forests

MDPI

Article

Spatial Pattern of the Mitochondrial and Chloroplast Genetic Variation in Poland as a Result of the Migration of *Abies alba* Mill. from Different Glacial Refugia

Monika Litkowiec [1,*], Andrzej Lewandowski [1] and Grzegorz Rączka [2]

[1] Laboratory of Molecular Biology, Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, Kórnik 62-035, Poland; alew@man.poznan.pl

[2] Department of Forest Management, University of Life Sciences, Wojska Polskiego 71C, Poznan 60-625, Poland; g.raczka@wp.pl

* Correspondence: milt@man.poznan.pl; Tel.: +48-61-817-0033

Academic Editors: Feihai Yu and Timothy A. Martin
Received: 28 September 2016; Accepted: 9 November 2016; Published: 17 November 2016

Abstract: Currently, the information on the gene pool of silver fir (*Abies alba* Mill.) at the northeastern edge of its distribution in Poland is scarce and insufficient. Using the advantage provided by markers with different modes of inheritance, a hypothesis that gene flow via both seeds and pollen contributed to the genetic structure across the entire analyzed region was investigated. The geographic distribution of maternally inherited mitochondrial DNA (mtDNA, *nad5-4*) and paternally inherited chloroplast DNA (cpDNA, *psbC*) variation was studied in 81 Polish populations and three reference populations from Ukraine and Romania. The spatial pattern of mtDNA haplotypes (dispersed via seeds) indicated that the Apennine Peninsula was the only maternal glacial refugium for the entire territory of Poland and also the Ukraine no 1 population, whereas the other two populations—Ukraine no 2 and Romania—had the haplotype representing the Balkan origin. By contrast, the cpDNA haplotypes (dispersed via pollen) from all studied Polish and reference populations showed that *A. alba* colonized the current natural range from two genetically distinct glacial refugia located on the Apennine and Balkan peninsulas. The occurrence of cpDNA haplotypes varied among the studied populations. Additionally, statistical analyses were used to infer the genetic structure of examined populations. Two distinct groups of *A. alba* populations were identified showing the postglacial geographic distribution of haplotypes of both mtDNA and cpDNA. *A. alba* is an important ecological and economic component of forest ecosystems in Europe. An understanding of the Holocene history of this species is relevant for planning sustainable forest management, and acquired data can contribute to strategies of conservation and restoration.

Keywords: hybrid zone; organelle DNA markers; phylogeography; silver fir

1. Introduction

The modern geographical distribution and pattern of genetic diversity of forest tree populations within their natural range are, to a large extent, a consequence of the particularly strong climatic fluctuations of the Quaternary, with a dominant series of cold and dry glacial periods that alternated with shorter intervals of warmer and moister interglacial periods [1–3]. On the scale of Europe, many temperate trees survived these glacial periods as small, low-density populations that retreated into favorable microenvironments and distinct refugia located in deep valleys among the mountains of southern Europe [4–6]. In particular, with their isolation in these refugia for many tens of thousands of years, populations had opportunities to differentiate through selection and genetic drift. Additionally,

postglacial migrations northwards and the associated demographic processes, created durable genetic imprints on the genetic structure (e.g., variation in allelic frequency and genetic diversity) of extant tree populations [7,8].

Molecular markers are extremely useful tools to assess the genetic resources of forest trees by improving our understanding of the distribution of genetic diversity within and among populations and of the structure of the populations in the Northern Hemisphere after the last glacial maximum (LGM) [9]. In *Pinaceae*, the organelle genomes, mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA), are inherited maternally and paternally respectively [10], and they possess different rates of point mutations [11]. Hence, because of the contrasting modes of inheritance, gene exchange transmitted via seeds only (mtDNA) or via pollen and subsequently fertilized seeds (cpDNA) can be tracked. Spatiotemporal gene flow is characterized via seeds and pollen to a different and an asymmetric degree within outcrossing and wind-pollinated conifer species (e.g., [12,13]), and the expected different patterns of mtDNA and cpDNA in geographically distinct regions has been confirmed (e.g., [14,15]).

Silver fir (*Abies alba* Mill.), a conifer species in the family *Pinaceae*, is a key species of the mountainous forests of central and southern Europe. From the Pyrenees to the Balkans, this species is an important ecological and economic component of forest ecosystems. The current understanding of the glacial and postglacial history of *A. alba* is based on paleoecological and genetic studies (e.g., [4,16–18]). During the last ice age, *A. alba* survived in refugia in the central Apennine, the south of the Balkan Peninsula and the central massif in France, and these refuges contributed to the northwards colonization of *A. alba*, which began approximately 11,000 years ago (y.a.) (Figure 1). However, two refugia—one in Calabria in the south of the Apennine and one situated in the Pyrenees—remained isolated, and the populations that originated in these areas experienced only limited expansions and remain genetically distinct from other populations. Furthermore, the most likely routes of postglacial recolonization of *A. alba* have been identified, in addition to the zones of introgression between these routes [19].

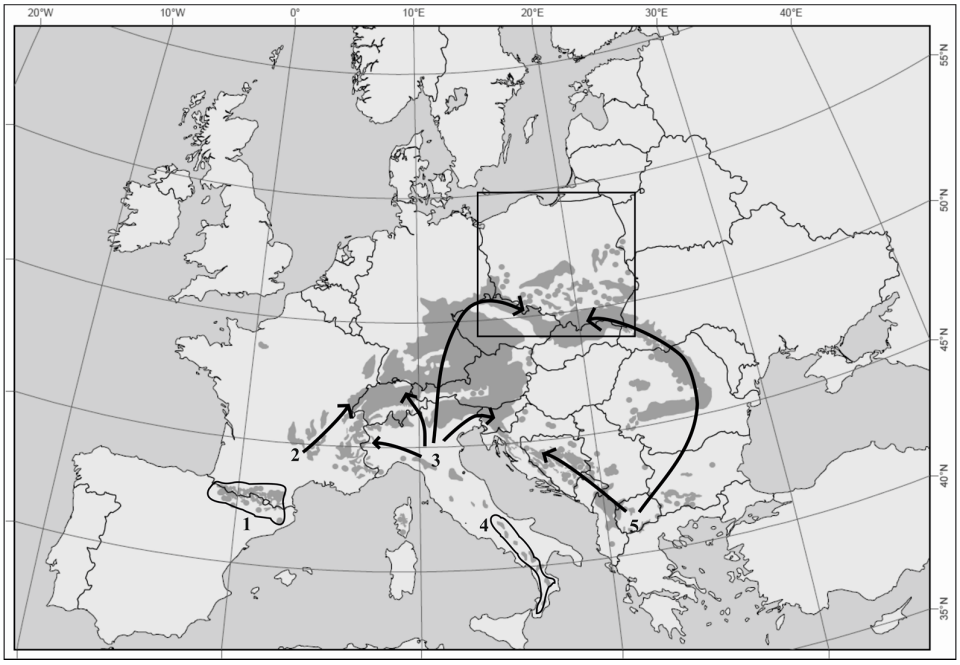

Figure 1. Map of the current natural range (grey areas), the location of glacial refugia (1—Iberian Peninsula, Pyrenees; 2—Iberian Peninsula, southwestern France; 3—Apeninne Peninsula, northwestern Italy; 4—Apennine Peninsula, southern Italy-Calabria; 5—Balkan Peninsula, the southern Balkans Massif) and the most likely routes of postglacial recolonization (arrows) of *Abies alba* in Europe.

