ORIGINAL RESEARCH PAPER

Could clonality contribute to the northern survival of grey alder [*Alnus incana* (L.) Moench] during the Last Glacial Maximum?

Monika Dering[1]*, Małgorzata Latałowa[2], Krystyna Boratyńska[1],
Piotr Kosiński[1,3], Adam Boratyński[1]

[1] Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland
[2] Laboratory of Palaeoecology and Archaeobotany, Department of Plant Ecology, University of
Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland
[3] Department of Botany, Faculty of Horticulture and Landscape Architecture, Poznań University of
Life Sciences, Wojska Polskiego 71c, 60-625 Poznań, Poland

* Corresponding author. Email: mdering@man.poznan.pl

Abstract

Reconstruction of the glacial and postglacial history of a species, including life-
history traits, provides valuable insights into the relationships between ecologi-
cal and genetic factors shaping phylogeographic patterns. Clonality appears as a
trait of high importance for survival in northern refugia. In the present study, the
phylogeographic structure of 24 *Alnus incana* (grey alder) populations and clonal
structure in seven populations were analyzed utilizing four microsatellites mark-
ers. Palaeobotanical data were collected and combined with the genetic results in
order to support the possibility that this species survived in northern refugia. Our
study indicated that: (*i*) Balkan populations are the most divergent, which likely
reflects their long-term in-situ existence, (*ii*) Western Alpine populations are ge-
netically different from other European populations, which corresponds with pal-
aeobotanical data, suggesting that this region served as a refugium for this species,
(*iii*) the macrofossil data indicate that the Scandinavian and northeastern Polish
populations are likely derived from the refugia located in northern latitudes, (*iv*)
Western and Eastern Carpathian populations form separate groups, which indi-
cate that both regions could serve as refugia. Clonality was an important factor
in allowing grey alder to survive in northern regions during the last glaciation.
However, this mode of reproduction has also influenced the population genetic
structure, as we noted rather low level of gene diversity, $H_E = 0.386$ and low allelic
variability, $A = 3.8$, in this species.

Keywords

clonality; genetic diversity; grey alder; macrofossils; northern refugia

Introduction

In the recent past, it was generally assumed that during the Last Glacial Maximum
(LGM; 18–25 ka), suitable environmental conditions for tree species survival were
restricted to the refugia located on the Balkan, Iberian, and Italian Peninsulas [1]. An
increasing number of phylogeographic studies have made it more evident that the
premise of southern refugia and their key role in the postglacial history of trees is an
oversimplification. For example, some species had the highest level of genetic diver-
sity and allelic richness in the middle or northern latitudes, rather than in southern
refugia as assumed by the established paradigm of the pattern of postglacial coloniza-
tion [2]. Additionally, macrofossils of the boreal trees such as *Picea abies* (L.) Karst.

(Norway spruce), *Pinus sylvestris* L. (Scots pine), and *Betula pendula* Roth (silver birch), as well as some mesophilous *Quercus* spp. (oaks) or *Fagus sylvatica* L. (common beech) suggest the persistence of tree stands close to the ice-sheet margins [3]. Currently, the theory of Steward and Lister [4] on the role of cryptic northern refugia located in microenvironmentally favorable sites explains much of the observed distribution and genetic diversity of tree species.

Bhagwat and Willis [5] found that tree species that likely survived in northern micro-refugia were wind-dispersed, small-seeded, and habitat-generalist. Additionally, all those species were capable of clonal growth. Clonal reproduction can be considered as an alternative option in a life cycle in a situation when the sexual reproduction is reduced or absent due to environmental conditions and may have profound influence on spatial genetic structure [6]. Clonal plant species dominate in the composition of plant communities of harsh environments, e.g., high latitudes and altitudes [7]. The long-term survival of the Australian shrub, *Erythroxylum pusillum* Clarkson, was possible due to clonality [8]. Population separated from the range core by the Gulf of Carpentaria (ca. 500 km) is derived from a single ancestral genetic lineage established ca. 12 000 years ago. Another example comes from the Hawaiian population of *Sphagnum palustre* L., which was probably founded by a single founding episode ca. 49–54 ka ago and clonal reproduction is the sole means of population growth and persistence [9]. Also, in the early successional phase of modern glacier forelands, after initial seed recruitment, clonality is the major mode of reproduction and colonization [10]. Öberg and Kullman [11] have stated, that the ability of Norway spruce to clonal reproduction was the major factor that enabled and enhanced the survival of this species during the early immigration phase into the Scandes at the very beginning of the Holocene.

Alnus incana (L.) Moench (grey alder) is a light-demanding, cold-tolerant, anemophilous and anemochoric tree species. Its natural range extends from the Fennoscandia, throughout the northern part of the Eastern European Plain, the Alps, the mountains of Central Europe, the Caucasus, and as far as to the Ural Mountains [12] (Fig. 1). It has been reported that grey alder from tree-limit and forest-limit in the Scandes reproduces only but extensively by root shoots and epicormic stem shoots [13]. Palaeobotanical studies on the genus *Alnus* support the idea of northern refugia formed by the species [14]. Recent reconstruction of the postglacial history of grey alder made with genetic markers indicated that the species was able to survive beyond the classic southern refugia in areas of Central Europe [15]. Those populations were the main source for subsequent colonization of Fennoscandia and Eastern Europe. We assumed that the ability for clonal reproduction could largely facilitate the northern survival of *A. incana* during the LGM. Using nuclear microsatellite markers we aimed to analyze the phylogeographic pattern of the species in relation to the clonal structure of populations. Next, to test the obtained phylogeographic pattern we combined genetic results with palaeobotanical data collected in published sources.

Material and methods

Sampling and genetic analysis

Grey alder was sampled in 24 locations covering the Central European part of the species range from the Scandinavian Peninsula to the Balkans (Tab. 1, Fig. 1). Leaves were collected from 28–32 individuals per population. In total, 721 individuals were subjected to genetic analysis. The clonal structure was investigated in seven populations: Bulgaria, Balkans, (B_1), the Austrian Alps (A_1), the French Alps (A_2), the Italian Alps (A_3), the Scandinavian Peninsula, Finland (S_2, S_3), and the Scandinavian Peninsula, Norway (S_6). Grey alder in those localities formed homogenous groves/thickets along the streams and the ramets were sampled along a transect. Within the transect, each ramet was separated from another by ca. 10 m and the transect was expanded until the number of ramets approached 30.

