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Genetic Consequences of Hybridization in Relict Isolated Trees *Pinus sylvestris* and the *Pinus mugo* Complex

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Abstract: Scots pine (*Pinus sylvestris* L.) and the taxa from the *P. mugo* complex can hybridize in the contact zones and produce fertile hybrids. A unique example of an early Holocene relict population of *P. sylvestris* and *P. uliginosa* (a taxon from the *P. mugo* complex) growing on the tops of Jurassic sandstone rocks is located in Błędne Skały (Sudetes). Phenotypically, there are trees resembling *P. sylvestris*, *P. uliginosa* and intermediate forms between them. We expected that some of *P. sylvestris* and/or *P. uliginosa*-like trees could be in fact cryptic hybrids resembling one of the parental phenotypes. To address this question, we examined randomly sampled individuals, using a set of plastid (cpDNA), nuclear (nDNA) and mitochondrial (mtDNA) markers as well as biometric characteristics of needles and cones. The results were compared to the same measurements of allopatric reference populations of the *P. sylvestris* and the *P. mugo* complex (*Pinus mugo s.s*, *P. uncinata* and *P. uliginosa*). We detected cpDNA barcodes of the *P. mugo* complex in most individuals with the *P. sylvestris* phenotype, while we did not detect cpDNA diagnostic of *P. sylvestris* within *P. uliginosa*-like trees. These results indicate the presence of cryptic hybrids of the *P. sylvestris* phenotype. We found only three typical *P. sylvestris* individuals that were clustered with the species reference populations based on needle and cone characteristics. Most trees showed intermediate characteristics between *P. sylvestris* and *P. uliginosa*-like trees, indicating intensive and probably long-lasting hybridization of the taxa at this area and subsequent gene erosion of parental species.

Keywords: biostatistics; hybridization; gene flow; genetic variation; molecular markers; morphological variation; plant variation

1. Introduction

Hybridization and introgression are important evolutionary factors that increase species variation and may lead to speciation [1–6]. These two processes concern mostly related species, which do not express isolation mechanisms such as phenological barriers [7] and post-pollination barriers to fertilization [8]. The interbreeding between different species of *Pinaceae* was found to be quite

frequent [9–12]. Considering their long generation time and lifespan, the relatively recent divergence between dwarf mountain pine (*P. mugo* Turra) and Scots pine (*P. sylvestris* L.) [13] resulted in their similar genetic variation at neutral loci [14]. The hybrids between the taxa of the *P. mugo* complex and *P. sylvestris* in the wild state have been traditionally reported as morphologically intermediate individuals ([15,16] and the literature cited herein). The biometric studies were conducted to verify the presence of hybrid individuals or even populations [17–25]. However, the unequivocal identification of hybrids is difficult based on morphological and anatomical characteristics alone.

During the last few decades, isoenzymatic and molecular analyses were used to estimate the potential hybridization between the taxa of the *P. mugo* complex and *P. sylvestris* [26–33]. The possibility of hybridization was also investigated by artificial crossing experiments ([10,34,35] and the literature cited herein). The results indicated the higher success of pollination, when taxa of the *P. mugo* complex were the pollen donors, and possible back-crossing of hybrids with both parental species. However, a more complete picture of interspecific gene flow within natural populations of hybridizing taxa could be obtained by combining biometric, biochemical and molecular methods [19,30,36–38].

To date, studies on hybridization between the *P. mugo* complex and *P. sylvestris* have been conducted in different types of the species contact zones including the small and isolated populations of one taxon surrounded by extensive, massive populations of the other [10,25,26,30] or between relatively large populations of both species [31]. However, to our knowledge, hybridization has not been investigated in small, relict and isolated populations of the taxa. Such stands are rare but are especially valuable, as relict populations may conserve products of hybridization and introgression repeated over many generations without or with only very restricted gene inflow from outside. A population of this type is located in Błędne Skały in the Stołowe Mountains (Sudetes). Pines grow there on the tops of Jurassic sandstone rocks, in hardly accessible places, which have not been exploited for wood and are unsuitable for pasturing [39,40]. This mixed stand of pines originated most probably from the Boreal period of the Holocene. The pollen of *P. sylvestris* type, which may also include very similar and indistinguishable pollen of the *P. mugo* complex [41], appeared during that time in high frequencies in the Sudetes [42,43], and was detected also on peat-bogs nearest to the Stołowe Mountains [44,45]. The early Holocene expansion of broad-leaved trees and Norway spruce (*Picea abies* L.), which formed dense and shady forests, reduced occurrence of light demanding *P. sylvestris* and *P. mugo* to the sites where conditions were inaccessible for other forest tree species such as *Corylus*, *Ulmus, Alnus* and *Picea* [44–46]. Consequently, pines could survive in the Sudetes only on the rocks at the tops of the mountains and/or the rocky edges of their plateau. The best examples of such sites in the Stołowe Mountains are the sandstone labyrinths on the tops of Szczeliniec Wielki and Skalniak mounts. Regarding Błędne Skały location, there are currently growing individuals phenotypically resembling *P. sylvestris*, *P. mugo s.s.* and *P. uliginosa* G. E. Neumann [47]. The pollen record of *P. sylvestris* type proves the persistent character of pine population in the Stołowe Mountais during the entirety of the Holocene [44,45,48], and even reflects the temporal expansion of pines after fires in the High Middle Ages and Early Modern period (see Table 2 and Figure 5 in [48]).

Pines have high pollen production and dispersal ability [49–51], however, most pollen grains fall in a distance up to a few hundred meters from the source tree [52]. Therefore, considering the species biology, success of pollination and the recognized mating system [52,53], the inflow of pollen from outside Błędne Skały population was most likely very limited from the boreal time in the case of rare *P. uliginosa* (the species from the *P. mugo* complex) and at least significantly reduced in the case of much more common *P. sylvestris*. Thus, currently the observed mixed population of both taxa, which consist of individuals phenotypically resembling typical species and intermediate forms, was formed and has persisted locally over the last 7000–8000 years. In this context, the population in Błędne Skały is a unique and very interesting model for hybridization and gene flow studies.

In the presented study, we investigated the hybridization processes in the pine population in Błędne Skały to verify (i) if there are any hybrids among *P. sylvestris*-like and/or *P. uliginosa*-like trees which resemble one of the parental trees; (ii) if there are any patterns of successful hybridization

as compared to some earlier works (iii) to what extent the level of genetic variation of this small and isolated population is comparable to variation in much bigger reference populations of pure species. To address those questions, we sampled individuals across the phenotypic forms present in this population and performed genetic and biometric analyses to verify taxonomic relationships between the samples and to compare morphology of trees from the mixed population to the reference populations.

2. Materials and Methods

2.1. Material

The mixed pine population in Błędne Skały (BS) occurs on the upper parts of sandstone rocks and is located at the summit of the north-western part of the Skalniak ridge in the Stołowe Mountains, at an elevation of 850 m a.s.l. It consists of dispersed pine individuals of *P. sylvestris* and *P. uliginosa* phenotype, with a few individuals resembling *P. mugo s.s.* and a number of individuals of intermediate phenotype. The pine population is surrounded by extensive *Picea abies* forests, which isolate it from direct contact with other pine populations.

Material for the study was taken randomly from 64 individuals in an area of about 2 ha. The sampled individuals were about 20 m distance from each other or growing on separate rocks. During sampling, part of the individuals was preliminary identified in the field based on growth form, bark color on the upper part of stem, needle color and shape and setting angle of the one-year-old conelet [15,16]. The sampling included 15 individuals of *P. sylvestris*-like phenotype (BS_S), 17 individuals resembling the taxa from the *P. mugo* complex namely *P. uliginosa* (BS_M), 7 individuals of intermediate character between *P. sylvestris* and *P. uliginosa* classified as hybrids (BS_H) and another 25 unclassified individuals (BS_N). As the sampled trees could potentially represent a genetic background of different pine species, we included a set of reference populations taken from across current natural ranges of *P. sylvestris* and the taxa of the *P. mugo* complex (*P. mugo s.s.*, *P. uliginosa* and *P. uncinata* Ramond (Table 1; Figure 1). These populations have been used in previous morphometric [54–61] and genetic [30,62–65] studies. Particular analyses were conducted using all taxa, but not always the same reference populations.

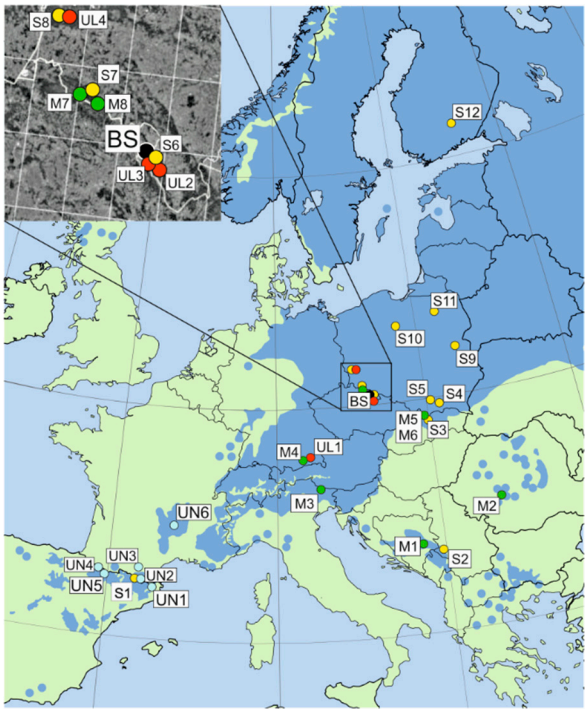

Figure 1. Geographic position of Błędne Skały mixed population (BS) and samples of the reference species—*Pinus sylvestris* (S), *P. mugo s.s.* (M), *P. uliginosa* (UL) and *P. uncinata* (UN); population acronyms as in Table 1.