The present-day natural range of *A. alba* is the most extensive of all European *Abies* species, as the limit of the distribution reached approximately 6000 Before Present (BP). In the Holocene, *A. alba* migrated to Poland very late and reached the northeastern border of its current natural range approximately 2000 BP. The distribution of this species in Polish territory is relatively small and usually scattered. The species occurs primarily in southern Poland, particularly in the Carpathians, the Sudetes and the Holy Cross Mountains, and also in minor proportions in the Roztocze region. Apart from a continuous distribution at the northern edge of the range, small, isolated patches occur in the lowlands, such as in the eastern part of the Białowieża Primeval Forest and near Warsaw in which a reserve area was created [20]. The northern limit of the distribution of this species is correlated with an annual precipitation of 600 mm [21].

To date, according to studies based on isozymes and DNA markers, the Polish populations of *A. alba* are characterized by lower levels of genetic variation within populations and higher genetic differentiation among populations than those of other coniferous species, however, these studies are not comprehensive [17,22,23]. Based on these studies, a hypothesis is proposed for two distinct gene pools of *A. alba* in Poland. Nevertheless, a recent study of Polish populations of *A. alba* using a strictly maternally inherited marker indicated that it migrated to Polish territory from only one refugium located in western Europe [24]. However, the possible participation of the pollen gene pool in the formation of Polish populations of *A. alba* was not considered in that study. Currently, *A. alba* is threatened by human impact and future climate change in large parts of its natural range, including the northern edge of its distribution in Poland [25,26]. Thus, a precise assessment of the Holocene history of *A. alba* might help in developing appropriate conservation strategies because, in plants with a different post-glacial origin, significant differences in the levels of genetic variation, and thus growth variability and climate sensitivity, are displayed [27].

In this study, we used markers with contrasting modes of inheritance (mtDNA and cpDNA) to infer the Holocene history for many *A. alba* populations from the entire natural range in Poland. Using markers with contrasting modes of inheritance permitted the verification of the hypothesis that the colonization of the Polish territory by *A. alba* was from two separate refugia. Therefore, the spatial distribution of mtDNA and cpDNA haplotypes was used to determine (1) the contribution of particular refugia to the colonization of Polish territory and adjacent areas; and (2) whether there was a crossroads of migratory routes from different refugia.

2. Materials and Methods

2.1. Plant Material and Genetic Analyses

In this study, 81 populations of *Abies alba* from across the entire range of the species in Poland were examined, including 13 that represented Natural Reserves and two that represented National Parks. Additionally, two populations of *A. alba* from Ukraine and one population from Romania were analyzed as reference populations (Figures 1 and 2, Table 1). The sample size of each population ranged from eight to 36 individuals, with a total of 1558 individuals analyzed (Table 1). Genomic DNA was extracted from the needles of each sample using a modified CTAB (Cetyltrimethylammonium bromide) protocol [28].

In this study, two organelle DNA markers (mitochondrial (mtDNA) and chloroplast (cpDNA)) were examined with contrasting modes of inheritance, maternal and paternal, respectively, developed by Liepelt et al. [17].

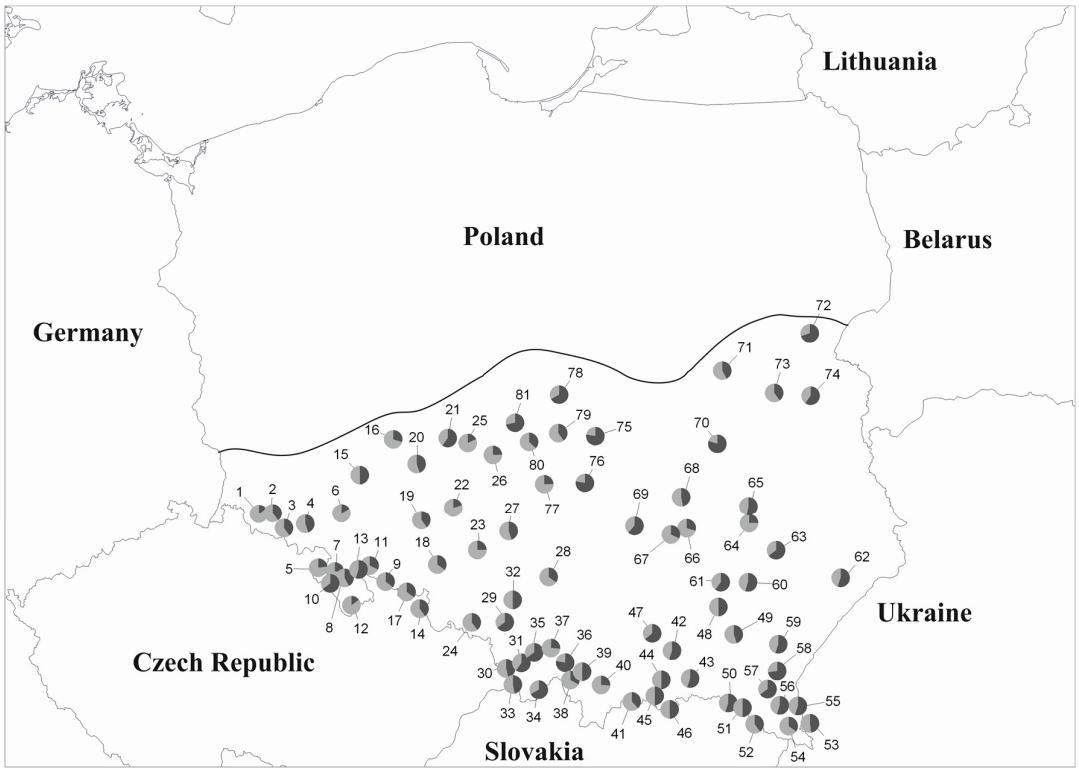

Figure 2. Locations of the 81 populations of *Abies alba* included in this study and the distribution of the haplotypes in the chloroplast *psbC* marker. The population codes and details on the frequency of occurrence of haplotypes A (dark circle, Balkan refugium) and B (grey circle, Apennine refugium) are shown in Table 1. The border of the natural distribution of *A. alba* in Poland is indicated with a wavy black line.

