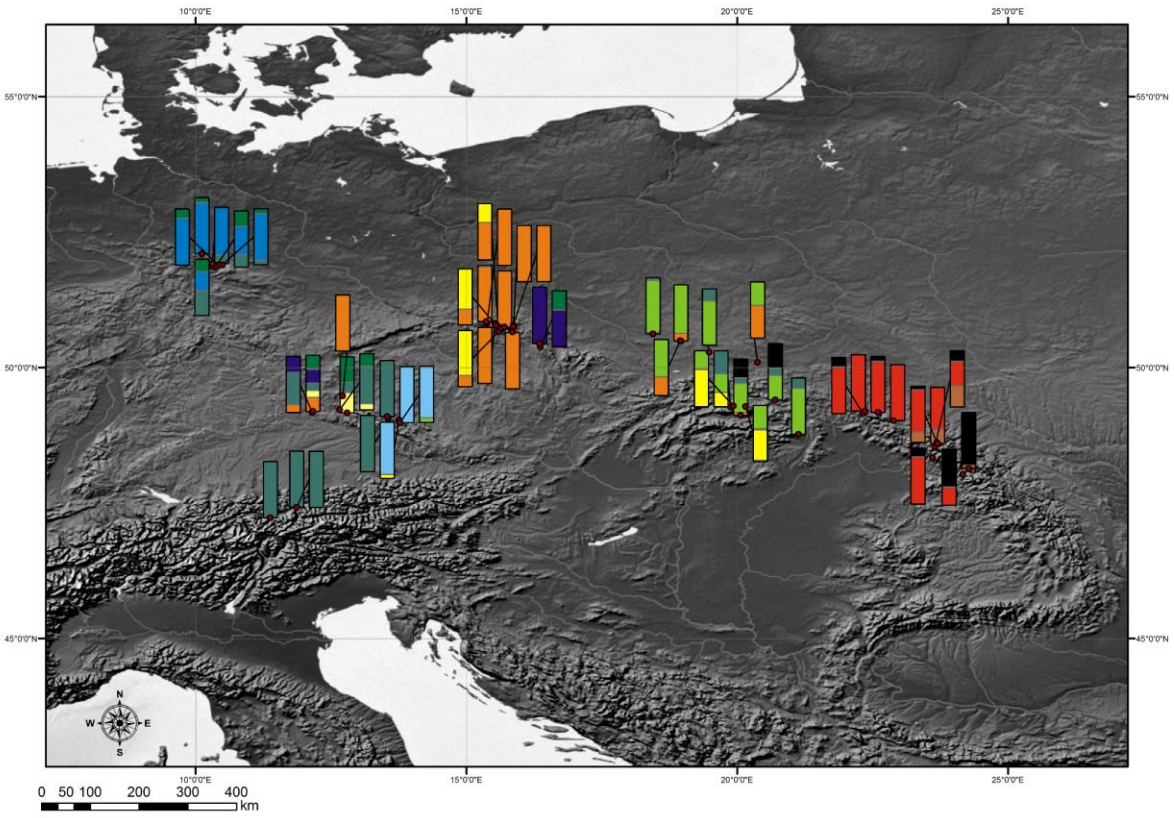

Fig 2. Geographic distribution of cpDNA haplotypes present in the investigated populations of A. halleri. Bar charts represent the frequency of each haplotype in each investigated population.