DNA was extracted from leaves using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A total of 10 nuclear microsatellite markers (nSSR) from *Betula pendula*
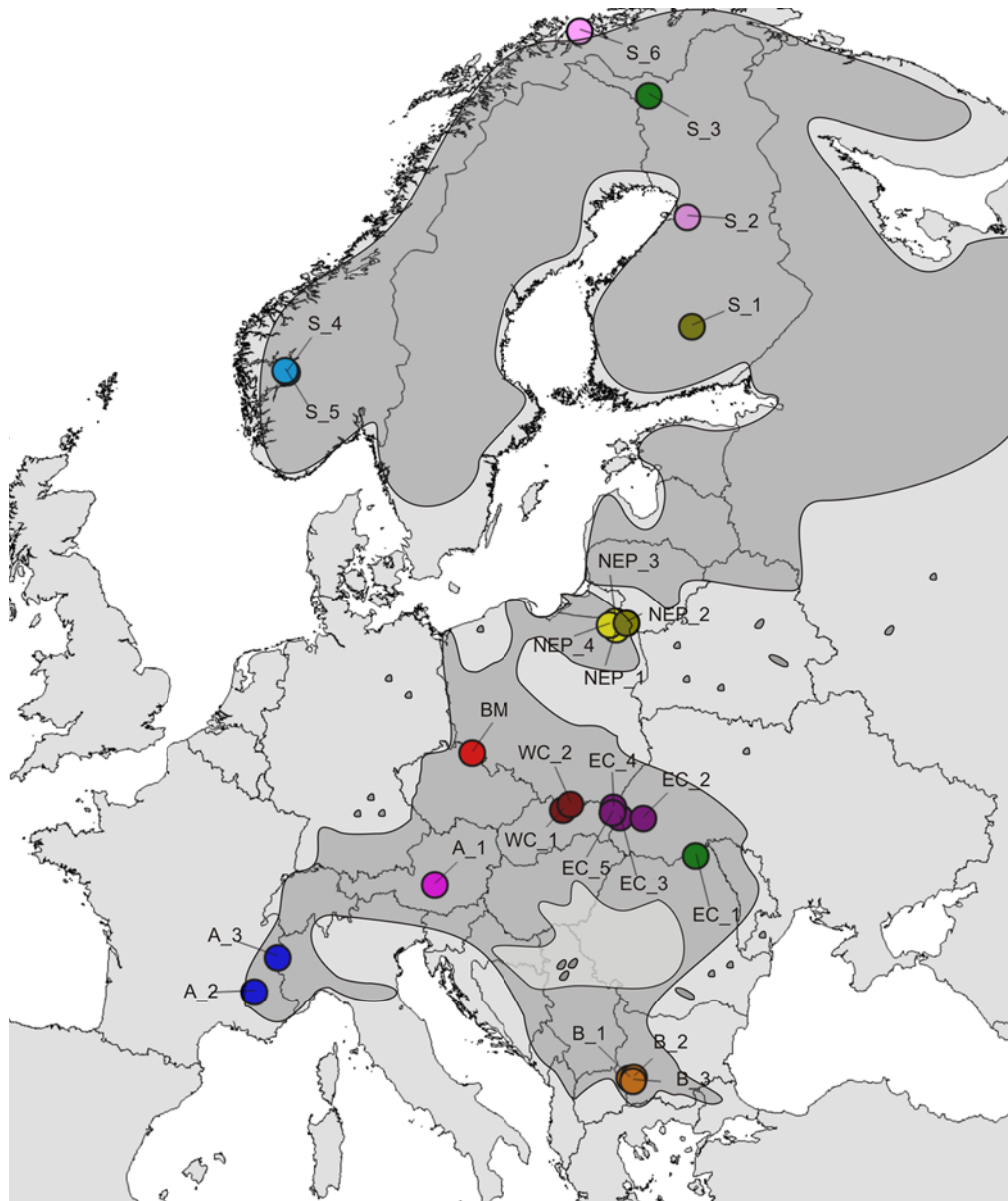
Fig. 1 Geographic location and Bayesian clustering of the 24 *Alnus incana* populations analyzed in the current study; abbreviations of populations names are in Tab. 1.

were tested in *A. incana*: L1.10, L2.2, L2.3, L2.7, L7.3, L13.1, L3.1. L5.4, L5.5, and L022 [16]. Only four primer sets for the microsatellites: L3.1, L5.4, L5.5, and L022, were selected for use in the analysis of *A. incana* as they gave repeatable, high quality amplification products (Tab. 2). nSSR were amplified in a single reaction with a Multiplex PCR Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. PCR products labeled with different fluorescent dyes (6-FAM, VIC, PET, and NED) were loaded on the 3130 Genetic Analyzer (Applied Biosystems, USA) and analyzed with internal size standard, GeneScan-LIZ 500. Fragments were scored with GeneMapper v. 4.0 software (Applied Biosystems).

Genetic diversity, differentiation, and clonality

Genetic diversity measured as expected heterozygosity (H_E), observed heterozygosity (H_O), and average number of alleles per locus (A) were estimated in Fstat v. 2.9.3.2 [17]. Null allele frequencies were obtained following the expectation–maximization (EM) algorithm using software INEst 1.0 [18]. Conformity to Hardy–Weinberg (H–W) genotypic proportions was tested using the exact test in GenePop v. 4.0 [19].

Tab. 1 Location and parameters of genetic variability of 24 *Alnus incana* populations analyzed in this study.

Population name and acronym	Voucher	Longitude	Latitude	Altitude (m a.s.l.)	n	A	H_O	H_E	F_{IS}	F_{IS} (null)	Null
Balkans, Rila, Beli Iskar, Bulgaria (B_1)	KOR 45516	23°08'	42°07'	1150	30	3.50	0.392	0.619	0.367***	0.197	0.173
Balkans, Rila, Bulgaria, Rilsky Monastyr (B_2)	KOR 47348	23°21'	42°08'	1180	32	3.75	0.469	0.516	0.091	0.025	0.071
Balkans, Pirin, Blagoevgrad, Bulgaria (B_3)	KOR 11673	23°17'	42°02'	990	31	4.25	0.540	0.549	0.016	0.022	0.063
Alps, Niedere Tauern, Solkpass, Austria (A_1)	KOR 49946	14°04'	47°16'	1500	28	2.75	0.339	0.348	0.025*	0.030	0.083
Alps, Sauveur, France (A_2)	KOR 49956	05°39'	44°25'	750	30	2.25	0.450	0.388	−0.159	0.014	0.062
Alps, Beaulard, Italy (A_3)	KOR 45725	06°45'	45°02'	1150	30	2.50	0.367	0.382	0.041	0.036	0.078
Eastern Carpathians, Chernistvi, Ukraine (EC_1)	KOR 49950	26°12'	48°03'	273	30	3.25	0.350	0.380	0.078	0.038	0.080
Eastern Carpathians, Bolekhiv, Ukraine (EC_2)	KOR 49951	23°46'	49°03'	370	30	3.25	0.425	0.433	0.020	0.028	0.072
Eastern Carpathians, Poland, Bieszczady Mts (EC_3)	KOR 49949	22°40'	49°04'	710	30	4.25	0.425	0.433	0.018	0.025	0.067
Eastern Carpathians, Poland, Bieszczady Mts (EC_4)	KOR 49947	22°24'	49°20'	440	30	3.25	0.408	0.425	0.040*	0.023	0.085
Eastern Carpathians, Poland, Bieszczady Mts (EC_5)	KOR 49957	22°20'	49°11'	549	30	3.25	0.400	0.422	0.051	0.025	0.072
Western Carpathians, Tatra Mts, Poland (WC_1)	KOR 4344	20°03'	49°15'	1000	30	3.25	0.342	0.311	−0.099	0.020	0.077
Western Carpathians, Pieniny Mts, Poland (WC_2)	KOR 0046	20°22'	49°24'	850	30	3.75	0.300	0.340	0.117***	0.022	0.097
Bohemian Massif, Sudetes, Giant Mts, Poland (BM)	KOR 49948	15°48'	50°46'	800	30	4.00	0.433	0.507	0.145*	0.027	0.095
Northern European Plain, Olecko Wielkie, Poland (NEP_1)	KOR 49953	22°31'	54°03'	200	30	3.50	0.375	0.378	0.007	0.018	0.071
Northern European Plain, Maków, Poland (NEP_2)	KOR 49955	22°41'	54°19'	222	30	3.50	0.425	0.344	−0.235	0.011	0.058
Northern European Plain, Jedrzejow, Poland (NEP_3)	KOR 49954	22°29'	54°17'	220	30	3.00	0.325	0.307	−0.058	0.022	0.073
Northern European Plain, Czerwony Dwor, Poland (NEP_4)	KOR 49952	22°13'	54°11'	140	30	3.00	0.313	0.339	0.076	0.037	0.082
Scandinavian Peninsula, Taipole, Finland (S_1)	KOR 46386	25°44'	62°12'	90	30	3.50	0.408	0.386	−0.058	0.018	0.065
Scandinavian Peninsula, Oulu, Finland (S_2)	KOR 49958	25°49'	65°00'	100	30	3.00	0.258	0.242	−0.066	0.032	0.087
Scandinavian Peninsula, Ketomella, Finland (S_3)	KOR 46350	24°02'	68°16'	260	30	3.00	0.333	0.358	0.069	0.025	0.085