Table 1. Sample descriptions of *Abies alba* populations included in this study and the distribution of frequency (%) of mitochondrial (*nad5-4*) and chloroplast (*psbC*) haplotypes in these populations.

CODE	NAME	Longitude° E/Latitude° N	N	mtDNA		cpDNA	
POLAND				180 bp (%)	250 bp (%)	A (%)	B (%)
1	Świeradów	15.46/50.96	19		100.0	40.0	60.0
2	Lwówek	15.64/50.95	20		100.0	15.0	85.0
3	Śnieżka	15.82/50.81	20		100.0	40.0	60.0
4	Jawor	16.11/50.88	20		100.0	45.0	55.0
5	Szczeliniec	16.34/50.48	31		100.0	23.0	77.0
6	Świdnica	16.60/50.96	20		100.0	15.0	75.0.
7	Wolary	16,52/50,44	20		100.0	25.0	75.0
8	Bystrzyca	16.61/50.42	20		100.0	42.0	58.0
9	Lądek	17.13/50.38	20		100.0	39.0	71.0
10	Zdroje	16.51/50.40	14		100.0	65.0	35.0
11	Wójtówka	16.99/50.50	19		100.0	32.0	68.0
12	Międzylesie	16.72/50.22	20		100.0	15.0	85.0
13	Bardo	16.78/50.49	20		100.0	55.0	45.0
14	Prudnik	17.64/50.15	20		100.0	40.0	60.0
15	Reserve Jodłowice	16.81/51.29	20		100.0	50.0	50.0
16	Milicz	17.24/51.58	20		100.0	30.0	70.0
17	Prudnik	17.47/50.29	20		100.0	35.0	65.0
18	Pruszków	17.87/50.54	20		100.0	35.0	65.0
19	Brzeg	17.65/50.89	20		100.0	40.0	60.0
20	Syców	17.61/51.36	20		100.0	45.0	55.0
21	Reserve Majówka	18.02/51.59	20		100.0	60.0	40.0
22	Siemianice	18.11/51.02	20		100.0	20.0	80.0
23	Zawadzkie	18.43/50.68	20		100.0	25.0	75.0
24	Rybnik	18.34/50.05	20		100.0	40.0	60.0
25	Reserve Olbina	18.27/51.56	18		100.0	18.0	82.0

Table 1. *Cont.*

CODE	NAME	Longitude° E/Latitude° N	N	mtDNA		cpDNA	
POLAND				**180 bp (%)**	**250 bp (%)**	**A (%)**	**B (%)**
26	Reserve Nowa Wieś	18.61/51.45	8		100.0	25.0	75.0
27	Herby	18.82/50.82	20		100.0	45.0	55.0
28	Siewierz	19.35/50.41	20		100.0	35.0	65.0
29	Pszczyna	18.78/50.07	20		100.0	65.0	35.0
30	Czarne	18.82/49.70	26		100.0	46.0	54.0
31	Wisła	18.98/49.72	20		100.0	60.0	40.0
32	Dobka	18.89/50.22	14		100.0	50.0	50.0
33	Bukowiec	18.88/49.55	21		100.0	47.0	53.0
34	Ujsoły	19.21/49.48	15		100.0	67.0	33.0
35	Bielsko	19.14/49.82	20		100.0	65.0	35.0
36	Sucha Beskidzka	19.57/49.71	9		100.0	78.0	22.0
37	Andrychów	19.39/49.85	19		100.0	26.0	74.0
38	Babiogórski Park Narodowy	19.68/49.60	21		100.0	33.0	67.0
39	Nowy Targ	19.80/49.65	20		100.0	50.0	50.0
40	Stańcowa	20.06/49.54	19		100.0	26.0	74.0
41	Pieniński Park Narodowy	20.46/49.40	13		100.0	38.0	62.0
42	Gromnik	21.04/49.84	36		100.0	55.0	45.0
43	Gorlice	21.26/49.61	20		100.0	55.0	45.0
44	Nawojowa	20.87/49.58	20		100.0	50.0	50.0
45	Piwniczna	20.77/49.46	14		100.0	50.0	50.0
46	Majdan	20.98/49.33	16		100.0	50.0	50.0
47	Dąbrowa Tarnowska	20.73/49.98	11		100.0	64.0	36.0
48	Tuszyma	21.63/50.17	16		100.0	50.0	50.0
49	Strzyżów	21.86/49.96	20		100.0	45.0	55.0
50	Rymanów	21.79/49.39	20		100.0	55.0	45.0
51	Moszczaniec	21.97/49.36	20		100.0	50.0	50.0
52	Komańcza	22.13/49.21	20		100.0	40.0	60.0
53	Bieszczadzki Park Narodowy	22.88/49.23	21		100.0	48.0	52.0
54	Lutowiska	22.57/49.21	20		100.0	35.0	65.0
55	Brzegi Dolne	22.71/49.36	15		100.0	55.0	45.0
56	Baligród	22.48/49.36	20		100.0	65.0	35.0
57	Lesko	22.30/49.49	20		100.0	65.0	35.0
58	Reserve Krępak	22.45/49.68	15		100.0	73.0	27.0
59	Kańczuga	22.46/49.87	20		100.0	55.0	45.0
60	Rudnik	22.04/50.38	20		100.0	55.0	45.0
61	Buda Stalowa	21.67/50.39	20		100.0	60.0	40.0
62	Wólka Husińska	23.30/50.40	18		100.0	61.0	49.0
63	Janów Lubelski	22.42/50.64	17		100.0	65.0	35.0
64	Gościeradów	22.05/50.86	20		100.0	25.0	75.0
65	Reserve Natalin	22.06/51.00	14		100.0	53.0	47.0
66	Łagów	21.20/50.84	20		100.0	30.0	70.0
67	Łagów	21.00/50.76	19		100.0	32.0	68.0
68	Marcule	21.17/51.08	17		100.0	47.0	53.0
69	Kielce	20.51/50.85	13		100.0	62.0	38.0
70	Zwoleń	21.62/51.53	20		100.0	80.0	20.0
71	Reserve Jedlina	21.69/52.14	24		100.0	42.0	58.0
72	Radzyń Podlaski	22.85/52.45	10		100.0	70.0	30.0
73	Reserve Jata	22.38/51.95	25		100.0	40.0	60.0
74	Reserve Jata	22.87/51.92	22		100.0	60.0	40.0
75	Kruszewiec	19.99/51.59	14		100.0	78.0	22.0
76	Reserve Jata	19.85/51.21	18		100.0	78.0	22.0
77	Reserve Łuszczanowiec	19.31/51.19	12		100.0	25.0	75.0
78	Reserve Zabrzeżna	19.50/51.93	18		100.0	67.0	33.0
79	Reserve Molenda	19.49/51.61	20		100.0	40.0	60.0
80	Reserve Jodły Łaskie	19.11/51.54	16		100.0	37.0	63.0
81	Reserve Jamno	18.90/51.70	19		100.0	72.0	28.0
OTHER							
82	Ukraine1	24.56/48.43	8		100.0	75.0	25.0
83	Ukraine2	25.81/47.66	11	100.0		55.0	45.0
84	Romania	25.82/47.64	13	100.0		46.0	54.0
Average				**2.4**	**97.6**	**53.0**	**47.0**