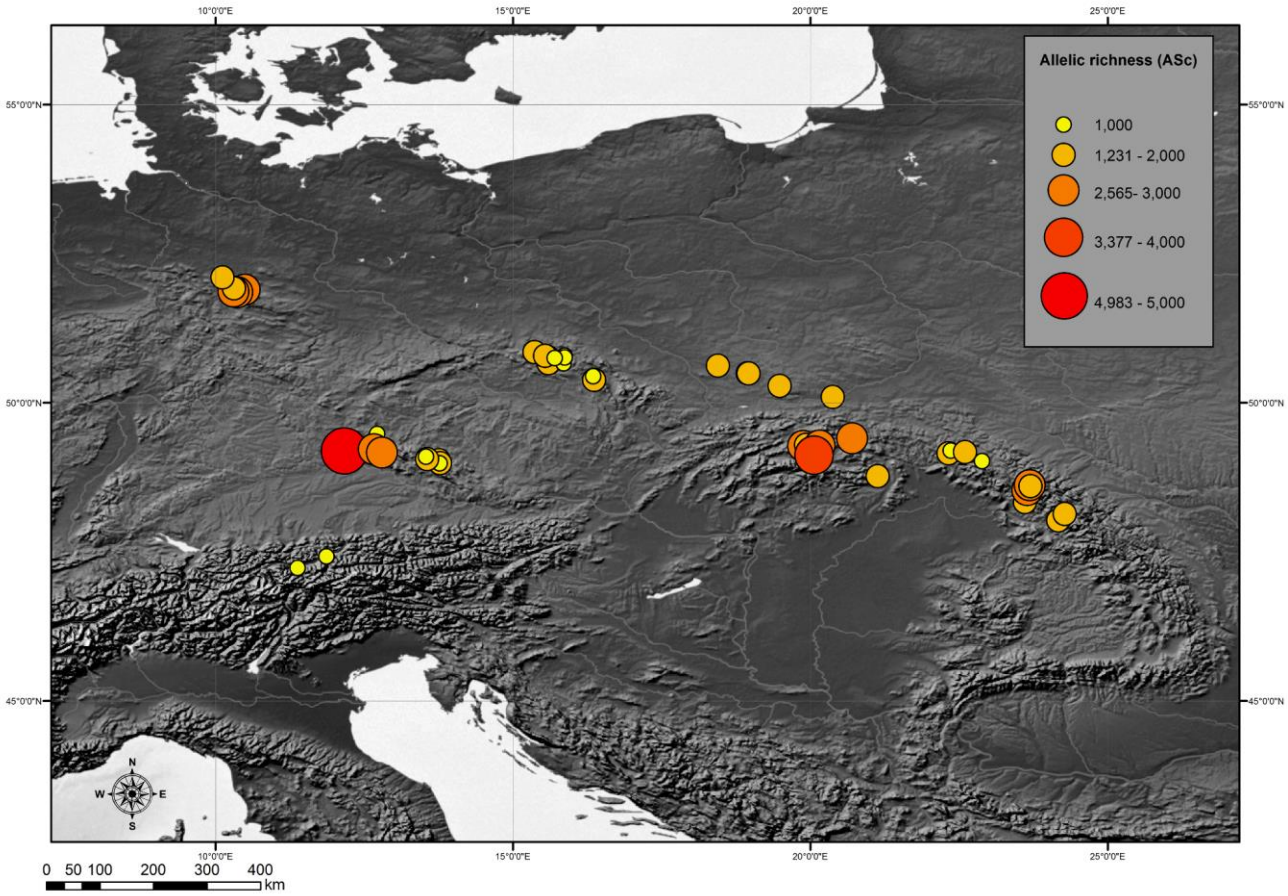

Fig 3. Geographic distribution of within-population cpDNA allelic richness (ASc) in the populations investigated.