Table 1. Plant material used in the study.

Species	Code	Location	Latitude N	Longitude E	Altitude (m)	N_b (needles/cones)	N_g
Mixed BS	BS	Błedne Skały, Stołowe Mountains (Poland)	50.48	16.29	850	64 (320/315)	64
Pinus sylvestris S	S1	St. Miguel d'Engolasters (Andorra)	42.51	1.56	1550	-	32
	S2	Divčibare Mts. (Serbia)	44.10	19.99	957	-	26
	S3	Tatra National Park (Poland)	49.27	19.83	1000	-	12
	S4	Beskid (Poland)	49.40	20.82	600	-	45
	S5	Pieniny National Park (Poland)	49.42	20.36	800	-	21
	S6	Stołowe Mountains (Poland)	50.43	16.23	900	30 (300/0)	
	S7	Sudetes, Karkonosze (Poland)	50.83	15.64	600	-	20
	S8	Silesian Lowland, Węgliniec (Poland)	51.28	15.23	170	34 (340/46)	
	S9	Liski Nature Reserve (Poland)	51.93	22.82	150	-	11
	S10	Bory Tucholskie Forest, (Poland)	53.32	17.94	90	50 (0/50)	
	S11	Sosny Taborskie Reserve (Poland)	53.78	20.02	115	-	13
	S12	Joutsa (Finland)	61.76	26.10	125	-	25
Pinus mugo M	M1	Dinaric Alps (Bosnia and Herzegovina)	43.72	18.24	2020	-	25
	M2	Carpathians, Fagaraş (Romania)	45.61	24.60	1900	-	25
	M3	Alps, Carnic Alps, Pramollo (Italy)	46.56	13.28	1600	-	25
	M4	Ammergau Alps (Germany)	47.53	10.92	1870	-	25
	M5	Tatra National Park (Poland)	49.12	20.05	1700	30 (300/330)	12
	M6	Tatra National Park, Grześ (Poland)	49.23	19.77	1620	-	25
	M7	Sudetes (Poland)	50.78	15.58	1350	33 (330/310)	30
	M8	Sudetes, Śnieżka (Poland)	50.74	15.74	1435	-	30
Pinus uliginosa UL	UL1	Alps, Mittelwalde (Germany)	47.48	11.27	860	-	20
	UL2	Zieleniec Nature Reserve (Poland)	49.13	20.03	700	30 (300/50)	
	UL3	Large Batorów Peatland (Poland)	50.45	16.38	750	50 (500/50)	31
	UL4	Węgliniec Nature Reserve (Poland)	51.29	15.23	190	52 (520/230)	30
Pinus uncinata UN	UN1	Vall de Nuria (Spain)	42.22	02.10	2200	28 (280/50)	
	UN2	Vall de Ransol (Andorra)	42.38	01.37	2000	32 (320/49)	
	UN3	Coll de Jau (France)	42.69	2.25	1500	-	24
	UN4	Belagua (Spain)	42.97	-0.77	1760	-	24
	UN5	La Trapa near Jaca (Spain)	42.69	-0.54	1720	-	22
	UN6	Col de la Croix-Morand (France)	45.60	2.85	1400	-	14

N_b—number of trees used in biometric analysis; N_g—number of trees used in genetic analysis.

2.2. DNA Extraction, Amplification and Marker Genotyping

Genomic DNA was isolated from dry needles, using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA quality was evaluated using a BioPhotometer (Eppendorf, Hamburg, Germany). The samples were analyzed with a set of organellar and nuclear markers that show different modes of inheritance. Polymorphisms at 14 mitochondrial regions, inherited in pines in the maternal line and distributed at short geographical distances by seeds, were investigated including 13 markers [66], and the insertion–deletion (IN–DEL) locus *nad1* intron B/C [67]. The standardized 15 µL PCR reaction mix contained 1 × PCR buffer, 1X bovine serum albumin (BSA), 1.5 µM $MgCl_2$, 40 µM dNTP mix, 0.2 µM of both forward and reverse primers (Genomed, Warsaw, Poland), 0.15 U Taq DNA polymerase (Novazym, Poznań, Poland) and 15–20 ng of DNA template. Reaction conditions were unified with 3 min of initial denaturation at 94 °C, 35 cycles with 30 s denaturation at 94 °C, 30 s annealing at 57°C and 1 min 20 s

elongation at 72 °C, with the final extension for 5 min at 72 °C. Variants were genotyped by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and digested in 10 µL reaction volume, containing 3 µL of the PCR product, 2X buffer and 2.5 U of the relevant restriction enzyme [68]. Samples were incubated for at least 10 h, following the manufacturer´s recommendations for respective enzymes (Thermo Fisher Scientific, Waltham, MA, USA), and electrophoretically separated on 1.5%–2% agarose gel, stained with GelRed in UV. The insertion–deletion polymorphisms in intron B/C of the *nad1* locus was amplified and scored according to the methods described in Soranzo et al. [67]. The region contains shorter variant *a* that appears to be mostly fixed in *P. mugo* s.s. populations and it segregates with different frequency across the geographical distribution of *P. sylvestris*. The polymorphisms at *nad1* B/C region was scored by 2% agarose gel electrophoresis of the corresponding PCR-amplified fragment.

Variation at the plastid genome, which is paternally inherited in these taxa and transmitted by pollen, was studied at a set of 12 microsatellite markers. Two multiplex reactions of the chloroplast simple sequence repeats (cpSSR) loci were run, including regions PCP1289, PCP26106, PCP30277, PCP36567, PCP41131, PCP45071, PCP87314, PCP102652 (multiplex 1) and Pt15169, Pt26081, Pt30204, Pt71936 (multiplex 2) [69]. Additionally, a species-diagnostic plastid (cpDNA) intergenic *trn*F-t*rn*L region that discriminates *P. sylvestris* from the taxa of the *P. mugo* complex was used to determine the origin of the plastid genome in samples from the focal population in Błędne Skały. This PCR-RFLP marker has previously been developed based on a single-nucleotide polymorphism that leads to an undigested PCR product for *P. sylvestris* and a digested PCR product for *P. mugo* when treated with *Dra*I enzyme [70]. The PCR was run in a total volume of 15 µL, containing 15 ng of template DNA, 10 µM of each of dNTP, 0.2 µM each of forward and reverse primer, 0.15 U Taq DNA polymerase, 1X BSA, 1.5 µM of $MgCl_2$ and 1X PCR buffer (Novazym, Poznań, Poland). Standard amplification conditions were applied, including initial denaturation at 94 °C for 3 min, followed by 35 cycles with 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 1 min 30 s extension at 72 °C, with a final 5 min extension at 72 °C.

Variation at the biparentally inherited nuclear genome was investigated using a set of nine simple sequence repeats (nSSR), including psyl2, psyl25, psyl36, psyl42, psyl44, psyl57 and ptTX3025, ptTX4001, ptTX4011, following two multiplex reactions [71]. The fluorescently labelled PCR products, obtained for the studied cpDNA and nuclear (nDNA) microsatellite regions, along with a size standard GeneScan 500 LIZ (Applied Biosystems, Foster City, CA, USA), were separated on a capillary sequencer ABI 3130 (Thermo Fisher Scientific, Waltham, MA, USA). The SSR allele size was determined using the GeneMapper software ver. 4.0 (Applied Biosystems, Foster City, CA, USA).

2.3. Diversity and Differentiation Measures

Alleles identified within each mitochondrial (mtDNA) marker were concatenated, and connections among the obtained haplotypes were analyzed by a Median Joining network constructed in PopART 1.7 [72,73]. The number of segregating sites (S), number of private variants (S_P) and the mean within group distance (d) were calculated in DnaSP V6 [74] and MEGA 7 [75] for groups of samples defined as BS and for the reference populations. Similarly, the numbers of haplotypes (N_h) and private haplotypes (N_p) present in populations and species were calculated. To better assess the scale of genetic variation at mtDNA segregating in the taxa, the haplotype frequencies and population gene diversities (H_d) were estimated in DnaSP. The relationships among samples were evaluated based on the mean distances between populations (d_{xy}), using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as implemented in MEGA. The distribution of genetic variation among populations, and among genetic structures detected in the population groups were checked by F_{st} and G_{st} calculated in DnaSP. The differences between populations were verified by hierarchical analysis of molecular variance (AMOVA), using Arlequin 3.5.22 [76].