The studied sequence of the mtDNA marker was located within the fourth intron of the mitochondrial NAD dehydrogenase subunit 5 (*nad5-4*). Amplification was conducted in a total volume of 25 µL that contained 15–20 ng of DNA template, 1× PCR, 25 mM $MgCl_2$ at a pH of 8.6 (Novazym, Poznan, Poland), 0.2 mM dNTPs, 0.2 µM of each primer (primer sequences: F-5′-GGACAATGACGATCCGAGATA-3′; R-5′-CATCCCTCCCATTGCATTAT-3′), 10 ng/µL BSA (Bovine Serum Albumin), and 0.5 U of AllegroTaq polymerase (Novazym, Poznan, Poland). The *nad5-4* PCR cycling condition included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 1 min at 95 °C, 1 min at annealing temperature 52.5 °C, 1 min 20 s extension at 72 °C, and a final extension step of 8 min at 72 °C. Amplified fragments of *nad5-4* were separated by electrophoresis on a 1.2% agarose gel in 1× TBE (Tris-Borate-EDTA) buffer for 2 h at 5 V/cm and stained with GelView (Novazym, Poznan, Poland). To establish the size of the PCR products, a 100-bp DNA ladder size standard (Novazym, Poznan, Poland) was run on each gel. Gels were visualized and documented using an ultraviolet (UV) camera and the documentary system BioCaptMw (Vilber Lourmat, Marne-le-Vallée Cedex, France).

The cpDNA marker is a restriction site polymorphism in the photosystem II CP43 protein gene (*psbC*). The PCR mixture contained 15–20 ng of DNA template, 1× PCR, 25 mM $MgCl_2$ at a pH of 8.6 (Novazym, Poznan, Poland), 0.2 mM dNTPs, 0.2 µM of each primer (primer sequences: F-5′-GGTCGTGACCAAGAAACCAC-3′ (see [29]); R-5′-GGACAGGTTCGAAATCACGA-3′ (see [30])), 10 ng/µL BSA, and 0.5 U of AllegroTaq polymerase (Novazym, Poznan, Poland) in a total volume of 25 µL. The *psbC* thermocycler program consisted of an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of 1 min at 93 °C, 1 min at annealing temperature 57 °C, 2 min extension at 72 °C, and a final extension step of 10 min at 72 °C. In the restriction step, the products of amplification of *psbC* were digested using the FastDigest™ restriction enzyme HaeIII (BsuRI) (Fermentas, Waltham, MA, USA) at 37 °C for 15 min. For each digestion, 5 µL of PCR product was added to 9 µL of digestion mixture containing 8.5 µL of H_2O, 1 µL of FastDigest™ Green Buffer and 0.5 µL of restriction enzyme. The restriction fragment profiles were electrophoresed on a 1.5% agarose gel and stained with GelView (Novazym, Poznan, Poland) for 2 h at 5 V/cm. Gels were visualized and documented using a UV camera and the documentary system BioCaptMw (Vilber Lourmat, Marne-le-Vallée Cedex, France).

2.2. Data Analyses

To estimate their frequencies and to identify the postglacial history of the *A. alba* populations, the haplotypes obtained from mtDNA and cpDNA markers were analyzed separately.

Additionally, the statistical analysis of the geographical structure of genetic variation of mtDNA and cpDNA haplotypes was performed when they were encoded together.

The analysis was conducted using the program SAMOVA (spatial analysis of molecular variation) [31]. This approach uses a simulated annealing procedure to identify K groups of populations that are geographically homogenous and maximally differentiated from one another. Based on this simulated annealing procedure, the SAMOVA algorithm iteratively seeks the population composition of a user-defined a priori number of groups (K) that aims to maximize the proportion of total genetic variance (F_{CT}) due to differences between population groups. The software was run with default parameters. The number of initial conditions was set to 500 with K = 2–4. The largest F_{CT} was used to determine the most likely K.

The genetic relationship among populations was further analyzed by principle coordinate analysis (PCoA) using GenAlEx v. 6 [32] based on Fst values generated by ARLEQUIN [33]. The hierarchical distribution of genetic variation was characterized using analysis of molecular variance (AMOVA) as implemented in ARLEQUIN 3.11.

Three level AMOVAs were conducted between the groups of populations defined by SAMOVA and the significance was tested via 10,000 ranom permutations of samples between groups.

To check for isolation by distance (IBD), a Mantel correlation test [34] was used. Significance of correlations between pairwise geographic distances and pairwise genetic distance was tested with 9999 permutations, as implemented in GenAlEx v. 6 [32].

Regression analyses used to infer relationships between longitude and frequency of occurrence of haplotypes A and B of cpDNA in studied populations were conducted using GraphPad software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

After mapping the variation at both *nad5-4* (mitochondrial DNA, mtDNA) and *psbC* (chloroplast DNA, cpDNA) within the studied area (Poland, Ukraine, Romania), a total of four different haplotypes were identified. These haplotypes of both mtDNA and cpDNA were geographically specific for *A. alba* individuals from the entire natural range, as was previously described by Liepelt et al. [17].

3.1. mtDNA

According to Liepelt et al. [17], the analysis of polymorphisms in mitochondrial *nad5-4* fragments is based upon the insertion/deletion of 80 bp, which identified two haplotypes of different lengths: 230 bp and 150 bp. In individuals of the *A. alba* populations from the western natural range, representing the Apennine refuge, the allele was 80 bp longer than that detected in the individuals of fir populations from the eastern part of the range, representing the Balkan refugium. Based on the distribution of both mtDNA haplotypes (allele 150 bp and allele 230 bp), the origin of fir populations was determined within Polish territory and also the Ukrainian and Romanian territory. Our study using the maternally inherited marker clearly showed that *A. alba* migrated into the Polish area only from the Apennine refugium. Thus, all Polish populations of *A. alba* were fixed for the 230 bp haplotype that was specific to this refugium. Additionally, only the 230 bp haplotype was identified in the Ukraine1 population, whereas the other two populations—Ukraine2 and Romania—had only the 150 bp haplotype, representing the Balkan origin (Table 1).