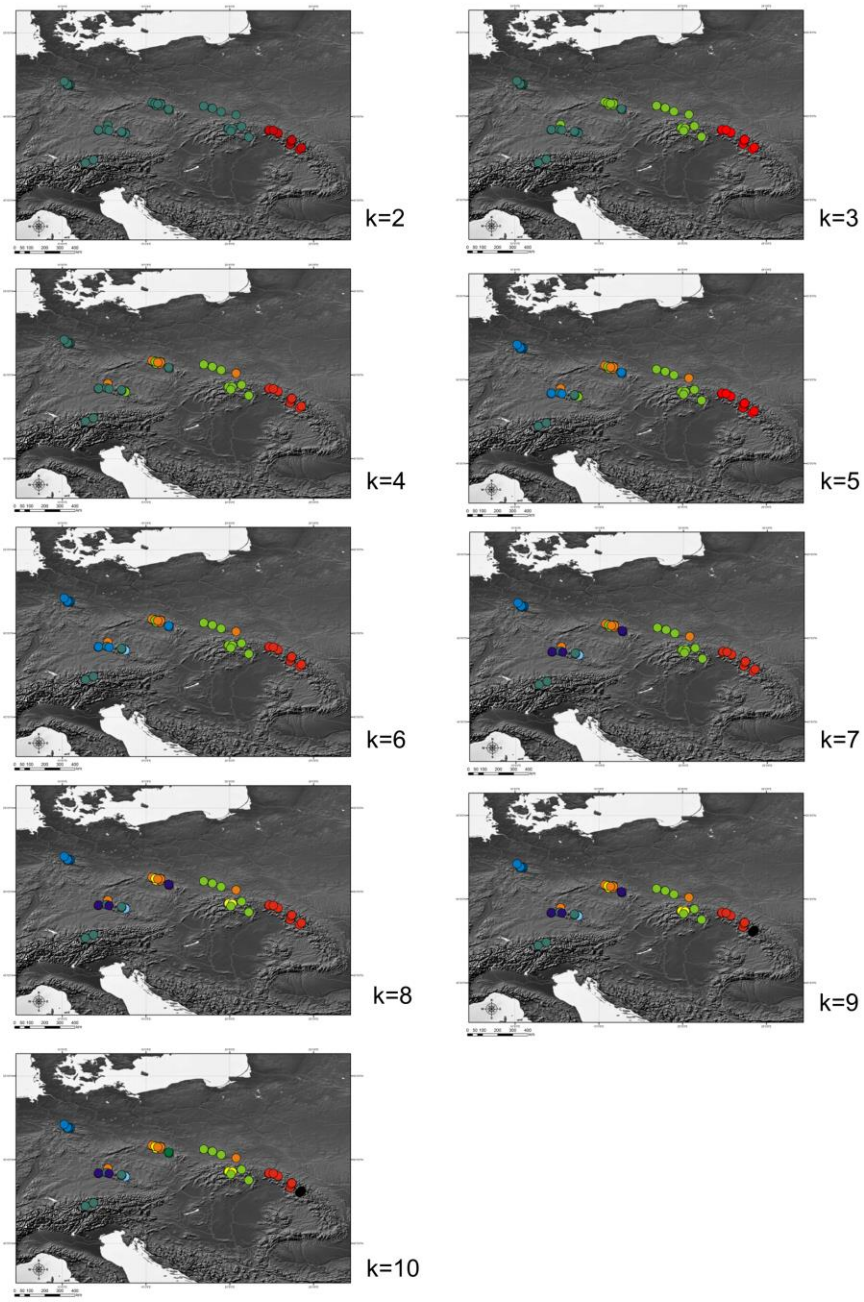

Fig 4. Results of spherical k-means clustering (SKMC). SKMC of investigated populations carried out using Shperikm [30]. Different levels of data structure are presented from k=2 (two groups) to k=10 (ten groups of populations). Population differentiation was inferred from a data matrix of chlorotype frequencies.

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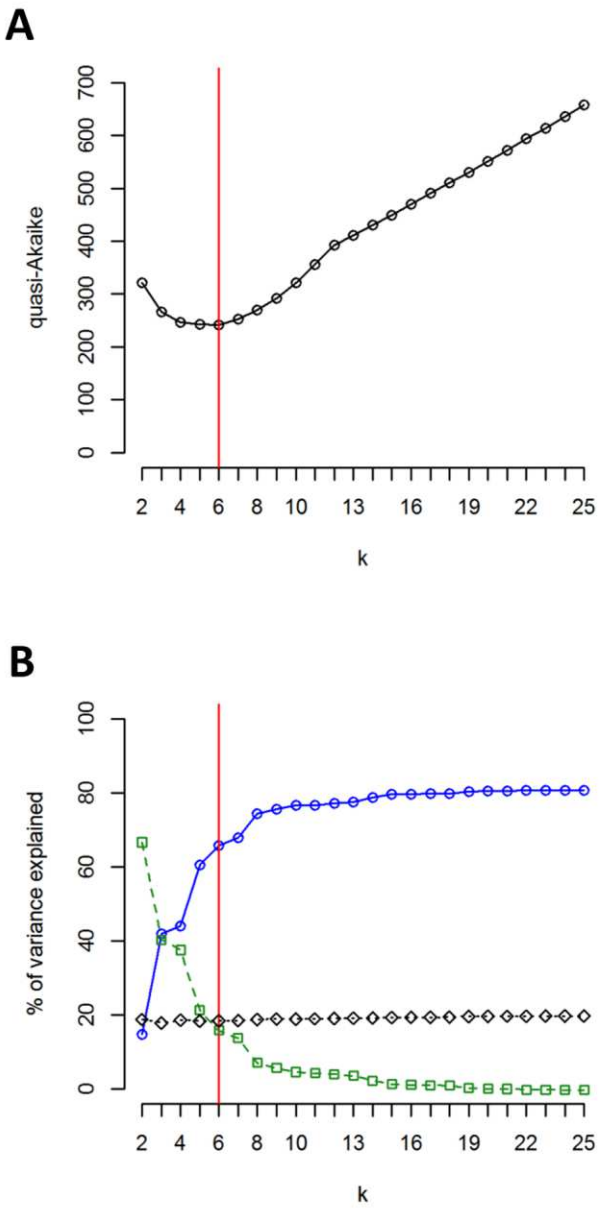