Tab. 1 Continued

Population name and acronym	Voucher	Longitude	Latitude	Altitude (m a.s.l.)	N	A	H_O	H_E	F_{IS}	F_{IS} (null)	Null
Scandinavian Peninsula, Aurlansdalen, Norway (S_4)	KOR 44949	07°12'	60°54'	100	30	3.25	0.250	0.253	0.011*	0.025	0.086
Scandinavian Peninsula, Udredalen, Norway (S_5)	KOR 44950	07°06'	60°57'	300	30	3.25	0.267	0.316	0.156***	0.043	0.095
Scandinavian Peninsula, Birtavarre, Norway (S_6)	KOR 46307	20°49'	69°29'	30	30	3.25	0.300	0.289	−0.037	0.024	0.071
Average						3.31	0.371	0.386	0.257***	0.033	0.081

n – No. of individuals; A – average number of alleles per locus; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient and significance at: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); F_{IS} (null) – inbreeding coefficient calculated in FREENA with INA correction; Null – frequency of null alleles averaged across four loci.

The multilocus inbreeding coefficient F_{IS} was evaluated using Fstat, and INEst was used to calculate the corrected value of F_{IS} which considers null alleles.

A multilocus unbiased estimate of Wright's fixation index (F_{ST}) was calculated as a measure of genetic differentiation in Fstat [20]. Null alleles may lead to overestimation of F_{ST}, thus the global F_{ST} of Weir using the ENA correction [21] was calculated in FreeNA. Pairwise genetic differentiation (F_{ST}) between all pairs of populations was calculated using Arlequin 3.5 and the significance of the differentiation was tested using 3000 permutations [22].

Clonal structure in selected sites was assessed by genotype diversity according to the formula $R = (G − 1)/(N − 1)$ [23], where G is the number of total genotypes detected and N is the number of individual ramets collected along a transect. GenAlEx 6.5 software was used for inferring the clonal structure, that is, the number of unique multilocus genotypes, the number of multiplied genotypes, and the probability of the identical genotype arising in the result of independent sexual reproduction (p_{gen}) [24].

Geographic structure

Principal coordinate analysis (PCoA) was performed using GenAlEx. Changes in the diversity parameters values within populations were estimated by linear regressions for H_E, A, and F_{IS} with latitude, longitude, and altitude (Statistica v.11). Bayesian methods implemented in Geneland v. 2.13.1 were used to investigate geographic structure [25]. Georeferenced genotypes were used as prior information in the estimation procedure which included 100 independent runs, 200 000 iterations each, saving every 50th with a K set from 1 to 25. Owing to the presence of null alleles, a null allele model was used. The optimum number of K for the dataset is displayed by Geneland as the modal value along the MCMC chain. Analysis of molecular variance (AMOVA) for groups defined by Geneland was performed in Arlequin and significance was verified using 3000 permutations [22].

Collection of palaeobotanical data

In palaeobotanical analysis we considered mainly macrofossils that have more reliable taxonomical resolution than pollen data [26], which is especially crucial in case of alders. The species determination of pollen within alders is not routine, although possible [27]. However, separation of the pollen of *A. incana* and *A. glutinosa* (*A. glutinosa* / *A. incana*-type) is very difficult and is practiced only by some authors [28].

The *A. incana* / *Alnus* sp. palaeobotanical dataset was restricted chronologically to the last full glacial (including LGM), LG (Late Glacial) and early Holocene records derived from published literature and from the publicly available Eurasian Macrofossil Database at the University of Oxford [29]. If there were several records of *A. incana* / *Alnus* sp. for a site, only the oldest one was included in the dataset and shown on the generated map. Due to limited access and problems with exploring original publications used by Tallantire for a map of the distribution of alders fossil remains in Fennoscandia, these data were not included in our map [30]. Those who are interested may refer to his paper.

Three different categories of data were collected to produce a map illustrating the distribution of fossil records: 1 – macrofossils specifically determined as *A. incana*, 2 – macrofossils determined as *Alnus* sp., 3 – selected pollen records of *A. glutinosa* / *A. incana*-type. Categories 2 and 3 are less confident in representing *A. incana* because they may be related to *A. glutinosa* as well. Macrofossils determined as *Alnus* sp. are mostly wood

Tab. 2 Characteristic of four microsatellite loci used in the study of the genetic structure of *Alnus incana* populations in Europe.

Locus	Allele size range	N alleles	H_O	H_E	F_{IS}	F_{ST}	F_{ST} (ENA)	Null
L3.1	219–237	9	0.348	0.393	0.110***	0.053	0.052	0.094
L5.4	236–242	4	0.209	0.244	0.106*	0.119	0.128	0.098
L5.5	118–130	7	0.674	0.670	−0.008	0.081	0.080	0.055
L022	178–202	5	0.251	0.237	−0.056	0.102	0.101	0.078
Average		6.25	0.370	0.386	0.066	0.0836	0.0843	0.081

H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient and significance at: $p < 0.05$ (*), $p < 0.001$ (***); F_{ST} – Weir and Cockerham [20] and F_{ST} (ENA) calculated in FREENA with ENA correction (Chapuis and Estoup [21]); Null – frequency of null alleles.

or charcoal fragments which are not identifiable to the species level [31]. A small number of the records were fruits, fruit scales, cones, or other remains that are badly preserved and thus devoid of diagnostic features. This category of data was included in our study, especially if *A. incana* was proposed as the most probable species by the author reporting on a site.