3.2. cpDNA

The restriction polymorphism of the paternal inheritance marker located in the *psbC* gene of the chloroplast genome, as *nad5-4*, was characterized by a specific restriction pattern, which defined the origins of the postglacial geographical distributions of *A. alba* individuals [17]. Participation of particular haplotypes of cpDNA was very different in the studied populations, and across all tested populations, both A and B haplotypes were observed simultaneously (Table 1, Figure 2). The frequency of the occurrence of haplotype A, characteristic for a Balkan refugium, contributed in the range from 15% to 85%, whereas the contribution of haplotype B, specific for an Apennine refugium, ranged from 20% to 80%. Each haplotype occurred at a similar average frequency: 47% for the Balkan refugium and 53% for the Apennine refugium. Significant correlations were found between the longitude and the frequency of occurrence of haplotype A ($r^2 = 0.1788$, $p < 0.0001$) and between the longitude and the frequency of occurrence of haplotype B ($r^2 = 0.1722$, $p < 0.0001$).

3.3. Genetic Structure

To investigate the genetic structure of *A. alba* populations, SAMOVA was applied to define the groups and to identify any locations of genetic uniqueness among the 84 populations (81 polish populations, two Ukrainian populations, one Romanian population). For the combination of mtDNA and cpDNA haplotypes, an assumption with two groups (K = 2) displayed the greatest value of F_{CT} and the maximum variance ($F_{CT} = 0.664$; $p < 0.001$). The groups at K = 2 exactly matched the geographical distribution of haplotypes of both mtDNA and cpDNA in accordance with their refugium origin. The Polish populations and the Ukraine1 population formed one group, and the Ukraine2 and Romania populations formed the other.

With this grouping, AMOVA partitioned 66.4% ($p < 0.001$) of the mitochondrial and chloroplast genetic variation between the groups, 1.66% ($p < 0.001$) among the populations within the groups and 31.94% ($p < 0.001$) within the populations; ϕST between the two groups was 0.680 ($p < 0.001$).

The relationships between examined populations of *A. alba* are well illustrated in the PCoA plot where, two distinct groups were identified, such as the SAMOVA analysis (Figure 3). The first and second principal coordinates accounted for 62.08% and 27.03% of the total variation, respectively. Populations from Poland (1–81) and Ukraine1 (82) were grouped together because these populations had only allele 230 bp representing Apennine refugium, while the populations Ukraine2 (83) and Romanian (84) are supposed to originate from the Balkan refugium and allele 150 bp was fixed; due to that, the separate group was created.

Moreover, the Mantel test of isolation by distance (IBD) showed that among populations, differentiation increased significantly with the logarithm of geographical distance expressed in km ($R = 0.465$, $p = 0.01$), although the linear regression explained only 21% of the total variance ($R^2 = 0.210$).

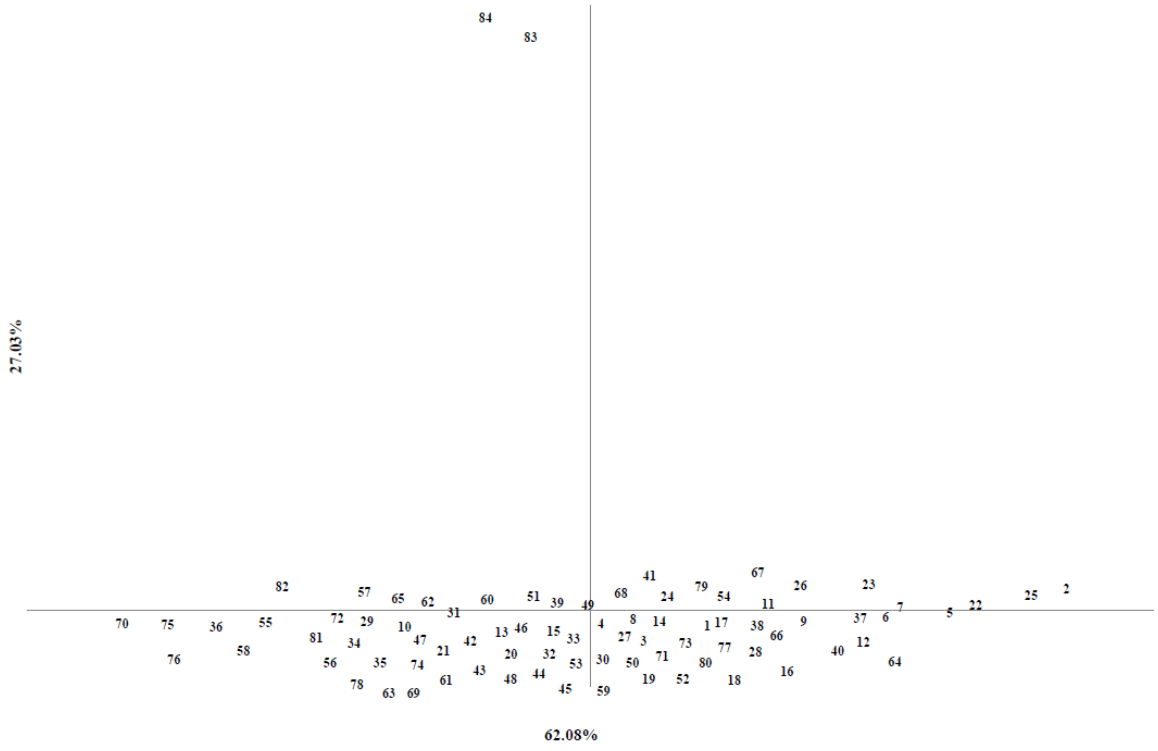

Figure 3. Results of Principal Component Analysis based on mitochondrial and chloroplast haplotypes in the study populations of *Abies alba.* The population codes are shown in Table 1.

4. Discussion

To better understand the genetic structure of extant populations, the historical processes that shaped the current natural range, and the adaptive potential of a species in the future, under the intensification of global climate change and the fragmentation of natural habitats caused by human activities, and the conservation of the genetic resources of a species, it is therefore important to have information concerning the postglacial phylogeography, evolution and expansion. In this study, more detailed and comprehensive information was provided for the distribution of the postglacial lineage of *Abies alba* in the entire territory of Poland. The results were compared with previously inferred postglacial histories of *A. alba* (e.g., [16,17,19,22,35]) and analyzed in relation to predictions regarding the direction and intensity of introgression using markers experiencing different rates of gene flow [10].