To describe the variation of cpDNA SSR loci, for each defined sample group we calculated mean number of haplotypes (A_h), mean effective number of haplotypes (A_e), number of private haplotypes (A_P), haplotype richness (A_R), haplotype diversity (H_d) and mean genetic distance of individuals

within populations (D^2sh) using GenAlEx 6 [77]. Variation at the diagnostic *trn*F-*trn*L cpDNA region was scored as a presence or absence of a *Dra*I restriction site.

Genetic variation at nSSR of the mixed population and the reference populations was analyzed based on mean number of alleles per locus (N_a), mean effective number of alleles per locus (Ne), number of private alleles (A_p), mean observed heterozygosity (H_o), mean expected heterozygosity (H_e), mean fixation index (F). The analysis of molecular variance (AMOVA) on the R_{st} distances, as implemented in Arlequin 3.5.22, was used to evaluate the differentiation among defined groups of populations from BS contact zone and reference populations at nSSR loci. Afterward, the principal coordinates analysis (PCoA) on Nei's genetic distance was applied to visualize the genetic structure of the populations and BS group. The Bayesian clustering method implemented in STRUCTURE 2.3.4 [78–80] was used to assign individuals and populations into genetically distinct groups without a priori assumption of their origin. The STRUCTURE software uses the algorithm of Markov chain Monte Carlo (MCMC) to assign particular individuals to a number of genetic clusters (K) whose members share similar patterns of variation. It does not consider samples' origins but assumes that each cluster is in optimal Hardy–Weinberg and linkage equilibria. The parameter sets, that assumed correlated allele frequencies and the used admixture model, together allowed attribution of mixed recent ancestry of individuals and assignment of the proportion of inferred clusters' origin within each individual genome. The "recessive alleles" option was used, with 20 runs for each K, from $K = 1$ to 10, and with burn-in lengths of 500,000 and 100,000 iterations. The data probability distributions (Ln $P[D]$) and the ΔK values [81] were calculated using STRUCTURE HARVESTER Web v0.6.94 application and were used to determine optimal K for given dataset [82].

2.4. Biometrics

We characterized two-year-old needles using a set of traits (Table S5), following the procedures described by Boratyńska et al. [56] and Boratyńska and Boratyński [23]. Every individual was characterized using the average measurements of five needles, each from a different dwarf shoot (i.e., the morphological structure in Conifers from which needles in fascicles of 2–5 grow together). The anatomical characteristics were measured and/or evaluated on the preparations from the central part of the needle [21,23,55,56,83].

Cones were characterized using 11 measured characteristics and five proportions (Table S6) describing cone and cone scale shape [17,54,84]. Measurement procedures were adopted from Marcysiak [54] and Marcysiak and Boratyński [57]. Length, width, diameter and circumference measurements were taken from closed cones after soaking, while the number of cone scales was determined for open cones after drying. The scale characteristics were measured on one scale taken from the convex side of each cone at the maximum diameter. All measurements were done manually, using an electronic caliper.

2.5. Statistical Analyses of Biometric Data

Prior to multivariate comparisons, the data distribution and homoscedasticity of the variances were inspected, applying the Shapiro–Wilk's and Brown–Forsythe tests, respectively [85,86]. The arcsine transformation of percentage data was applied prior to statistical analyses. The data were standardized using the STATISTICA 9 software (StatSoftPolska, Kraków, Poland) to avoid a possible influence of their different types on the results of the multivariate analyses.

We determined the minimum and maximum values of the characteristics, arithmetic means, and variation coefficients. These parameters were calculated and analyzed for every individual in the BS population, and for reference populations of *P. sylvestris*, *P. mugo*, *P. uliginosa* and *P. uncinata*. Principal component analysis (PCA) was used to detect grouping of samples from the BS mixed population and their position among referenced populations of *P. sylvestris*, *P. mugo* and *P. uncinata.* Subsequently, discrimination analysis was conducted on these characteristics, except for the ones which were strongly biased (SRC, SRF, VB and RC – see Tables S5 and S6 for traits description) [86,87].

To assess the significance of differences for specific measured traits between groups preliminary determined in the BS population during sampling, Tukey's HSD test for traits with normal distribution and the Kruskal–Wallis test for traits with biased distribution were applied [86,88], using STATISTICA. The taxonomic position of each individual from the BS mixed population, assessed via macro-morphological traits during sampling, was verified using characteristics of the needles and cones. Finally, the results from the biometric study were compared with the results of the genetic analyses to distinguish individuals of *P. sylvestris* from the taxa from the *P. mugo* complex and their hybrids.

3. Results

3.1. Genetic Diversity and Differentiation

In total, 39 mitotypes were identified across the four pine species (Figure 2, Table S1). Species-specific combination of haplotypes was observed only in *P. uncinata*, which was distinct when compared to the other species (Figure 2, Figure S1). Different frequencies of mitotypes were observed in individual reference populations. The highest frequency of shared haplotypes was found in individuals from the mixed BS population (Table S2). In the BS pine mixed population, we observed mitotypes of *P. sylvestris*, *P. mugo s.s.* and *P. uliginosa* reference populations, but not of *P. uncinata*. Unique mtDNA haplotypes of pines from BS population were found for five individuals from hybrid or unclassified sample groups. The remaining pines shared numerous mitotypes present in individuals from BS and reference populations. The most frequent mitotypes at the BS population were found at high frequency in the *P. uliginosa* population from Węgliniec Nature Reserve (Table S1). Some unique mitotypes were found at low frequency in every examined species and BS population. The samples including four reference species and a BS population formed each separate group in the UPGMA tree based on Nei's genetic distance at mtDNA regions (Figure S2).

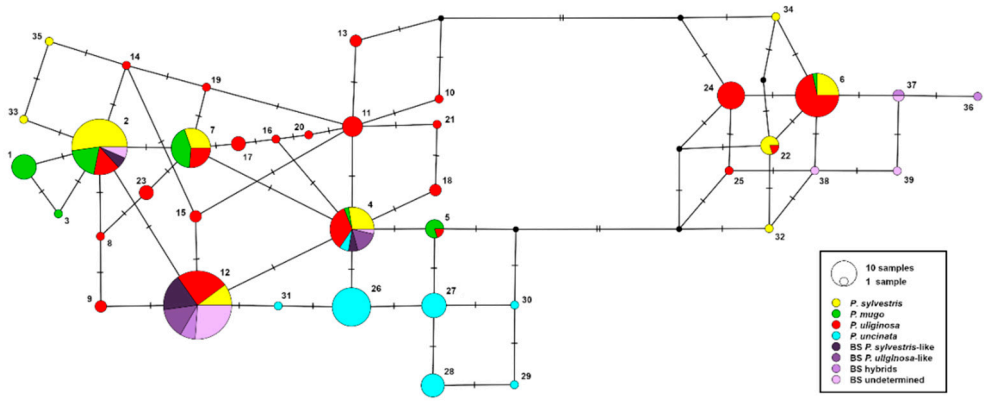

Figure 2. Median Joining network of the mitotypes of pines from the mixed population in Błędne Skały (BS) and the reference four pine species populations based on 14 mtDNA regions. Circle sizes represent relative frequencies of haplotypes in a combined sample, lines between two circles represent singular mutation events, and black dots substitute absent haplotypes (population acronyms as in Table 1).

Only three individuals from the BS population had diagnostic a cpDNA marker characteristic for *P. sylvestris*. Those three individuals were indicated as pure *P. sylvestris* based on STRUCTURE analysis of cpSSR loci. The remaining individuals from BS had plastid DNA specific to the *P. mugo* complex (i.e., *P. uliginosa*), independently of the phenotype.

Individuals from BS showed reduced genetic variation at cpSSRs, with a considerably lower number of haplotypes and lower haplotype diversity as compared to the reference species (Table 2A) and individual populations (Table S3). The individuals unclassified taxonomically and those defined as *P. sylvestris* from the BS mixed pine population showed the highest mean genetic distance between

individuals within populations (D^2sh range 32.1–51.9) as compared to the other groups in this area (7.3–9.1) and the reference populations (4.4–13.6). Haplotype and gene diversity at mtDNA markers were also lowest in the BS population (Table 2B). However, in contrast to organelle markers, variation at biparentally inherited nSSR loci was similar in the BS population as in the reference samples (Table 2C, Table S4). There was no evidence of reduced heterozygosity among samples in the BS population (Table 2C).

Table 2. Genetic variation of samples from mixed pine population in Błędne Skały and four reference pine species based on: (**A**) chloroplast microsatellite (cpSSR) loci; (**B**) mitochondrial (mtDNA) markers; (**C**) nuclear microsatellite (nSSR) loci.