We used some selected pollen records of *A. glutinosa* / *A. incana*-type. We chose only those stands where the author of the data attributed them specifically to the local presence of *A. incana* supporting such statements by well-substantiated arguments.

Results

Genetic diversity and differentiation

Tests for linkage disequilibrium between pairs of microsatellite loci in each population did not indicate significant relationships. Genetic variation at nSSR loci was low with the average expected heterozygosity (H_E) 0.386 and only 6.25 alleles per locus (Tab. 2). Twenty-five different alleles were noted in total for 721 individuals. The highest gene diversity was reported for locus L5.5 ($H_E = 0.670$) and lowest for locus L022 ($H_E = 0.237$). The number of alleles per locus ranged from 9 to 4 in Locus L3.1 and Locus L5.4, respectively. In case of two loci, L3.1 and L5.4, significant departure from Hardy–Weinberg genotypic proportions was noted. However, these two loci were affected by the null alleles at most (Tab. 2). Highest population differentiation was noted for the Locus L5.4 ($F_{ST} = 0.119$) and lowest for L3.1 ($F_{ST} = 0.053$).

Gene diversity (H_E) and number of alleles per locus (A) exhibited high variation among the studied populations. H_E ranged from 0.619 in Population B_1 (Balkans, Rila) to 0.242 in Population S_2 (Scandinavian Peninsula, Finland), and A from 4.25 in B_3 (Balkans, Pirin Mts) and EC_3 (Eastern Carpathians, Bieszczady Mts) to 2.25 in A_2 (Alps, France) (Tab. 1). The substantial frequency of null alleles and generally high values for the inbreeding coefficient (F_{IS}) were very evident. The corrected estimation of F_{IS} in INEst, however, resulted in a decrease in the value of F_{IS} in most of the populations (Tab. 1).

The overall level of genetic differentiation, based on sequence variation at nSSR loci, was low but highly significant ($F_{ST} = 0.0836$; $p < 0.001$). The majority of the pairwise F_{ST} were also significantly greater than zero (data not shown). The highest value of pairwise F_{ST} was 0.3246 and it was found in the population from Rila, Balkans (B_2) and the population from the western coast of Norway (S_4); the lowest differentiation was noted between two Finnish populations (S_2 and S_3). A high level of genetic differentiation was also observed between most of the populations from the Central and Northern Europe vs. Western Alpine populations, as well as between Balkan populations and the remaining stands in Europe.
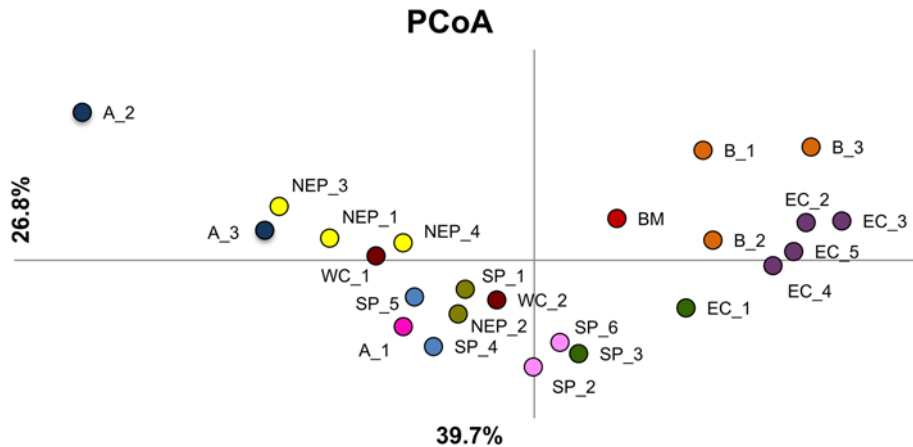
Fig. 2 Principal coordinate analysis (PCoA) of the 24 populations of *Alnus incana*; colors of the populations as in Bayesian clustering and abbreviations as in Tab. 1.

Geographic structure

A PCoA analysis conducted at the level of population resulted in the first two axes accounting for 39.7% and 26.8% of the total variation, respectively (Fig. 2). The ordination of the studied populations according to the first axis underlined the distinct character of the Alpine Population A_2 and revealed two major groups of populations. The first group contained Carpathian and Balkan populations with a single stand from Bohemian Massif, while the second one comprised all of the remaining stands from Central Europe and Scandinavian Peninsula. Testing the relationship between genetic structure coefficients and geographic distances (latitude, longitude, and altitude) revealed a significant decrease in gene diversity (H_E) with increasing latitude ($R^2 = 0.46$; $p < 0.001$) and altitude ($R^2 = 0.30$; $p < 0.01$). Generally, the most-southerly located populations presented the highest level of gene diversity (Tab. 1). No significant relationships with geographic location were noted for the remaining parameters, i.e. average alleles per locus (A) and inbreeding coefficient (F_{IS}).

Bayesian analysis detected 11 groups with a log of posterior probability of -1637 (Fig. 1). The inferred clusters were as follows: Cluster I – two Western Alpine populations (A_2 and A_3), Cluster II – three Balkan populations (B_1, B_2, and B_3), Cluster III – two Eastern Carpathian populations (EC_2 and EC_5), Cluster IV – one population from Finland (S_2) and one from Norway (S_6), Cluster V – population from the North European Plain (NEP_2) and population from Finland (S_1), Cluster VI – two Western Carpathian populations (WC_1 and WC_2), Cluster VII – two populations from western Norway (S_4 and S_5), Cluster VIII – population from Giant Mountains (BM), Cluster IX – three populations from the North European Plain (NEP_1, NEP_3, and NEP_4), Cluster X – Eastern Carpathian population (EC_1) and population from Finland (S_3), and Cluster XI – eastern Alpine population (A_1). AMOVA indicated that 6.4% of total variation resided between these groups and the differentiation was highly significant ($p < 0.001$).

Clonal structure

A considerable amount of vegetative reproduction was noted in seven of the studied populations (Tab. 3). The smallest genotypes/individuals ratio was recorded for the Finnish population (S_2; $R = 0.414$) where only 13 unique genotypes among 30 sampled ramets were found. The largest number of unique genotypes (27) was found in the population from Balkans (B_1; $R = 0.896$). The size of genets varied among studied populations with the average number of ramets per genet being 3.15. The most frequent number of ramets within the genet was 2 (17.3%). The largest genet with 13 ramets was detected in Population S_2 (Finland). The values of probability of emerging of multiplied genotypes in result of independent generative reproduction

Tab. 3 The clonal structure of seven analyzed populations of *Alnus incana*.

Population	N	N_{gen}	R
Balkans, Rila, Beli Iskar, Bulgaria (B_1)	30	27	0.896
Alps, Niedere Tauern, Solkpass, Austria (A_1)	28	17	0.551
Alps, Sauveur, France (A_2)	30	19	0.620
Alps, Beaulard, Italy (A_3)	30	25	0.827
Scandinavian Peninsula, Oulu, Finland (S_2)	30	13	0.414
Scandinavian Peninsula, Ketomella, Finland (S_3)	30	16	0.517
Scandinavian Peninsula, Birtavarre, Norway (S_6)	30	20	0.655

N – number of individuals; N_{gen} – number of unique genotypes detected in population; R – genotype diversity according to Dorken and Eckert [23].

events were very variable in particular populations (Tab. S1). They were ranging from low ($p_{gen} = 6.6 \times 10^{-3}$) to considerably high ($p_{gen} = 2.528 \times 10^{-1}$), which reflects low number of loci used and their moderate polymorphism.