Within the Quaternary history of trees, the Mazovian and Eemian interglacials were significant periods of dominance for *A. alba* in Europe and also in Poland, and the species was widely dispersed,

although this was never repeated in subsequent periods [36]. Seeds of *A. alba* are heavier than those of most other European conifers, which was most likely one reason that in the Holocene, *A. alba* was one of the last components to enter the developing spruce–beech forests in Poland. Based on palynological data, the primary postglacial *A. alba* migration to Polish territory began towards the end of the Atlantic period, approximately 3500 Before Present (BP), from the southwest refugial region and also from the southeast refugium somewhat later, with most migration between 2500 BP and 2000 BP [21,37,38].

The distributions of the haplotypes of maternally inherited markers are informative and suggest areas that may have served as glacial refugia and the subsequent dispersal routes from them. When discussing the outcomes generated by genetic analyses using only maternally inherited mitochondrial DNA (mtDNA) *nad5-4*, what is actually tracked is only the local dispersion of seeds from one generation to the next generation. In this study, the Polish gene pool indicated that *A. alba* was derived from the Apennine Peninsula as the only putative maternal glacial refugium. The results of this study confirmed those obtained by Pawlaczyk et al. [24]. However, those authors analyzed only 10 populations of *A. alba* from southwestern Poland, which is not a representative sample. Our study was much more comprehensive because many populations of *A. alba* trees were surveyed, providing continuous coverage of the entire natural range in Poland. Additionally, the trends observed in this study corresponded closely with those observed in previous studies that used the large extent of complementary genetic data from a wide-range of *A. alba* populations using this same maternally inherited mitochondrial marker [17,39–41]. In those studies, in mixed populations of this species, the two haplotypes corresponding to the postglacial origins (Apennine and Balkans) were fixed and identified Croatia, Slovenia and northeastern Italy [17] and the northern Carpathians [40]. By contrast, in the populations of *A. alba* to the west and east of the mixing zone, a single haplotype was preserved. Those authors concluded that the participation of the western refugium in the recolonization process was greater than that of the eastern one. Despite the two maternal lineages, two or even more refugial populations might have existed during the last glacial maximum (LGM) on the Balkan Peninsula, as indicated by the strong differentiation between the southern Balkans and the Romanian Carpathians and the occurrence of regionally specific alleles [19,42,43].

However, analyses based on only variation in a mitochondrial marker are not sufficient because they do account for the possible participation of a pollen pool of origin in creating the Polish populations of *A. alba*. Because the wind-borne pollen of *A. alba* typically moves farther than the seeds, the potential for gene flow is higher for paternally inherited chloroplast markers (cpDNA) and bipaternally inherited nuclear genes (nDNA) than that for mtDNA, which is low. The analyses of the variation of cpDNA across study populations of *A. alba* depicted co-existing postglacial origins on the Apennine and Balkan peninsulas, with similar levels of participation in creating the gene pool of this species in Poland. The haplotype characterized for the Balkan Peninsula was fixed at 100% in only the reference populations from Ukraine2 and Romania. It was linked with the clear geographic trend that corresponded to the Holocene history of *A. alba*, as observed by Liepelt et al. [17].

During the postglacial migration of many taxa, including tree species, the mixing of divergent genetic lineages is a common phenomenon, which is increasingly regarded as an important evolutionary force that creates new opportunities for range expansion (see review [44,45]). Nevertheless, the importance of genetic admixture has been the subject of recent debate because nonadmixed or even severely bottlenecked populations often maintain the same level of genetic variation as an admixed one (see review [44]). To date, the broad introgression zones of divergent lineages of *A. alba* have been identified employing different systems of markers [17,19,40]. In areas both west and east of the Great Hungarian Plane in the Ukrainian Carpathians and in Bosnia and Croatia, individuals of *A. alba* representing the Apennine and Balkan lineages came into contact and interbred. In our study, with the data from the combination of two genetic markers with different variability and modes of inheritance (mtDNA and cpDNA), we reconstructed detailed patterns of postglacial colonization of Polish populations of *A. alba*. Apparently, the significant geographical structuring across studied *A. alba* populations as indicated by SAMOVA and PCoA, and also the positive Mantel

test, was consistent with the scenario of its postglacial migration to Polish, Ukrainian, and Romanian territories from two glacial refugia. The Polish populations that were fixed for one mitochondrial haplotype were most likely settled from the Apennine refugium, which indicated that migration of seeds did not contribute to the introgression. By contrast, the results in this study for cpDNA were consistent with strong gene flow via pollen, and the secondary contact between the two lineages of *A. alba* populations led to the homogenization of genetic structure primarily by the transport of only pollen from the Balkan refugium after isolation during the LGM. Such an exchange of genetic information between refugia would produce hybrid individuals with the creation of new genotypic combinations resulting in transgressive phenotypes well outside the paternal norms that are widely considered to be better adapted to new, local environmental conditions [46–48]. Moreover, the results of this study did not support the prior presumption concerning the distinctiveness of the Sudeten and Carpathian *A. alba* populations based on previous studies using isoenzyme markers [22,23] and provenance experiments [49–51]. Hence, the Sudeten area was colonized by *A. alba* individuals derived from the refugium located in northern Italy and the eastern Carpathians by the populations from the Balkan refugial region [37].

As mentioned previously, according to earlier studies of the Holocene history of *A. alba*, a broad suture zone and repeated contact during postglacial expansion are indicated (e.g., [17,19,40]). However, the novel results obtained by Bosela et al. [27] based on the combination of dendroecological and genetic data (mtDNA) from many *A. alba* sites suggest that the pollen-mediated gene flow between lineages may not have been sufficient to significantly influence the phenotypic traits associated with the climate sensitivity of the populations near the contact zone. These authors suspect that the migration pathway was narrow in the contact zone and that *A. alba* populations on both sides were highly fragmented, with the mixing of lineages only recently. This scenario is consistent with the conclusions of Gömory et al. [41]. Moreover, they found that the Balkan populations possessed a higher degree of genetic variation and more regular growth dynamics and also resistance to air pollution compared with the Apennine populations. Therefore, to promote resilience and adaptation and to ensure and preserve the sustainability of forest ecosystems, an understanding of the suture zone is essential and of practical relevance, not only for gene conservation but also for the transfer of forest reproductive materials and reforestation.

5. Conclusions

The present geographical distribution of forest tree species, as well as their genetic structure, are the consequence of the last ice age and postglacial migration from refugia in southern Europe [1].

The use of two different types of molecular markers, mitochondrial (mtDNA) and chloroplast (cpDNA), with opposite modes of inheritance, allowed us to explore the Holocene history of *Abies alba* in Poland and the adjacent regions (as reference populations). The mtDNA and cpDNA data clearly demonstrated that the Polish gene pool of this species is a mix of *A. alba* individuals derived from two distinct refugia located in the Apennine and Balkan Peninsulas. However, the mixing of gene pools took place in a specific way. In the maternal line (seed dispersion), *A. alba* is derived only from the Apennine refugium, while in the paternal line (dispersion of pollen) from two refugia—Apennine and Balkan. This result points to the distant and efficient transport of pollen originating from the Balkan refugium (Romanian Carpathian region), especially at the preliminary stage of formation of the gene pool of *A. Alba* in Poland after the last glaciation.