(**A**)

Sample Name	Measures of Genetic Variation						
	N	**A_h**	**A_e**	**A_P**	**A_R**	**H_d**	**D^2sh**
Błędne Skały	64	19	13	15	7.56	0.881	32.1
P. mugo	105	100	99	46	95.87	0.999	13.5
P. sylvestris	128	118	118	46	109.28	0.999	5.6
P. uncinata	48	33	32	32	20.57	0.972	7.2
P. uliginosa	81	40	32	28	22.70	0.968	8.2

(**B**)

Sample Name	Measures of Genetic Variation							
	N	**S**	**S_p**	**d**	**H_n**	**H_p**	**H_d (SD)**	**$_uH$**
Błędne Skały	64	9	0	1.83	7	4	0.487 (0.071)	0.495
P. mugo	34	10	2	4.03	7	2	0.786 (0.034)	0.810
P. sylvestris	61	9	0	3.31	10	4	0.796 (0.038)	0.809
P. uncinata	44	5	1	1.06	7	6	0.687 (0.055)	0.703
P. uliginosa	101	11	1	1.54	23	16	0.904 (0.014)	0.913

(**C**)

Sample Name	Measures of Genetic Variation					
	N	**N_a**	**N_e**	**H_o**	**H_e**	**F**
Błędne Skały	63	4.78	2.34	0.425	0.467	0.046
P. mugo	105	4.78	2.06	0.386	0.460	0.142
P. sylvestris	128	7.33	2.37	0.416	0.451	0.076
P. uncinata	48	3.89	2.17	0.372	0.410	0.051
P. uliginosa	81	4.78	2.09	0.393	0.438	0.101

N—sample size, A_h—mean number of haplotypes, A_e—mean effective number of haplotypes, A_P—number of private haplotypes, A_R—haplotype richness, H_d—haplotype diversity, D^2sh—mean genetic distance of individuals within populations, S—number of segregating sites, S_P—number of private polymorphisms, d—average number of nucleotide differences between two sequences, H_n—number of haplotypes, H_p—number of private haplotypes, $_uH$—unbiased gene diversity, N_a—mean number of alleles per locus, N_e—mean effective number of alleles per locus, H_o—mean observed heterozygosity, H_e—mean expected heterozygosity, F—mean fixation index.

According to the PCoA based on genetic distance at nSSR loci, all defined groups of individuals from BS showed close genetic similarity to each other, to *P. uliginosa* from the Węgliniec Nature Reserve and to *P. uncinata* samples (Figure 3). This group of samples was distinct from the reference populations of *P. mugo s.s.* and *P. sylvestris*. Among two outlier populations detected appeared one *P. uliginosa* from the Large Batorów Peatland, with high genetic similarity to group of three *P. mugo s.s.* populations and the fourth *P. mugo* stand localized in Sudetes (M8), which was distinct as compared to all other samples analyzed (Figure 3).

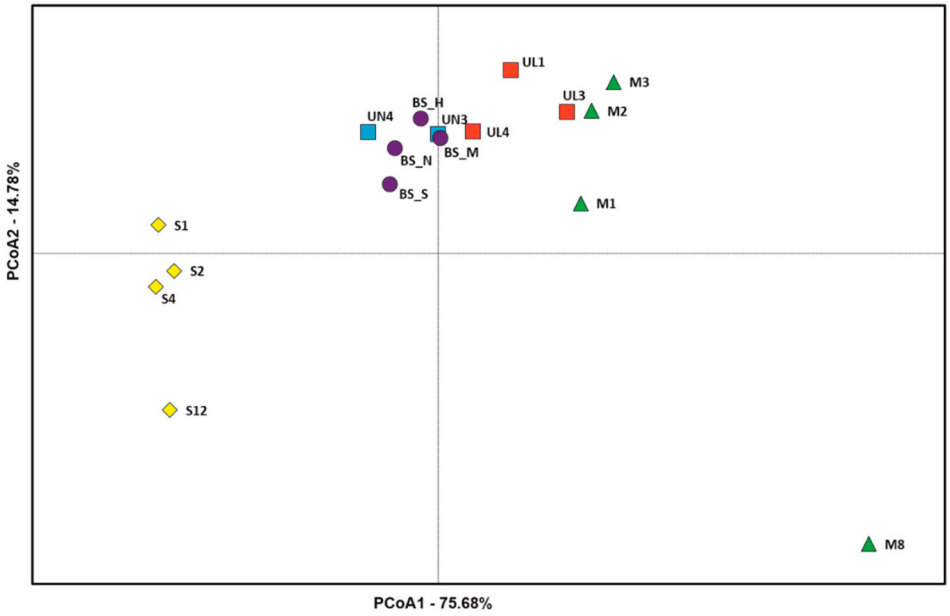

Figure 3. Principal coordinates analysis (PCoA) based on Nei genetic distance at nSSR loci showing genetic relationships between groups of samples defined at the mixed pine population from Błędne Skały (BS) and reference populations of the four pine species (population acronyms as in Table 1).

Structure analysis at nSSR loci indicated the presence of five genetically distinct groups corresponding approximately to the four reference species and one sample group from BS (Figure 4, Figure S3). However, the pattern was not homogenous, and the samples showed varying levels of admixture among different clusters. Three genetic clusters, including *P. sylvestris*, *P. mugo s.s.* and a mixture of samples from populations of other taxa, were found at cpSSR loci (Figure S4).

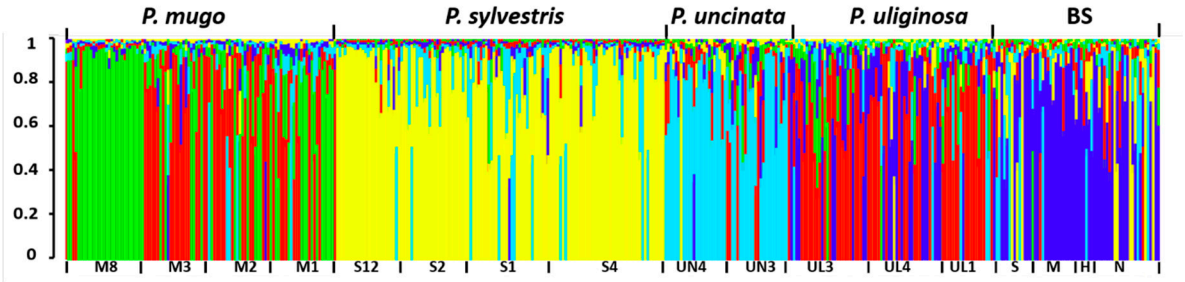

Figure 4. STRUCTURE results indicating five groups of samples based on genetic variation at nSSR loci of the mixed pine population from Błędne Skały (BS) and reference populations of the four pine species (population acronyms as in Table 1).

3.2. Phenotypic Variability and Differentiation

The level of the intraspecific morphological variation differed among the taxa and populations from BS in terms of needle (Table S5) and cone characteristics (Table S6). The highest variability in most of the needle characteristics showed *P. uliginosa* and the population from BS; reference taxa variation was at a lower level (Table S5). Regarding the cone characteristics, the most variable ones were *P. sylvestris* and *P. uliginosa* (Table S6).

Most of the analyzed needle and cone characteristics differed significantly ($p \leq 0.01$) between samples from BS and reference populations. The BS population significantly differed in terms of needle characteristics from every reference species, while in terms of cone characteristics it differed significantly from *P. uncinata* and *P. mugo* ($p \leq 0.01$). The groups of individuals described in the putative hybrid population during material collection based on macro-morphology as *P. sylvestris*-like

(BS_S), *P. uliginosa*-like (BS_M), hypothetical hybrids intermediate between these two taxa (BS_H) and individuals not determined (BS_N), appeared close to each other in terms of needle and cone characteristics. Each of these four groups did not reveal significant pairwise differences neither in terms of needle nor in terms of cone characteristics. The individuals determined during sampling as *P. uliginosa*-like (BS_M) in terms of needle characteristics revealed a relatively low number of traits differentiating them from reference populations of that species, but a relatively high number of characteristics differentiating them from *P. mugo s.s.* and *P. uncinata*. Twice as many characteristics differentiated the group BS_M from *P. sylvestris* populations (Table S7). However, there was no general rule in terms of cone characteristics (Table S8). Surprisingly, there were no differences between each group from BS and the *P. uliginosa* population from the Zieleniec Nature Reserve (UL2).

The centroids of four groups from BS were close to each other on the scatterplots of the PCA and fell between reference populations of *P. sylvestris* and the taxa representing the *P. mugo* complex.

The group assigned to *P. sylvestris* during sampling appeared only slightly similar to these species reference populations, and only in terms of needle characteristics (Figure 5). The needle characteristics that mostly contributed to group differentiations were NRC, SC, SF, TN/WN, VBF and VBL, while the most important cone characteristics were CD, CL, CDM, DAU, TA, CVX and WA (Figure S5, code descriptions in Tables S5 and S6).

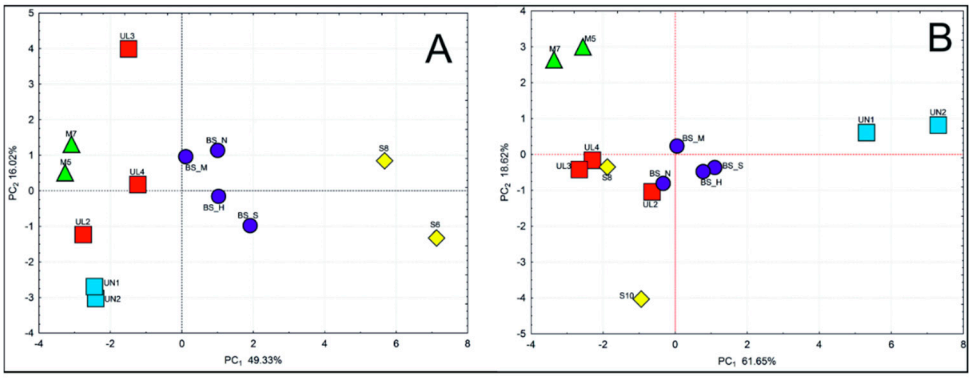

Figure 5. Principal component analysis (PCA) plot showing relationships between groups of samples defined at the mixed pine population in Błędne Skały as *P. sylvestris* (BS_S), *P. uliginosa* (BS_M), hybrids (BS_H), undetermined (BS_N) and reference populations of the four pine species (population acronyms as in Table 1): (**A**) based on needle traits; (**B**) based on cone traits.