Distribution of fossil records

Definitive fossil remains of *A. incana*, and those with a high probability of being grey alder, were not frequent in the palaeoecological records (Fig. 3, Tab. S2). Our survey indicated the presence of this species in the higher latitudes of the Russian Plain during the last full glacial and probably (*Alnus* sp.) in the Hungarian Plain, Bohemia, and northern Carpathians (Slovakia); convincing arguments for the potential presence of grey alder in the LGM in southern Britain (the Bodmin Moore site) have been previously presented [32]. An indication of the possible survival of *A. incana* in Central Europe and even further northward can be drawn from the location of the sites in which this species has been documented for the LG: Kašučiai Lake in Lithuania and Starunia in NW Ukraine. These findings led us to the opinion that several records of *Alnus* sp. macrofossils (mainly wood) of the same age from the area of Poland probably belong to grey alder as well. Pollen data also support the presence of this species in the LG in the Alps and in the mountains of Scandinavia [28,33]

The early-Holocene appearance of *A. incana* in the middle and high latitudes deserves special consideration as well. In Kreekrak (the Netherlands), grey alder grew at about 11.4 ka cal. BP [34], wood of *Alnus* sp. dated to ca. 10.3 ka cal. BP was found at the KH1 site (Estonia) [35] and several occurrences of *A. incana* mega- and macrofossils dating to the 10th millennium cal. BP was reported from Scandinavia [36,37].

Discussion

Geographic structure and fossil records: glacial survival of grey alder

Grey alder habitat requirements, particularly in thermal and precipitation parameters would potentially enable it to survive the LGM in stands located further to the north in Europe. In Scandinavia, fossil remains of boreal trees dated to the LGM and LG, and evidence of an early postglacial appearance of some mesophilous taxa, have been the subject of vivid debate for more than a decade [37–42]. Based on analysis of modern and ancient mtDNA from Scandinavia, Parducci et al. [43] suggested that *Picea abies* and *Pinus sylvestris* survived the last glaciation as far as northwestern Norway. This result was contested by Birks et al. [42]. The oldest fossil remains of grey alder in
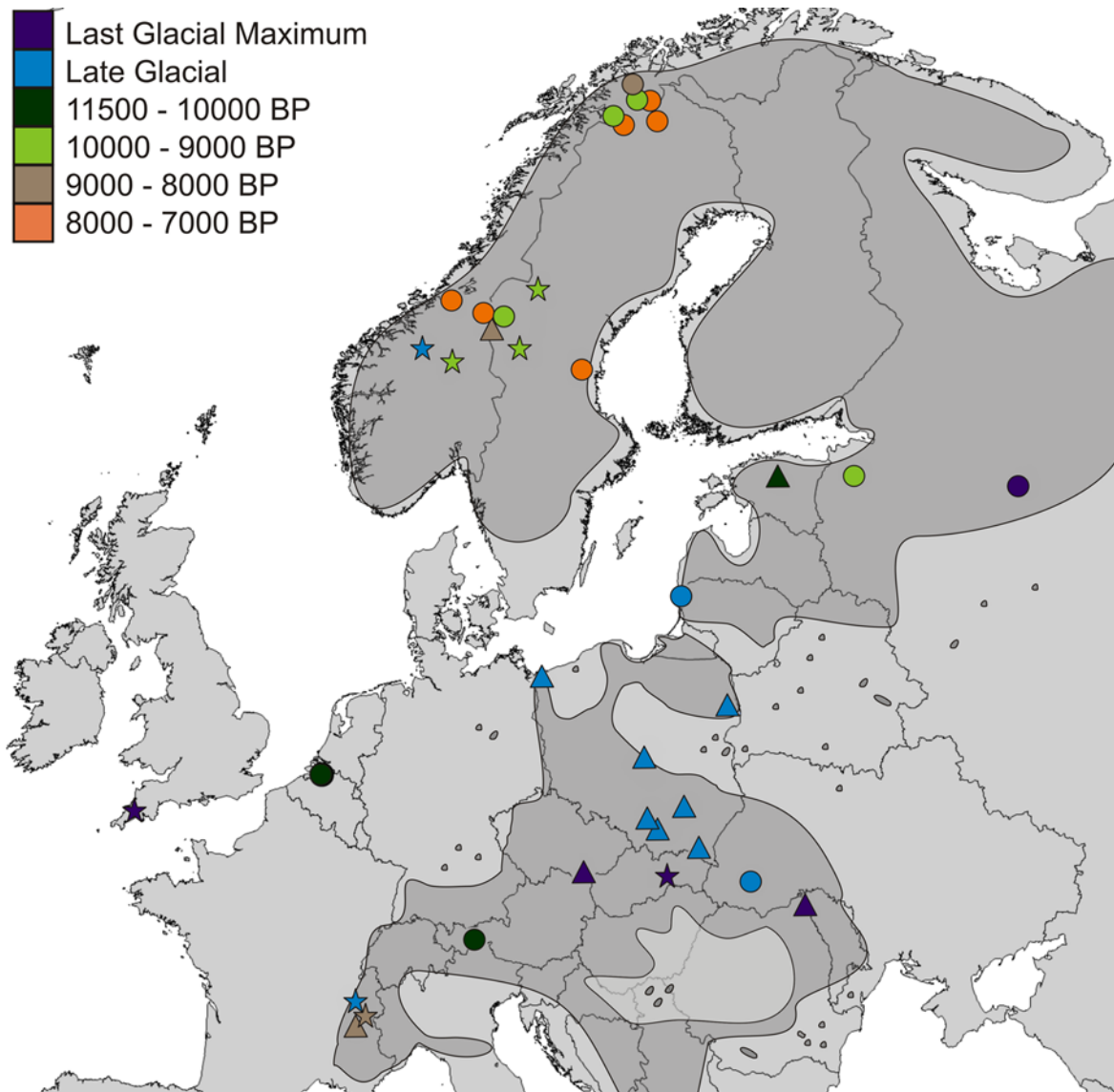
Fig. 3 Distribution of *Alnus* fossil records. Macrofossils: circle – *Alnus incana*, triangle – *Alnus* sp. with high probability of *Alnus incana*. Pollen: asterisk – *Alnus glutinosa* /*Alnus incana*-type with high probability of *Alnus incana*. See Tab. S2 for details; the map does not include data collected for Scandinavia by Tallantire [30].

the Scandes are dated to 9.3 ka cal. BP [36,37]. A pollen analysis of sediments from a nunatak lake located in central Norway indicated the possible occurrence of *A. incana* during the LG interstadial [33]. The palaeobotanical data from NE Europe suggest its presence in the period of full glacial [29] and LG [44] in the vicinity of the ice-sheet. The presence of grey alder macroremains in the Kreekrak site (SW Netherlands) dating from the beginning of the Holocene may be another argument for the potential persistence of small, scattered populations of *A. incana* in a periglacial zone of Western Europe [34]. Recently, Tzedakis et al. [45] have proposed a model of patchily distributed populations of trees that would be present up to 49° N in the Carpathians and in higher latitudes in the East European Plain during the LGM, which was possible due to a milder climate in the eastern part of the continent at the LGM.