Additionally, a precise assessment of the Holocene history of *A. alba* populations might help to develop appropriate conservation strategies, because plants of a different post-glacial origin display significant differences in the levels of genetic variation and, consequently, such differences are also observed in their growth variability and climate sensitivity.

Acknowledgments: The National Centre of Science (Poland) (grant no. N N309 79430) supported this study. The Institute of Dendrology of the Polish Academy of Sciences provided additional funding. The authors thank M. Ratajczak and A. Misiorny for providing expert technical support. All authors have read and approved the final

manuscript. We also thank Euforgen network for providing distribution map of *Abies Alba* Mill. and anonymous reviewers for constructive comments on the paper.

Author Contributions: M.L. and A.L. conceived and designed the experiments; M.L. performed the experiments; M.L. analyzed the data; G.R. contributed and designed the collection of plant materials; M.L. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hewitt, G.M. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* **1996**, *58*, 247–276. [CrossRef]
2. Hu, F.S.; Hampe, A.; Petit, R.J. Paleoecology meets genetics: Deciphering past vegetational dynamics. *Front. Ecol. Environ.* **2009**, *7*, 371–379. [CrossRef]
3. Magri, D.; Vendramin, G.G.; Comps, B.; Dupanloup, I.; Geburek, T.; Gömöry, D.; Latałowa, M.; Litt, T.; Paule, L.; Roure, J.M.; et al. A new scenario for the quaternary history of European beech populations: Palaeobotanical evidence and genetic consequences. *New Phytol.* **2006**, *171*, 199–221. [CrossRef] [PubMed]
4. Huntley, B.; Birks, H.J. *An Atlas of Past and Present Pollen Maps Europe: 0–13000 Years Ago*; Cambridge University Press: Cambridge, UK, 1983.
5. Bennett, K.D.; Tzedakis, P.C.; Willis, K.J. Quaternary refugia of north European trees. *J. Biogeogr.* **1991**, *18*, 103–115. [CrossRef]
6. Willis, K.J.; Niklas, K.J. The role of Quaternary environmental change in plant macroevolution: The expectation or the rule? *Philos. Trans. R. Soc. B* **2004**, *359*, 159–172. [CrossRef] [PubMed]
7. Hewitt, G.M. Genetic consequences of climatic oscillations in the Quaternary. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2004**, *359*, 183–195. [CrossRef] [PubMed]
8. Avise, J.C. Phylogeography: Retrospect and prospect. *J. Biogeogr.* **2009**, *36*, 3–15. [CrossRef]
9. Hewitt, G.M. The genetic legacy of the quaternary ice ages. *Nature* **2000**, *405*, 907–913. [CrossRef] [PubMed]
10. Wagner, D.B. Nuclear, chloroplast, and mitochondrial DNA polymorphisms as biochemical markers in population genetic analyses of forest trees. *New For.* **1992**, *6*, 373–390. [CrossRef]
11. Palmer, J.D. Mitochondrial DNA in plant systematics: Applications and limitations. In *Molecular Systematics of Plants*; Springer: New York, NY, USA, 1992; pp. 36–49.
12. Hamrick, J.L.; Godt, M.J.W.; Sherman-Broyles, S.L. Factors influencing levels of genetic diversity in woody plant species. *New For.* **1992**, *6*, 95–124. [CrossRef]
13. Jaramillo-Correa, J.P.; Beaulieu, J.; Bousquet, J. Contrasting evolutionary forces driving population structure at expressed sequence tag polymorphisms, allozymes and quantitative traits in white spruce. *Mol. Ecol.* **2001**, *10*, 2729–2740. [CrossRef] [PubMed]
14. Petit, R.J.; Duminil, J.; Fineshi, S.; Hampe, A.; Salvini, D.; Vendramin, G.G. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Mol. Ecol.* **2005**, *14*, 689–701. [CrossRef] [PubMed]
15. Litkowiec, M.; Dering, M.; Lewandowski, A. Utility of two mitochondrial markers for identification of *Picea abies* refugial origin. *Dendrobiolgy* **2009**, *61*, 65–71.
16. Terhürne-Berson, R.; Litt, T.; Cheddadi, R. The spread of *Abies* throughout Europe since the last glacial period: Combined macrofossil and pollen data. *Veg. Hist. Archaeobot.* **2004**, *13*, 257–268. [CrossRef]
17. Liepelt, S.; Bialozyt, R.; Ziegenhagen, B. Wind-dispersed pollen mediates gene flow among refugia. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14590–14594. [CrossRef] [PubMed]
18. Litkowiec, M.; Lewandowski, A. Postglacial history of silver fir (*Abies alba* Mill.) in Poland as a result of migration from different refugia—The current state of knowledge. *Sylwan* **2015**, *159*, 109–116.
19. Konnert, M.; Bergmann, F. The geographical distribution of genetic variation of silver fir (*Abies alba*, *Pinaceae*) in relation to its migration history. *Plant Syst. Evol.* **1995**, *196*, 19–30. [CrossRef]
20. Zając, A.; Zając, M. *Atlas of Distribution of Vascular Plants in Poland*; W. Szafer Institute of Botany PAN: Kraków, Poland, 2001.
21. Obidowicz, A.; Szczepanek, K.; Madeyska, E.; Nalepka, D. *Abies alba* Mill.—Fir. In *Late Glacial and Holocene History of Vegetation in Poland Based on Isopollen Maps*; Ralska-Jasiewiczowa, M., Latałowa, M., Wasylikowa, K., Tobolski, K., Madeyska, E., Wright, H.E., Jr., Turner, C., Eds.; W. Szafer Instiute of Botany PAN: Kraków, Poland, 2004; pp. 31–38.