The needle characteristics on the dispersion diagram between the two first discrimination variables, responsible for 80% of the total variation, indicate the intermediate position of BS samples between *P. uncinata* and individuals of *P. uliginosa* and *P. sylvestris*. Some of BS individuals intermixed with individuals of *P. uncinata*, *P. uliginosa* and *P. mugo*, and entered the 95% confidence intervals of these species. Interestingly, BS individuals did not intermix with *P. sylvetris* specimens (Figure S6A). In the cone characteristics, pines from BS partly intermixed with reference *P. uncinata*, *P. uliginosa* and *P. sylvestris*, but did not enter *P. mugo s.s.* at a 95% confidence interval (Figure S6B).

The dispersion of BS samples described by the two first PCA variables also indicates their intermediate character among the compared taxa. In the needle characteristics, a great part of BS individuals, determined during sampling as *P. uliginosa*-like, was located between *P. mugo s.s.*, *P. uliginosa* and *P. uncinata*, while the remaining ones were located between *P. uliginosa* and *P. sylvestris*. Individuals determined as BS_S were located between reference *P. sylvestris* and taxa of the *P. mugo* complex, while those determined as hybrids were dispersed among all the others (Figure 6A). In the cone characteristics, BS_M individuals mostly revealed close connection to reference *P. uliginosa*, *P. uncinata* and *P. mugo s.s.*, although some of them were located between *P. sylvestris* and taxa from the *P. mugo* complex, with high affinity to *P. uncinata.*

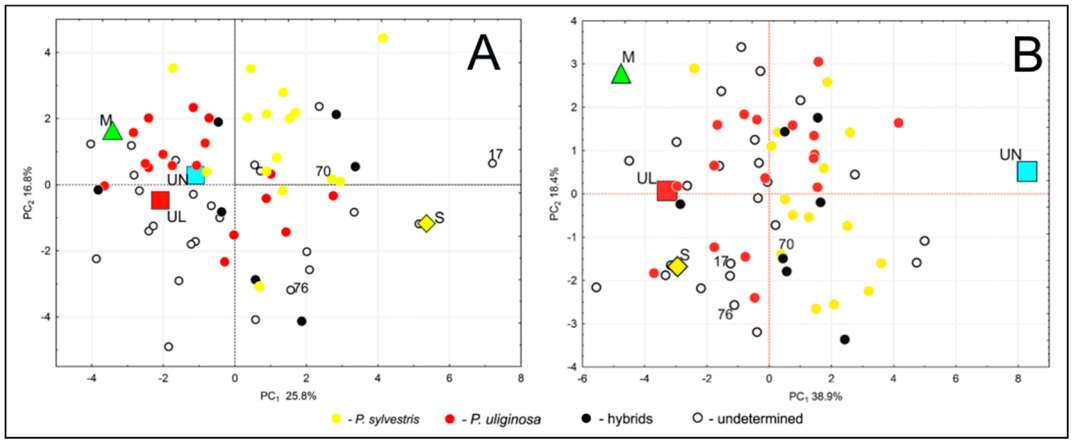

Figure 6. Principal component analysis PCA plot showing relationships between individuals from four groups defined at the mixed pine population in Błędne Skały (marked in circles) and reference species, including *P. sylvestris* (S), *P. mugo* (M), *P. uliginosa* (UL) and *P. uncinata* (UN); (numbers 17, 70 and 76 indicate individuals with cpDNA haplotype characteristic for *P. sylvestris*): (**A**) based on needle traits; (**B**) based on cone traits.

In PCA analysis, trees from BS assigned to *P. sylvestris* were located between reference *P. sylvestris* and *P. uncinata* populations, while the putative hybrids were more dispersed (Figure 6B). The three individuals identified as pure *P. sylvestris* in genetic analysis were close to this species´ reference populations on the PCA, both based on needle and cone characteristics (Figure 6) and in the discrimination analysis (Figure S6).

4. Discussion

Due to the relatively recent speciation history and the possible gene exchange during speciation [13], the taxa of the *P. mugo* complex and *P. sylvestris* show high genetic similarity at nuclear and organellar genomes [62–64,71]. So far, species diagnostic markers were found at cpDNA discriminating *P. sylvestris* from the taxa of the *P. mugo* complex [33,35,70]. In advances on earlier studies, we provide another diagnostic marker at mtDNA, which discriminates between *P. uncinata* and the remaining species from the *P. mugo* complex. However, the unique *P. uncinata* mitotype was not observed in the studied BS population, indicating that *P. uncinata* did not contribute, at least as a seed donor, to the development of the group of pines at the study area. Pines from the BS stand had most of the mtDNA mitotypes observed at high frequency in the small, relict population of *P. uliginosa* surrounded with extensive *P. sylvestris* stands in the WęgliniecNature Reserve. However, the discriminating power of the mtDNA is still too low to be conclusive about any further genetic relationships between the samples and between *P. mugo s.s.* and *P. uliginosa*.

The indication of a hybrid origin of the individuals comes from the analysis of the paternally inherited plastid and the biparentally inherited nuclear genome [30,62,64,71]. Interestingly, only three individuals carrying the cpDNA genome typical for *P. sylvestris* were identified among the 64 individuals sampled: one of them was assigned based on the macro-morphological phenotype to *P. sylvestris* (BS_S), while the other two remained undetermined during sampling (BS_N). All remaining individuals analyzed from BS possessed plastid DNA characteristic of the *P. mugo* complex, independently of phenotype.

The plastid genome derived from the *P. mugo* complex observed in the 14 out of 15 trees classified phenotypically as *P. sylvestris* means that those samples represent cryptic hybrids, which resemble pure species and cannot be distinguished in the field via macro-morphological characteristics. Furthermore, despite the vast diversity of phenotypic forms sampled from Błędne Skały and preliminarily classified into three distinct phenotypic groups, they all showed a uniform chloroplast background and high genetic similarity to each other at nuclear loci. For instance, they had the lowest genetic distance

and high similarity at the allelic frequency spectra that placed them together in clustering analysis as compared to the reference species populations. These results indicate that pines from the BS contact zone represent a highly hybridized population, and the presence of genetically pure individuals of *P. sylvestris* seems marginal, despite a number of macro-phenotypically trees deceptively resembling this species. Overall, pines from that area showed higher genetic similarity to *P. uliginosa* and *P. uncinata*, but not to *P. mugo s.s.* and *P. sylvestris* (Figure 2 and Figures S2 and S4). The predominance of hybrid individuals and their morphological differentiation could indicate either hybridization lasting for many generations with restricted inflow of genes of *P. sylvestris*, or more recent hybridization without competition of *P. sylvestris* individuals from the *P. mugo* complex. However, both hypotheses shall be verified in the special study applying more genetic markers at nuclear loci to reveal the proportion of genetic background from each parental species at much finer resolution.

Some discordance of needle characteristics and cpDNA markers in pines has been reported earlier for *P. uncinata.* Four individuals sampled as *P. sylvestris* appeared to be cryptic hybrids with cpDNA typical for the taxa of *P. mugo* and a set of needle characteristics that placed them at a marginal position of *P. sylvestris* individuals [31]. In the present biometric investigations, the majority of individuals determined during sampling as *P. sylvestris* did not resemble this species from the reference populations, neither in terms of needle (Figure 6A), nor in terms of cone characteristics (Figure 6B), falling between *P. sylvestris* and *P. uliginosa* reference populations (see also Figure S5). The only three individuals carrying *P. sylvestris* cpDNA also appeared close to *P. sylvestris* in the biometrical analyses on the needle and cone characteristics. During sampling, only one of them was classified as *P. sylvestris* based on the main phenotype traits used in the field of taxonomic classification of trees [15,16]. However, the remaining individuals classified as this species were intermediate between *P. sylvestris* and *P. uliginosa*, indicating a lack of correlation between habit form vs. needle and cone traits. Nevertheless, this observation has not been verified using statistical methods. The lack of correlation between morphological characteristics of hybrids has been reported earlier by Bobowicz et al. [89], but on restricted and relatively young material.

Interestingly, the individuals bearing cpDNA of *P. sylvestris* were close to the reference individuals of the species in terms of PCA and discrimination scatter plots for needle and cone characteristics (Figure 6A,B and Figure S6) showing that the results of the biometrical analyses are similar to those of cpDNA used for distinguishing between *P. sylvestris* and the *P. mugo* complex. In spite of that, the 64 individuals representing the BS mixed population formed one group situated between *P. sylvestris* and the taxa of the *P. mugo* complex, without a clear division between *P. sylvestris* and *P. uliginosa*, as detected by Jasińska et al. [31] for *P. sylvestris* and *P. uncinata*.