Fig 5. Results of SKMC and AMOVA. (A)Value of the quasi-Akaike information criterion as a function of k (number of groups identified by SKMC analysis). Statistically optimal solution (having the lowest value of the quasi-Akaike criterion) is marked with a red line. (B) Percent of genetic variance among groups of populations identified by SKMC (blue), among populations within groups (green) and within populations (black) calculated by AMOVA. Statistically optimal solution of SKMC is marked with a red line.

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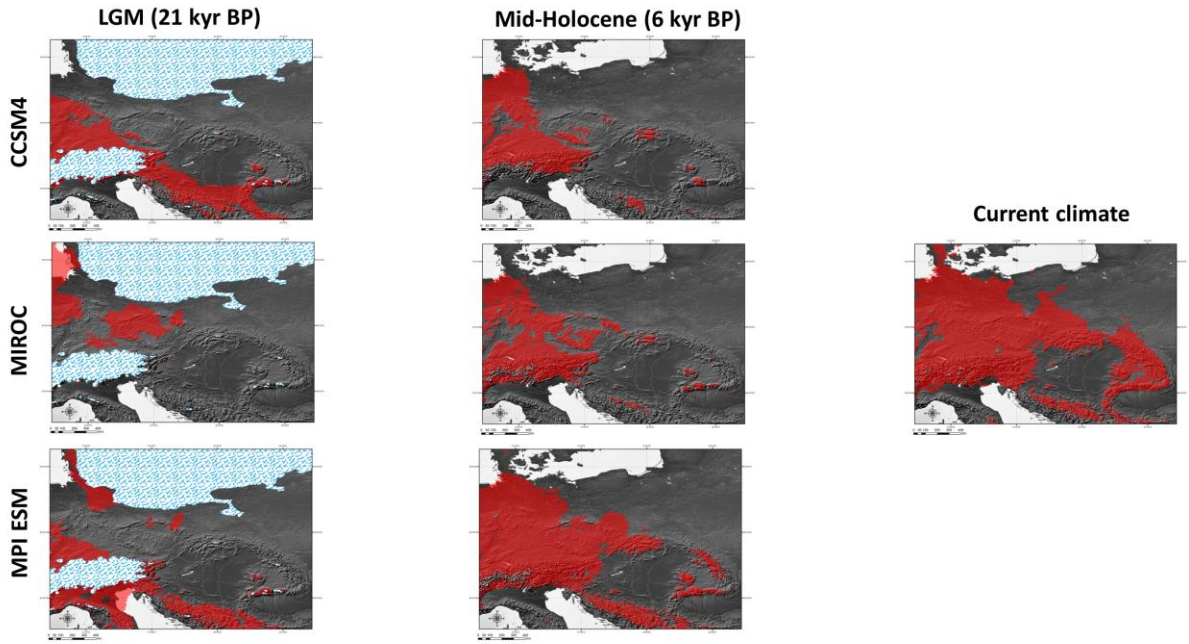

Fig 6. Results of modelling experiments. Binary maps of distributions based on the results of ensemble models (mean of probabilities) are shown. Results are based on the data from three different paleoclimate models: CCSM4, MIROC and MPI ESM, as well as current climate observations. Species range was reconstructed for two time periods: Last Glacial Maximum (LGM, ca. 21 kyr BP) and Mid-Holocene (ca. 6 kyr BP).

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