22. Lewandowski, A.; Filipiak, M.; Burczyk, J. Genetic Variation of *Abies alba* in polish part of Sudety Mts. *Acta Soc. Bot. Pol.* **2001**, *70*, 215–219. [CrossRef]
23. Mejnartowicz, L. Genetic Analysis of Silver fir Populations in the North Carpathian and Sudeten Mountains. *Acta Soc. Bot. Pol.* **2004**, *73*, 285–292. [CrossRef]
24. Pawlaczyk, E.M.; Kroplewska, I.; Bobowicz, M.A. Postglacial migration of silver fir (*Abies alba* Mill.) to Poland—Analysis on the basis of mitochondrial DNA polymorphism. *Sylwan* **2013**, *157*, 458–463.
25. Larsen, J.B. Das Tannensterben: Eine neue hypothese zur Klärung des Hintergrundes dieser rätselhaften Komplexkrankheit der Weißtanne (*Abies alba* Mill.). *Forstwiss. Cent.* **1986**, *105*, 381–396. [CrossRef]
26. Bergmann, F.; Gregorius, H.R.; Larsen, J.B. Levels of genetic variation in European silver fir (*Abies alba*). Are they related to the species' decline? *Genetica* **1990**, *82*, 1–10. [CrossRef]
27. Bosela, M.; Popa, I.; Gömöry, D.; Longauer, R.; Tobin, B.; Kyncl, J.; Kyncl, T.; Nechita, C.; Petras, R.; Sidor, C.G.; et al. Effects of post-glacial phylogeny and genetic diversity on growth variability and climate sensitivity of European silver fir. *J. Ecol.* **2016**, *104*, 716–724. [CrossRef]
28. Dumolin, S.; Demesure, B.; Petit, R.J. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theor. Appl. Genet.* **1995**, *91*, 1253–1256. [CrossRef] [PubMed]
29. Demesure, B.; Sodzi, N.; Petit, R.J. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* **1995**, *4*, 129–131. [CrossRef] [PubMed]
30. Wakasugi, T.; Tsudzuki, J.; Ito, S.; Nakashima, K.; Tsudzuki, T.; Sugiura, M. Loss of all ndh genes as determined by sequencing the entire chloroplast genome of black pine *Pinus thunbergii*. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 9794–9798. [CrossRef] [PubMed]
31. Dupanloup, I.; Schneider, S.; Excoffier, L. A simulated annealing approach to define the genetic structure of populations. *Mol. Ecol.* **2002**, *11*, 2571–2581. [CrossRef] [PubMed]
32. Peakall, R.; Smouse, P.E. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Resour.* **2006**, *6*, 288–295. [CrossRef]
33. Excoffier, L.; Laval, G.; Schneider, S. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinform. Online* **2005**, *1*, 47–50.
34. Mantel, N. The detection of disease clustering and a generalized regression approach. *Cancer Res.* **1967**, *27*, 209–220. [PubMed]
35. Liepelt, S.; Cheddadi, R.; de Beaulieu, J.L.; Fady, B.; Gömöry, D.; Hussendörfer, E.; Konnert, M.; Litt, T.; Longauer, R.; Terhürne-Berson, R.; 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*, 139–149. [CrossRef]
36. Srodoń, A. *Nasze Drzewa Leśne. Jodła Pospolita Abies alba Mill.*; Państwowe Wydawnictwo Naukowe: Poznań, Poland, 1983.
37. Ralska-Jasiewiczowa, M. *Late Glacial and Holocene History of Vegetation in Poland Based on Isopollen Maps*; W. Szafer Institute of Botany, Polish Academy of Science: Kraków, Poland, 2004.
38. Pidek, I.A.; Svitavská-Svobodová, H.; van der Knaap, W.O.; Magyari, E. Pollen percentage thresholds of *Abies alba* based on 13-year annual records of pollen deposition in modified Tauber traps: Perspective of applications to fossil situations. *Rev. Palaeobot. Palynol.* **2013**, *195*, 26–36. [CrossRef]
39. Ziegenhagen, B.; Fady, B.; Kuhlenkamp, V.; Liepelt, S. Differentiating groups of *Abies* species with a simple molecular markers. *Silvae Genet.* **2005**, *54*, 123–126.
40. Gömöry, D.; Longauer, R.; Liepelt, S.; Ballian, D.; Brus, R.; Kraigher, H.; Parpan, V.I.; Parpan, T.V.; Paule, L.; Stupar, V.; et al. Variation patterns of mitochondrial DNA *Abies alba* Mill. In suture zone of postglacial migration in Europe. *Acta Soc. Bot. Pol.* **2004**, *73*, 203–206. [CrossRef]
41. Gömöry, D.; Paule, L.; Krajmerová, D.; Romšáková, I.; Longauer, R. Admixture of genetic lineages of different glacial origin: A case study of *Abies alba* Mill. in the Carpathians. *Plant Syst. Evol.* **2012**, *298*, 703–712. [CrossRef]
42. Longauer, R. Genetic Diversity of European Silver Fir (*Abies alba* Mill.). Ph.D. Thesis, Technická Univerzita vo Zvolene, Zvolen, Slovakia, 1996.
43. Vendriamin, G.G.; Degen, B.; Petit, R.J.; Anzidei, M.; Madaghiele, A.; Ziegenhagen, B. High level of variation at *Abies alba* chloroplast microsatellite loci in Europe. *Mol. Ecol.* **1999**, *8*, 1117–1126. [CrossRef]
44. Rius, M.; Darling, J.A. How important is intraspecific genetic admixture to the success of colonising populations? *Trends Ecol. Evol.* **2014**, *29*, 233–242. [CrossRef] [PubMed]

45. Heuertz, M.; Hausman, J.F.; Hardy, O.J.; Vendramin, G.G.; Frascaria-Lacoste, N.; Velkemans, X. Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern European populations of the common ash (*Fraxinus excelsior* L.). *Evolution* **2004**, *58*, 976–988. [CrossRef] [PubMed]
46. Bell, M.A.; Travis, M.P. Hybridization, transgressive segregation, genetic covariation, and adaptive radiation. *Trends Ecol. Evol.* **2007**, *20*, 358–361. [CrossRef] [PubMed]
47. Żukowska, W.B.; Wachowiak, W. Utility of closely related taxa for genetic studies of adaptive variation and speciation: Current state and perspectives in plants with focus on forest tree species. *J. Syst. Evol.* **2016**, *54*, 17–28. [CrossRef]
48. Wachowiak, W.; Żukowska, W.B.; Wójkiewicz, B.; Cavers, S.; Litkowiec, M. Hybridization in contact zone between temperate European pine species. *Tree Genet. Genomes* **2016**, *12*, 48. [CrossRef]
49. Gunia, S. Próba oceny wartości genetycznej i hodowlanej jodły pospolitej (*Abies alba* Mill.) z Sudetów i Karpat polskich. *Sylwan* **1986**, *130*, 83–92.
50. Skrzyszewska, K. Zmienność wewnątrzgatunkowa jodły pospolitej w doświadczeniach proweniencyjnych. In *Elementy Genetyki i Hodowli Selekcyjnej Drzew Leśnych*; Sabor, J., Ed.; Centrum Informacyjne Lasów Państwowych: Warszawa, Poland, 2006.
51. Barzdajn, W. Adaptacja różnych pochodzeń jodły pospolitej (*Abies alba* Mill.) do warunków Sudetów. *Leśn. Pr. Badaw.* **2009**, *70*, 49–58.