The presence of *P. uliginosa* × *P. sylvestris* hybrids bearing the phenotype of *P. sylvestris* and the lack of a vice versa morphological combination indicates a potential unidirectional gene flow from *P. uliginosa* to *P. sylvestris*, without phenotype expression of the former taxon (e.g., [30,31] and the literature cited herein). The putative gene flow from *P. uliginosa* (also from other taxa of the *P. mugo* complex) to *P. sylvestris* could be a specific evolutionary attribute, which allow the taxa of the *P. mugo* complex to diverge from *P. sylvestris*. The known invasions due to gene flow by pollen are a frequent phenomenon in nature. The gene inflow of *Quercus petraea* (Matt.) Liebl. into *Q. robur* L. by pollen is a good example of such a process [90–92], which also takes place in the populations of other wind-pollinated central-European trees ([93,94] and the literature cited herein). Examples of invasion by hybridization and gene flow were described also for other plant species [95–97], but only certain types of hybrids have adaptive advantages over other hybrid types and parental species in diverse environments. This hypothesis, however, shall be verified in the study on the genetic basis of hybrid adaptation, which requires in-depth investigation of polymorphisms at various genomic regions [98].

Predominance of individuals bearing cpDNA barcodes of the *P. mugo* complex suggests long-lasting hybridization in isolation from other populations of *P. sylvestris*, as a result of vegetation changes during the Holocene [44,45,48]. The occurrence of pines in the Sudetes was strongly reduced during the Boreal period of the Holocene. This was mainly due to the expansion of *Picea*, which pushed pines

to specific sites, such as rocky ridges or rock tops and, consequently, involved the spatial isolation between their populations. Thus, in Błędne Skały we observe the result of hybridization between *P. sylvestris* and *P. uliginosa* without or with highly restricted gene inflow from other populations. Isolation together with unidirectional gene flow from the *P. mugo* complex to *P. sylvestris*, could explain the considerable presence of hybrid individuals there and the restricted number of specimens with a genome typical for the latter species.

Despite its hybrid origin, we detected a relatively low level of genetic variation in the mixed BS population in terms of mitochondrial and plastid DNA markers, lower than in the reference populations of *P. mugo s.s.*, *P. sylvestris* and *P. uliginosa* [63–65,68,99,100]. Some discrepancy between the level of genetic variation at the genomes of different inheritance modes in pines suggests that a limited number of paternal trees contributed to the reproductive success of the population. However, this observation and scale of possible inbreeding would need some additional investigations including genetic variation of the progeny derived from seeds sampled in the population.

5. Conclusions

The investigated population consists mostly of hybrids, and the presence of pure *P. sylvestris* individuals seems marginal. Hybridization between *P. sylvestris* and the taxa of the *P. mugo* complex is predominantly unidirectional from the latter species to *P. sylvestris*. The macro-morphological characteristics of the latter parent tree are frequently conserved in hybrids, but we did not find correlation of those traits with morphological and anatomical characteristics of the needles and cone traits in our study.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/10/1086/s1, Figure S1: Median Joining network of the mitotypes of pines from the mixed population in Błędne Skały and the reference four pine species populations based on 14 mtDNA regions, Figure S2: UPGMA tree based on Nei genetic distance at 14 mtDNA regions showing genetic relationships between defined groups of samples from the mixed pine population in Błędne Skały and reference pure species populations, Figure S3: ΔK and L(K) indices showing the most likely partitioning of the genetic variation at nSSR loci in Structure analysis, Figure S4: STRUCTURE results indicating three genetic groups of samples based on genetic variation at cpSSR loci (above) with optimal number of clusters detected using the method of Evanno's ΔK among compared pine populations (below), Figure S5: Influence of needle (A) and cone (B) characteristics on the dispersion of samples of four sample groups from Błędne Skały and reference pine taxa, Figure S6: Results of discrimination analysis for mixed populations from Błędne Skały (BS), *P. sylvestris* (PS), *P. mugo* (PM), *P. uliginosa* (PUL) and *P. uncinata* (PUN) based on the characteristic of needles (A) and cones (B), Table S1: mtDNA haplotypes and their frequencies in the four groups of pines from Błędne Skały, Table S2: Frequency of the 20 most frequent cpDNA haplotypes (present in > 3 individuals) out of 302 detected ones in the four groups of pines from Błędne Skały, Table S3: Genetic variation at individual populations based on cpSSR loci, Table S4: Genetic variation of samples from the mixed pine population in Błędne Skały and reference populations of the four pine species based on nDNA SSR loci, Table S5: Basic summary statistics of analysed needle traits of pines from the Błędne Skały (BS) and of the taxa compared—*P. sylvestris* (S), *P. mugo* (M), *P. uliginosa* (UL) and *P. uncinata* (UN), Table S6: Basic summary statistics of analysed cone characteristics of pines from Błędne Skały (BS) and of the taxa compared—*P. sylvestris* (S), *P. mugo* (M), *P. uliginosa* (UL) and *P. uncinata* (UN), Table S7: Needle characteristics differing at $p \leq 0.01$ and $p \leq 0.05$ in the Tukey's HSD and Kruskal–Wallis tests between subpopulations from Błędne Skały and compared populations of *P. sylvestris*, *P. mugo*, *P. uliginosa* and *P. uncinata*, Table S8: Cone characteristics differing at $p \leq 0.01$ and $p \leq 0.05$ in Tukey's HSD and Kruskal–Wallis tests between subpopulations from Błędne Skały and compared populations of *P. sylvestris*, *P. mugo*, *P. uliginosa* and *P. uncinata*.

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References

1. Rieseberg, L.H.; Ellstrand, N.C.; Arnold, M. What can molecular and morphological markers tell us about plant hybridization? *Crit. Rev. Plant Sci.* **1993**, *12*, 213–241. [CrossRef]
2. Arnold, M.L. *Natural Hybridization and Evolution*; Oxford University Press: Oxford, UK, 1997.
3. Arnold, M.L. *Evolution through Genetic Exchange*; Oxford University Press: Oxford, UK, 2007; pp. 1–252. [CrossRef]
4. Mallet, J. Hybridization, ecological races and the nature of species: Empirical evidence for the ease of speciation. *Philos. Trans. R Soc. B Biol. Sci.* **2008**, *363*, 2971–2986. [CrossRef] [PubMed]
5. Soltis, P.S.; Soltis, D.E. The role of hybridization in plant speciation. *Annu. Rev. Plant Biol.* **2009**, *60*, 561–588. [CrossRef] [PubMed]
6. Abbott, R.; Albach, D.; Ansell, S.; Arntzen, J.W.; Baird, S.J.E.; Bierne, N.; Boughman, J.; Brelsford, A.; Buerkle, C.A.; Buggs, R.; et al. Hybridization and speciation. *J. Evol. Biol.* **2013**, *26*, 229–246. [CrossRef]
7. Boratyński, A.; Boratyńska, K.; Lewandowski, A.; Gołąb, Z.; Kiciński, P. Evidence of the possibility of natural reciprocal crosses between *Pinus sylvestris* and *P. uliginosa* based on the phenology of reproductive organs. *Flora* **2003**, *198*, 377–388. [CrossRef]
8. Stacy, E.A.; Paritosh, B.; Johnson, M.A.; Price, D.K. Incipient ecological speciation between successional varieties of a dominant tree involves intrinsic postzygotic isolating barriers. *Ecol. Evol.* **2017**, *7*, 2501–2512. [CrossRef]
9. Gernandt, D.S.; López, G.G.; García, S.O.; Liston, A. Phylogeny and classification of Pinus. *Taxon* **2005**, *54*, 29–42. [CrossRef]
10. Kormuťák, A.; Ostrolucká, M.; Vooková, B.; Preťová, A.; Fečková, M. Artificial hybridization of *Pinus sylvestris* L. and *Pinus mugo* Turra. *Acta. Biol. Crac. Ser. Bot.* **2005**, *47*, 129–134.
11. Delgado, P.; Salas-Lizana, R.; Vazquez-Lobo, A.; Wegier, A.; Anzidei, M.; Alvarez-Buylla, E.R.; Vendramin, G.G.; Pinero, D. Introgressive hybridization in *Pinus montezumae* Lamb and *Pinus pseudostrobus* Lindl. (*Pinaceae*): Morphological and molecular (cpSSR) evidence. *Int. J. Plant Sci.* **2007**, *168*, 861–875. [CrossRef]
12. Krajmerová, D.; Paule, L.; Zhelev, P.; Voleková, M.; Evtimov, I.; Gagov, V.; Gömöry, D. Natural hybridization in eastern-Mediterranean firs: The case of *Abies borisii-regis*. *Plant Biosyst.* **2016**, *150*, 1189–1199. [CrossRef]
13. Wachowiak, W.; Palme, A.E.; Savolainen, O. Speciation history of three closely related pines *Pinus mugo* (T.), *P. uliginosa* (N.) and *P. sylvestris* (L.). *Mol. Ecol.* **2011**, *20*, 1729–1743. [CrossRef] [PubMed]
14. Wachowiak, W.; Zaborowska, J.; Labiszak, B.; Perry, A.; Zucca, G.M.; Gonzalez-Martinez, S.C.; Cavers, S. Molecular signatures of divergence and selection in closely related pine taxa. *Tree Genet. Genomes* **2018**, *14*. [CrossRef] [PubMed]
15. Christensen, K.I. Taxonomic revision of the *Pinus mugo* complex and *P. rhaetica* (*P. mugo sylvestris*) (*Pinaceae*). *Nord. J. Bot.* **1987**, *7*, 383–408. [CrossRef]
16. Businský, R.; Kirschner, J. *Pinus mugo* and *P. uncinata* as parents of hybrids a taxonomic and nomenclatural survey. *Phyton* **2010**, *50*, 27–57.
17. Staszkiewicz, J.; Tyszkiewicz, M. Variability of the natural hybrids of *Pinus sylvestris* L. x *Pinus mugo* Turra (= *P. x rotundata* Link) in south-western Poland and in some selected localities of Bohemia and Moravia. *Fragm. Flor. Geobot.* **1972**, *18*, 173–191.
18. Bobowicz, M.A. Differentiation of *Pinus sylvestris* L. and *Pinus mugo* Turra, pines from Bór na Czerwonem and from Zieleniec in traits of one- and two-year-old cones. *Bull. Soc. Amis. Sci. Lett. Pozn. Ser. D Sci. Biol.* **1988**, *26*, 99–108.
19. Neet-Sarqueda, C. Genetic differentiation of *Pinus sylvestris* L. *and Pinus mugo aggr. populations in Switzerland.* **1994**, *43*, 207–214.
20. Christensen, K.I.; Dar, G.H. A morphometric analysis of spontaneous and artificial hybrids of *Pinus mugo* ×*sylvestris* (*Pinaceae*). *Nord. J. Bot.* **1997**, *17*, 77–86. [CrossRef]
21. Boratyńska, K.; Bobowicz, M.A. *Pinus uncinata* Ramond taxonomy based on needle characters. *Plant. Syst. Evol.* **2001**, *227*, 183–194. [CrossRef]
22. Boratyńska, K.; Boratyński, A.; Lewandowski, A. Morphology of *Pinus uliginosa* (*Pinaceae*) needles from populations exposed to and isolated from the direct influence of *Pinus sylvestris*. *Bot. J. Linn. Soc.* **2003**, *142*, 83–91. [CrossRef]