In light of the above palaeobotanical data, populations from the Scandinavian Peninsula (S_1–S_6) and North European Plain (NEP_1–NEP_4) investigated in the present study could derive from micro-refugial populations located on mid-latitudes. However, our results indicate large genetic differentiation among those populations as they were grouped into different clusters. Based on our data we are not able to confirm a single refugium for those populations. According to Mandák et al. [15] grey alder inhabiting Northern Europe is derived from the ancestral populations that survived the LGM in Central Europe.

Although our search for macrofossil records of *A. incana* involved an extensive survey of palaeobotanical literature, we did not find any data on macroremains in Southern and Southeastern Europe for the period of interest. Recent study of Douda et al. [14] indicated for *Alnus* only low pollen percentages on Southern European peninsulas. We assume, however, that the presence of distinct *Alnus* pollen in sediments in Greece, Slovenia, and Italy from the LGM and LG reflects the presence of this species in the southern refugia [46,47]. Mandák et al. [15] reported a unique genetic diversity present in those southernmost European stands that exemplify the valuable "rear-edge populations" [48].

The Carpathians were probably another important refugium for *A. incana*. This has been previously suggested by Jankovská et al. [49] on the basis of a pollen analysis of glacial deposits in Slovakia and is supported by the LG macrofossil record from Starunia [50]. According to PCoA, Balkan and Carpathian populations are genetically more related to each other and similarly distinct from the remaining European stands. Also, it seems that they had limited impact on current grey alder gene pool that corresponds well to recent colonization scenario presented by Mandák et al. [15]. The authors also indicated that the Carpathian populations of *A. incana* derive from the Balkans, which may explain the close genetic affinity of both groups of populations revealed by PCoA.

PCoA and Bayesian clustering revealed also a split between the Western (WC_1, WC_2) and Eastern Carpathian (EC_1–EC_5) populations of *A. incana* (Fig. 1 and Fig. 2). A similar picture was shown in *Abies alba* Mill. [51] and *Pinus mugo* Turra [52]. Additionally, a single population from the Sudetes (BM), a mountain range located between the Alps and the Carpathians, appeared to be genetically distinct from the Carpathian populations (WC_1, WC_2, EC_1–EC_5) and exhibited relatively high genetic diversity (Tab. 1). This may indicate the long-term persistence of grey alder in this area, perhaps also during the LGM, as is suggested by recent genetic data [15]. A similar geographically-driven pattern for Carpathian and Sudetian populations has been observed for *Pinus mugo* [53].

We noted substantial genetic differentiation in pairwise genetic comparisons between two populations from the Western Alps (A_2 and A_3) and the remaining European populations; this result was supported by Bayesian clustering as well. PCoA analysis had lower resolution power and mainly revealed the very distinct character of the Western Alpine population, A_2. The Western Alps have been previously defined as a refugium for *Pinus sylvestris* [54] and *P. mugo* [55]. A continuous pollen presence for *A. glutinosa* / *A. incana*-type, starting from the Younger Dryas, has been reported in the northwestern Alps [56]. David [28] based on pollen records stated that *A. incana* refugial populations existed somewhere in the Western Alps. However, a recent study of Mandák et al. [15] based on cpDNA and SSR markers did not reveal the existence of the divergent genetic linage of *Alnus* species in the Western Alps.

Clonality

In all studied populations, significant clonal structure was present. It proves that clonal reproduction is an important component of grey alder population dynamics. The low genotypes/ramets ratio, observed in the northernmost populations, imply that in suboptimal habitats this mode of reproduction serves as an efficient strategy for survival [13]. The largest clone which comprised of 13 ramets was indeed found in population from the Scandinavian Peninsula, S_2. In our opinion, grey alder benefited from diverse advantages related to clonality, which was one of the important life-history traits enabling the species to survive the LGM in the northern micro-refugia, as it has been suggested with macrofossil data presented in this work.

It is generally expected that a lower level of genetic diversity will be found in clonal plants because sexual recombination, the major process generating genetic variation, is limited or temporarily absent. Theoretical predictions and field data show that clonal reproduction may negatively affect the genotypic diversity [57,58]. We observed an overall low level of genetic diversity in *A. incana*, $H_E = 0.386$, which was lowest in Northeastern European populations compared to the remaining stands ($H_E = 0.366$ and $H_E = 0.432$; $p = 0.009$). The allelic variability assessed with average number of

alleles per locus (A) was also rather low with only 3.31 alleles per locus. A similar level of allelic variability at eight SSR loci was found in *A. incana* (A = 3.88), by Zhuk et al. [59], while in *A. glutinosa* the authors noted a higher value (A = 5.13).

In our opinion, the cost of prolonged clonality assisting the northern survival of gray alder could be a partial loss of genetic variability, especially in the northernmost populations. In the situation of environmentally enhanced predominance of the vegetative spread, the intraspecific competition may be intensified [60] and locally less adapted genotypes may become outcompeted [61,62] that might lead to reduction of genetic variability; in the most extreme situation a single genet may become fixed.

Conclusions

The combined palaeobotanical and genetic data presented in our work indicate that the glacial and postglacial history of grey alder include existence of the northerly located refugia and differential input of the refugia into the processes of colonization. We believe that clonality may have been crucial in formation of an effective northern refugia and the successful early recolonization of the species. Gray alder populations inhabiting the southern part of the European continent, the so called "rear-edge populations" [48], constitute the important sources of species genetic diversity. These Balkan populations probably represent the oldest, ancestral gene pool of the species and need to be protected from genetic impoverishment.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3523/0:

Tab. S1   The clonal structure of seven populations of *Alnus incana*.

Tab. S2   Data sources for macrofossils of *Alnus incana*.

References

1. Bennett KD, Tzedakis PC, Willis KJ. Quaternary refugia of North European trees. J Biogeogr. 1991;18(1):103–115. https://doi.org/10.2307/2845248

2. Hewitt GM. Genetic consequences of climatic oscillations in the Quaternary. Philos Trans R Soc Lond B Biol Sci. 2004;359(1442):183–195. https://doi.org/10.1098/rstb.2003.1388

3. Willis K, van Andel T. Trees or no trees? The environments of Central and Eastern Europe during the Last Glaciation. Quat Sci Rev. 2004;23(23–24):2369–2387. https://doi.org/10.1016/j.quascirev.2004.06.002

4. Steward JR, Lister AM. Cryptic northern refugia and the origins of the modern biota. Trends Ecol Evol. 2001;16(11):608–613. http://dx.doi.org/10.1016/S0169-5347(01)02338-2

5. Bhagwat SA, Willis KJ. Species persistence in northerly glacial refugia of Europe: a matter of chance or biogeographical traits? J Biogeogr. 2008;35:464–482. https://doi.org/10.1111/j.1365-2699.2007.01861.x