23. Boratyńska, K.; Boratyński, A. Taxonomic differences among closely related pines *Pinus sylvestris*, *P. mugo*, *P. uncinata*, *P. rotundata* and *P. uliginosa* as revealed in needle sclerenchyma cells. *Flora* **2007**, *202*, 555–569. [CrossRef]
24. Boratyńska, K.; Jasińska, A.; Ciepłuch, E. Effect of tree age on needle morphology and anatomy of *Pinus uliginosa* and *Pinus silvestris*—Species-specific character separation during ontogenesis. *Flora Morphol. Distrib. Funct. Ecol. Plants* **2008**, *203*, 617–626. [CrossRef]
25. Venturas, M.; García Álvarez, S.; Alcantra, M.F.; Collada, C.; Gil, L. Species selection for reforestations: What happens with historic local extinctions and habitat protection zones? A case study in the Cantabrian Range. *Eur. J. Res.* **2013**, *132*, 107–120. [CrossRef]
26. Lewandowski, A.; Smoćko, J.; Boratynska, K.; Boratynski, A. Genetic differences between two Polish populations of *Pinus uliginosa*, compared to *P. sylvestris* and *P. mugo*. *Dendrobiology* **2002**, *48*, 51–57.
27. Wachowiak, W.; Celinski, K.; Prus-Glowacki, W. Evidence of natural reciprocal hybridisation between *Pinus uliginosa* and *P. sylvestris* in the sympatric population of the species. *Flora* **2005**, *200*, 563–568. [CrossRef]
28. Wachowiak, W.; Lewandowski, A.; Prus-Głowacki, W. Reciprocal controlled crosses between *Pinus sylvestris* and *P. mugo* verified by a species-specific cpDNA marker. *J. Appl. Genet.* **2005**, *46*, 41–43.
29. Kormutak, A.; Vookova, B.; Manka, P.; Salaj, J.; Camek, V.; Gömöry, D. Abortive embryogenesis in hybrid swarm populations of *Pinus sylvestris* L. and *Pinus mugo* Turra. *Trees* **2008**, *22*, 657–662. [CrossRef]
30. Wachowiak, W.; Prus-Glowacki, W. Hybridisation processes in sympatric populations of pines *Pinus sylvestris* L., *P.mugo* Turra and *P.uliginosa* Neumann. *Plant Syst. Evol.* **2008**, *271*, 29–40. [CrossRef]
31. Jasińska, A.K.; Wachowiak, W.; Muchewicz, E.; Boratyńska, K.; Montserrat, J.M.; Boratyński, A. Cryptic hybrids between *Pinus uncinata* and *P. sylvestris*. *Bot. J. Linn. Soc.* **2010**, *163*, 473–485. [CrossRef]
32. Wachowiak, W.; Cavers, S.; Żukowska, W.B. Interspecific gene flow and ecological selection in a pine (*Pinus sp.*) contact zone. *Plant Syst. Evol.* **2015**, *301*, 1643–1652. [CrossRef]
33. Wachowiak, W.; Zukowska, W.B.; Wojkiewicz, B.; Cavers, S.; Litkowiec, M. Hybridization in contact zone between temperate European pine species. *Tree Genet. Genomes* **2016**, *12*. [CrossRef]
34. Lewandowski, A.; Dering, M. Crossability between *Pinus uliginosa* and its putative parental species *Pinus sylvestris* and *Pinus mugo*. *Silvae Genet.* **2006**, *55*, 52–54. [CrossRef]
35. Kormutak, A.; Galgoci, M.; Manka, P.; Koubova, M.; Jopcik, M.; Sukenikova, D.; Bolecek, P.; Gőmőry, D. Field-based artificial crossings indicate partial compatibility of reciprocal crosses between *Pinus sylvestris* and *Pinus mugo* and unexpected chloroplast DNA inheritance. *Tree Genet. Genomes* **2017**, *13*, 68. [CrossRef]
36. Liston, A.; Parker-Defeniks, M.; Syring, J.; Willyard, A.; Cronn, R. Interspecific phylogenetic analysis enhances intraspecific phylogeographical inference: A case study in *Pinus lambertiana*. *Mol. Ecol.* **2007**, *16*, 3926–3937. [CrossRef]
37. Senjo, M.; Kimura, K.; Watano, Y.; Ueda, K.; Shimizu, T. Extensive mitochondrial introgression from *Pinus pumila* to *P. parviflora* var. *pentaphylla* (*Pinaceae*). *J. Plant Res.* **1999**, *112*, 97–105. [CrossRef]
38. Ma, X.-F.; Szmidt, A.E.; Wang, X.-R. Genetic structure and evolutionary history of a diploid hybrid pine *pinus densata* inferred from the nucleotide variation at seven gene loci. *Mol. Biol. Evol.* **2006**, *23*, 807–816. [CrossRef]
39. Staffa, M. *Słownik Geografii Turystycznej Sudetów: Góry Stołowe [Lexicon of the Turistic Geography of the Sudetes. 13. Stołowe Mountains]*; PTTK Kraj: Kraków, Poland, 1992.
40. Latocha, A.; Migoń, P. Geneza i przemiany krajobrazu kulturowego Gór Stołowych [Genezis and transformations of the cultural lanscape of the Stołowe Mountains]. In *Góry Stołowe—Przyroda i Ludzie [The Stołowe Mountains—Nature and People]*; Kabała, C., Ed.; Park Narodowy Gór Stołowych: Kudowa Zdrój, Poland, 2018; pp. 47–61.
41. Latałowa, M.; Tobolski, K.; Nalepka, D. *Pinus* L. subgenus *Pinus* (subgen. *Diploxylon* (Koehne) Pilger). In *Late Glacial and Holocene History of Vegetation in Poland Based on Isopollen Maps*; Ralska-Jasiewiczowa, M., Ed.; W. Szafer Institute of Botany: Kraków; Poland, 2004; pp. 165–177.
42. Treml, V.; Jankovská, V.; Petr, L. Holocene dynamics of the alpine timberline in the High Sudetes. *Biologia* **2008**, *63*, 73–80. [CrossRef]
43. Malkiewicz, M.; Waroszewski, J.; Bojko, O.; Egli, M.; Kabala, C. Holocene vegetation history and soil development reflected in the lake sediments of the Karkonosze Mountains (Poland). *Holocene* **2016**, *26*, 890–905. [CrossRef]