6. Dering M, Chybicki IJ, Rączka G. Clonality as a driver of spatial genetic structure in populations of clonal tree species. J Plant Res. 2015;128(5):731–745. https://doi.org/10.1007/s10265-015-0742-7

7. Silvertown J. The evolutionary maintenance of sexual reproduction: evidence from

the ecological distribution of asexual reproduction in clonal plants. Int J Plant Sci. 2008;169(1):157–168. https://doi.org/10.1086/523357

8. van der Merwe M, Spain CS, Rossetto M. Enhancing the survival and expansion potential of a founder population through clonality. New Phytol. 2010;188(3):868–878. https://doi.org/10.1111/j.1469-8137.2010.03396.x

9. Karlin EF, Hotchkiss SC, Boles SB, Stenøien HK, Hassel K, Flatberg KI, et al. High genetic diversity in a remote island population system: sans sex. New Phytol. 2012;193(4):1088–1097. https://doi.org/10.1111/j.1469-8137.2011.03999.x

10. Stöcklin J, Bäumler E. Seed rain, seedling establishment and clonal growth strategies on a glacier foreland. J Veg Sci. 1996;7(1):45–56. https://doi.org/10.2307/3236415

11. Öberg L, Kullman L. Ancient subalpine clonal spruces (*Picea abies*): sources of postglacial vegetation history in the Swedish Scandes. Arctic. 2011;64(2):183–196. https://doi.org/10.14430/arctic4098

12. Jalas J, Suominen J. Atlas Florae Europaeae: distribution of vascular plants in Europe Vol. 3. Salicaceae to Balanophoraceae. Helsinki: Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanario; 1976.

13. Kullman L. The ecological status of grey alder [*Alnus incana* (L.) Moench] in the upper subalpine birch forest of the central Scandes. New Phytol. 1992;120(3):445–451. https://doi.org/10.1111/j.1469-8137.1992.tb01085.x

14. Douda J, Doudová J, Drašnarová A, Kuneš P, Hadincová V, Krak K, et al. Migration patterns of subgenus *Alnus* in Europe since the Last Glacial Maximum: a systematic review. PLoS One. 2014;9(2):e88709. https://doi.org/10.1371/journal.pone.0088709

15. Mandák B, Havrdová A, Krak K, Hadincová V, Vít P, Zákravský P, et al. Recent similarity in distribution ranges does not mean a similar postglacial history: a phylogeographical study of the boreal tree species *Alnus incana* based on microsatellite and chloroplast DNA variation. New Phytol. 2016;210(4):1395–1407. https://doi.org/10.1111/nph.13848

16. Kulju KKM, Pekkinen M, Varvio S. Twenty-three microsatellite primer pairs for *Betula pendula* (Betulaceae). Mol Ecol Notes. 2004;4(3):471–473. https://doi.org/10.1111/j.1471-8286.2004.00704.x

17. Goudet J. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from: http://www2.unil.ch/popgen/softwares/fstat.htm

18. Chybicki IJ, Burczyk J. Simultaneous estimation of null alleles and inbreeding coefficients. J Hered. 2009;100(1):106–113. https://doi.org/10.1093/jhered/esn088

19. Rousset F. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour. 2008;8(1):103–106. https://doi.org/10.1111/j.1471-8286.2007.01931.x

20. Weir BS, Cockerham CC. Estimating *F*-statistics for the analysis of population structure. Evolution. 1984;38(6):1358–1370. https://doi.org/10.2307/2408641

21. Chapuis MP, Estoup A. Microsatellite null alleles and estimation of population differentiation. Mol Biol Evol. 2007;24(3):621–631. https://doi.org/10.1093/molbev/msl191

22. Excoffier L, Lischer HEL. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour. 2010;10(3):564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

23. Dorken ME, Eckert CG. Severely reduced sexual reproduction in northern populations of a clonal plant, *Decodon verticillatus* (Lythraceae). J Ecol. 2001;89(3):339–350. https://doi.org/10.1046/j.1365-2745.2001.00558.x

24. Peakall R, Smouse P. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics. 2012;28(19):2537–2539. https://doi.org/10.1093/bioinformatics/bts460

25. Guillot G, Santos F, Estoup A. Analysing georeferenced population genetics data with Geneland: a new algorithm to deal with null alleles and a friendly graphical user interface. Bioinformatics. 2008;24(11):1406–1407. https://doi.org/10.1093/bioinformatics/btn136

26. Birks HH, Birks HJB. Future uses of pollen analysis must include plant macrofossils. J Biogeogr. 2000;27(1):31–35. https://doi.org/10.1046/j.1365-2699.2000.00375.x

27. Erdtman OGE, Berglund B, Praglowski J. An introduction to a Scandinavian pollen flora. Stockholm: Almqvist & Wiksell; 1961.

28. David F. Développement des aulnes dans les Alpes françaises du Nord. Comptes Rendus de l'Académie des Sciences. Série 2, Mécanique, Physique, Chimie, Sciences de l'Univers, Sciences de la Terre. 1993;316(12):1815–1822.

29. Binney HA, Willis KJ, Edwards ME, Bhagwat SA, Anderson PM, Andreev AA, et al. The distribution of late-Quaternary woody taxa in northern Eurasia: evidence from a new macrofossil database. Quat Sci Rev. 2009;28(23–24):2445–2464. https://doi.org/10.1016/j.quascirev.2009.04.016

30. Tallantire PA. The palaeohistory of the grey alder [*Alnus incana* (L.) Moench.] and black alder [*A. glutinosa* (L.) Gaertn.] in Fennoscandia. New Phytol. 1974;73(3):529–546. https://doi.org/10.1111/j.1469-8137.1974.tb02131.x

31. SchochW, Heller I, Schweingruber FH, Kienast F. Wood anatomy of Central European species [Internet]. 2004 [cited 2016 Dec 14]. Available from: http://www.woodanatomy.ch/

32. Kelly A, Charman DJ, Newnham RM. A Last Glacial Maximum pollen record from Bodmin Moor showing a possible cryptic northern refugium in southwest England. Journal of Quaternary Sciences. 2010;25(3):296–308. https://doi.org/10.1002/jqs.1309

33. Paus A, Velle G, Berge J. The Lateglacial and early Holocene vegetation and environment in the Dovre mountains, central Norway, as signaled in two Lateglacial nunatak lakes. Quat Sci Rev. 2011;30:1780–1796. https://doi.org/10.1016/j.quascirev.2011.04.010

34. Bos JAA, Huisman DJ, Kiden P, Hoek WZ, van Geel B. Early Holocene environmental change in the Kreekrak area (Zeeland, SW-Netherlands): a multi-proxy analysis. Palaeogeogr Palaeoclimatol Palaeoecol. 2005;227:259–289. https://doi.org/10.1016/j.palaeo.2005.05.020

35. Koff T, Kangur M. Vegetation history in northern Estonia during the Holocene based on pollen diagrams from small kettlehole and lake sediments. In: Tonkov S, editor. Aspects of palynology and palaeoecology. Festschrift in honour of Elissaveta Bozilova. Sofia: Pensoft; 2003. p. 113–126.

36. Kullman L. Early Holocene tree growth at a high elevation site in the northernmost Scandes of Sweden (Lapland): a palaeobiogeographical case study based on megafossil evidence. Geografiska Annaler: Series A, Physical Geography. 1999;81(1):63–74. https://doi.org/10.1111/j.0435-3676.1999.00049.x

37. Kullman L. Non-analogous tree flora in the Scandes Mountains, Sweden, during the early Holocene – macrofossil evidence of rapid geographic spread and response to palaeoclimate. Boreas. 1998;27(3):153–161. https://doi.org/10.1111/j.1502-3885.1998.tb00875.x

38. Kullman L. Boreal tree taxa in the central Scandes during the Late Glacial: implications for Late Quaternary forest history. J Biogeogr. 2002;29(9):1117–1124. https://doi.org/10.1046/j.1365-2699.2002.00743.x

39. Birks HH, Larsen E, Birks HJB. Did tree-*Betula*, *Pinus* and *Picea* survive the last glaciation along the west coast of Norway? A review of the evidence, in light of Kullman (2002). J Biogeogr. 2005;32(8):1461–1471. https://doi.org/10.1111/j.1365-2699.2005.01287.x

40. Kullman L. Late-glacial trees from arctic coast to alpine tundra: response to Birks et al. 2005 and 2006. J Biogeogr. 2006;33(2):377–378. https://doi.org/10.1111/j.1365-2699.2005.01451.x

41. Kullman L. Early postglacial appearance of tree species in northern Scandinavia: review and perspective. Quat Sci Rev. 2008;27:2467–2472. https://doi.org/10.1016/j.quascirev.2008.09.004

42. Birks HH, Giesecke T, Hewitt GM, Tzedakis PC, Bakke J, Birks HJB. Comment on "Glacial survival of boreal trees in northern Scandinavia". Science. 2012;338(6108):742–742. https://doi.org/10.1126/science.1225345

43. Parducci L, Jørgensen T, Tollefsrud MM, Elverland E, Alm T, Fontana SL, et al. Glacial survival of boreal trees in northern Scandinavia. Science. 2012;335:1083–1086. https://doi.org/10.1126/science.1216043

44. Stančikaitė M, Šinkūnas P, Šeirienė V, Kisielienė D. Patterns and chronology of the Lateglacial environmental development at Pamerkiai and Kašučiai, Lithuania. Quat Sci Rev. 2008;27(1–2):127–147. https://doi.org/10.1016/j.quascirev.2007.01.014

45. Tzedakis PC, Emerson BC, Hewitt GM. Cryptic or mystic? Glacial tree refugia in Northern Europe. Trends Ecol Evol. 2013;28(12):696–704. https://doi.org/10.1016/j.tree.2013.09.001

46. Bennett KD, Tzedakis PC, Willis KJ. Quaternary refugia of north European trees. J Biogeogr. 1991;18(1):103–115. https://doi.org/10.2307/2845248

47. Drescher-Schneider R, de Beaulieu JL, Magny M, Walter-Simonnet AV, Bossuet G, Millet L, et al. Vegetation history, climate and human impact over the last 15 000 years at Lago dell'Accesa (Tuscany, central Italy). Veg Hist Archaeobot. 2007;16(4):279–299. https://doi.org/10.1007/s00334-006-0089-z

48. Hampe A, Petit RJ. Conserving biodiversity under climate change: the rear edge matters. Ecol Lett. 2005;8(5):461–467. https://doi.org/10.1111/j.1461-0248.2005.00739.x

49. Jankowská V, Chromy P, Nižnianská M. Šafárka – first palaeobotanical data of the character of Last Glacial vegetation and landscape in the West Carpathians (Slovakia). Acta Palaeobotanica. 2002;42(1):39–50.

50. Stachowicz-Rybka R, Galka M, Alexandrowicz WP, Alexandrowicz SW. Plant macrofossils and malacocoenoses of Quaternary mineral-organic sediments at Starunia palaeontological site and vicinity (Carpathian Region, Ukraine). Annales Societatis Geologorum Poloniae. 2009;79(3):297–313.

51. Liepelt S, Cheddadi R, de Beaulieu JL, Fady B, Gömöry D, Hussendörfer E, et al. Postglacial range expansion and its genetic imprints in *Abies alba* (Mill.) – a synthesis from palaeobotanic and genetic data. Rev Palaeobot Palynol. 2009;153(1–2):139–149. https://doi.org/10.1016/j.revpalbo.2008.07.007

52. Boratynska K, Muchewicz E, Drojma M. *Pinus mugo* Turra geographic differentiation based on needle characters. Dendrobiology. 2004;(51):9–17.

53. Dzialuk A, Boratynski A, Boratynska K, Burczyk J. Geographic patterns of genetic diversity of *Pinus mugo* (Pinaceae) in Central European mountains. Dendrobiology. 2012;68:31–41.

54. Cheddadi R, Vendramin GG, Litt T, François L, Kageyama M, Lorentz S, et al. Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. Global Ecology and Biogeography. 2006;15(3):271–282. https://doi.org/10.1111/j.1466-8238.2006.00226.x

55. Heuertz M, Teufel J, González-Martínez SC, Soto A, Fady B, Alía R, et al. Geography determines genetic relationships between species of mountain pine (*Pinus mugo* complex) in Western Europe. J Biogeogr. 2010;37(3):541–556. https://doi.org/10.1111/j.1365-2699.2009.02223.x

56. Ortu E, David F, Peyron O. Pollen-inferred palaeoclimate reconstruction in the Alps during the Lateglacial and the early Holocene: how to estimate the effect of elevation and local parameters. J Quat Sci. 2010;25(5):651–661. https://doi.org/10.1002/jqs.1335

57. Honnay O, Bossuyt B. Prolonged clonal growth: escape route or route to extinction? Oikos. 2005;108(2):427–432. https://doi.org/10.1111/j.0030-1299.2005.13569.x

58. Balloux F, Lehmann L, de Meeûs T. The population genetics of clonal and partially clonal diploids. Genetics. 2003;164(4):1635–1644.

59. Zhuk A, Veinberga I, Daugavietis M, Ruņģis. Cross-species amplification of *Betula pendula* Roth. simple sequence repeat markers in *Alnus* species. Balt For. 2008;14(2):116–121.

60. Wang P, Lei JP, Li MH, Yu FH. Spatial heterogeneity in light supply affects intraspecific competition of a stoloniferous clonal plant. PLoS One. 2012;7(6):e39105. https://doi.org/10.1371/journal.pone.0039105

61. Hartnett DC, Bazzaz FA. The genet and ramet population dynamics of *Solidago canadensis* in an abandoned field. J Ecol. 1985;73:407–413. https://doi.org/10.2307/2260483

62. Vandepitte K, Roldán-Ruiz I, Leus L, Jacquemyn H, Honnay O. Canopy closure shapes clonal diversity and fine-scale genetic structure in the dioecious understory perennial *Mercurialis perennis*. J Ecol. 2009;97:404–414. https://doi.org/10.1111/j.1365-2745.2009.01484.x