44. Marek, S. Rozwój Wielkiego Torfowiska Batorowskiego w świetle badań biostratygraficznych [Development of Wielkie Torfowisko Barowskie mire in the light of biostratygraphic investigations]. *Szczeliniec* **1988**, *2*, 49–88.
45. Madeyska, E. The history of the Zieleniec Mire and the surrounding areas based on the palynological research. *Monogr. Bot.* **2005**, *94*, 145–157.
46. Baranowska-Kącka, A. Historia roślinności Gór Stołowych w świetle badań palinologicznych Sudetów [The history of vegetation of the Stołowe Mts in the light of palynological studies]. In *Przyroda Parku Narodowego Gór Stołowych [Nature of National Park of the Stołowe Mts]*; Witkowski, A., Pokryszko, B.M., Ciężkowski, W., Eds.; National Park of the Stołowe Mountains: Kudowa Zdrój, Poland, 2013; pp. 130–136.
47. Boratyński, A. *Pinus uliginosa* Neumann in the Błedne Skały reserve in Stolowe Mountains. *Dendrobiology* **1978**, *23*, 261–267.
48. Glina, B.; Malkiewicz, M.; Mendyk, Ł.; Bogacz, A.; Woźniczka, P. Human-affected disturbances in vegetation cover and peatland development in the late Holocene recorded in shallow mountain peatlands (Central Sudetes, SW Poland). *Boreas* **2017**, *46*, 294–307. [CrossRef]
49. Sarvas, R. Investigations on the flowering and seed crop of *Pinus silvestris*. *Commun. Inst. Fenn.* **1962**, *53*, 198.
50. Sarvas, R. Investigations on the annual cycle of development of forest trees. Active period. *Commun. Inst. Fenn.* **1972**, *76*, 110.
51. Poska, A.; Pidek, I.A. Pollen dispersal and deposition characteristics of *Abies alba*, *Fagus sylvatica* and *Pinus sylvestris*, Roztocze region (SE Poland). *Veg. Hist. Archaeobot.* **2010**, *19*, 91–101. [CrossRef]
52. Robledo-Arnuncio, J.J.; Gil, L. Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Heredity* **2005**, *94*, 13–22. [CrossRef] [PubMed]
53. Burczyk, J.; Chalupka, W. Flowering and cone production variability and its effect on parental balance in a Scots pine clonal seed orchard. *Ann. Sci.* **1997**, *54*, 129–144. [CrossRef]
54. Marcysiak, K. Interpopulational variability of *Pinus uncinata* Ramond ex DC. in Lam. & DC (*Pinaceae*) cone characters. *Dendrobiology* **2004**, *51*, 43–51.
55. Marcysiak, K.; Boratyńska, K.; Mazur, M. Variability of *Pinus uliginosa* cones from the peat-bog in Węgliniec. *Dendrobiology* **2003**, *49*, 43–47.
56. Boratyńska, K.; Marcysiak, K.; Boratyński, A. *Pinus mugo* (*Pinaceae*) in the Abruzzi Mountains: High morphological variation in isolated populations. *Bot. J. Linn. Soc.* **2005**, *147*, 309–316. [CrossRef]
57. Marcysiak, K.; Boratyński, A. Contribution to the taxonomy of *Pinus uncinata* (*Pinaceae*) based on cone characters. *Plant Syst. Evol.* **2007**, *264*, 57–73. [CrossRef]
58. Sobierajska, K.; Boratynska, K. Variability of needle characters of *Pinus mugo* Turra populations in the Karkonosze Mountains in Poland. *Dendrobiology* **2008**, *59*, 41–49.
59. Boratynska, K.; Lewandowska, D. Differences among three populations of *Pinus uliginosa* and their relation to *P. sylvestris* as expressed by the needle characters. *Dendrobiology* **2009**, *61*, 37–46.
60. Sobierajska, K.; Boratyńska, K.; Marcysiak, K. Variation of cone characters in *Pinus mugo* (*Pinaceae*) populations in the Giant Mountains (Karkonosze, Sudetes). *Dendrobiology* **2010**, *63*, 33–41.
61. Jasińska, A.; Boratyńska, K.; Dering, M.; Sobierajska, K.; Ok, T.; Romo Diez, A.M.; Boratyński, A. Distance between south-European and south-west Asiatic refugial areas involved morphological differentiation: *Pinus sylvestris* case study. *Plant Syst. Evol.* **2014**, *300*, 1487–1502. [CrossRef]
62. Dzialuk, A.; Boratyńska, K.; Romo, A.; Boratyński, A. Taxonomic and geographic variation of the *Pinus mugo* complex on chloroplast microsatellite markers. *Syst. Biodivers.* **2016**, *15*, 464–479. [CrossRef]
63. Wachowiak, W.; Boratynska, K.; Cavers, S. Geographical patterns of nucleotide diversity and population differentiation in three closely related European pine species in the *Pinus mugo* complex. *Bot. J. Linn. Soc.* **2013**, *172*, 225–238. [CrossRef]
64. Łabiszak, B.; Zaborowska, J.; Wachowiak, W. Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). *AoB Plants* **2019**, *11*. [CrossRef]
65. Boratyńska, K.; Dzialuk, A.; Lewandowski, A.; Marcysiak, K.; Jasińska, A.; Sobierajska, K.; Tomaszewski, D.; Burczyk, J.; Boratyński, A. Geographic distribution of quantitative traits variation and genetic variability in natural populations of *Pinus mugo* in Central Europe. *Dendrobiology* **2014**, *72*, 65–84. [CrossRef]
66. Donnelly, K.; Cottrell, J.; Ennos, R.A.; Vendramin, G.G.; A'Hara, S.; King, S.; Perry, A.; Wachowiak, W.; Cavers, S. Reconstructing the plant mitochondrial genome for marker discovery: A case study using *Pinus*. *Mol. Ecol. Resour.* **2017**, *17*, 943–954. [CrossRef]

67. Soranzo, N.; Alia, R.; Provan, J.; Powell, W. Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insights into the postglacial history of European *Pinus sylvestris* populations. *Mol. Ecol.* **2000**, 9. [CrossRef] [PubMed]
68. Zaborowska, J.; Łabiszak, B.; Wachowiak, W. Population history of European mountain pines *Pinus mugo* and *Pinus uncinata* revealed by mitochondrial DNA markers. *J. Syst. Evol.* **2019**. [CrossRef]
69. Żukowska, W.B.; Boratyńska, K.; Wachowiak, W. Comparison of range-wide chloroplast microsatellite and needle trait variation patterns in *Pinus mugo* Turra (dwarf mountain pine). *iForest* **2017**, *10*, e1–e9. [CrossRef]
70. Wachowiak, W.; Lesniewicz, K.; Odrzykoski, I.; Augustyniak, H.; Prus-Głowacki, W. Species specific cpDNA markers useful for studies on the hybridisation between *Pinus mugo* and *P. sylvestris*. *Acta Soc. Bot. Pol.* **2000**, *69*, 273–276. [CrossRef]
71. Żukowska, W.B.; Wachowiak, W. Nuclear microsatellite markers reveal the low genetic structure of *Pinus mugo* Turra (dwarf mountain pine) populations in Europe. *Plant Syst. Evol.* **2017**, *303*, 641–651. [CrossRef]
72. Bandelt, H.-J.; Forster, P.; Röhl, A. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **1999**, *16*, 37–48. [CrossRef]
73. Leigh, J.W.; Bryant, D. Popart: Full-feature software for haplotype network construction. *Methods Ecol. Evol.* **2015**, *6*, 1110–1116. [CrossRef]
74. Librado, P.; Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **2009**, *25*, 1451–1452. [CrossRef]
75. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]
76. Excoffier, L.; Lischer, H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef]
77. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef] [PubMed]
78. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [PubMed]
79. Falush, D.; Stephens, M.; Pritchard, J.K. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Mol. Ecol. Notes* **2007**, *7*, 574–578. [CrossRef] [PubMed]
80. Hubisz, M.J.; Falush, D.; Stephens, M.; Pritchard, J.K. Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* **2009**, *9*, 1322–1332. [CrossRef]
81. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef]
82. Earl, D.A.; Vonholdt, B.M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
83. Boratyńska, K.; Tomaszewski, D.; Montserrat Martí, J.; Marek, S.; Boratyński, A. Taxonomic position of *Pinus ceciliae* (*Pinaceae*) endemic for Balearic Islands as revealed on needle characteristics. *Dendrobiology* **2019**, *82*, 8–16. [CrossRef]
84. Staszkiewicz, J. Morphological variation of leaves, cones and seeds. In *Scots Pine Biology*; Białobok, S., Boratyński, A., Bugała, W., Eds.; Instytut Dendrologii Polskiej Akademii Nauk: Poznań—Kórnik, Poland, 1993; pp. 33–44.
85. Zar, J.H. *Biostatistical Analysis*, 5th ed.; Prentice-Hall/Pearson: Upper Saddle River, NJ, USA, 2007.
86. Sokal, R.R.; Rohlf, F.J. *Biometry*, 4th ed.; W.H. FREEMAN & CO LTD: New York, NY, USA, 2012.
87. Watala, C. *Biostatystyka: Wykorzystanie Metod Statystycznych w Pracy Badawczej w Naukach Biomedycznych [Biostatistics: Using Statistic Methods in Biomedical Scientific Investigations]*; Alfamedica Press: Bielsko Biała, Poland, 2012.
88. Morrison, D.F. *Multivariate Statistical Methods*; Thomson/Brooks/Cole: Belmont, CA, USA, 2005.
89. Bobowicz, M.; Danielewicz, W. Isoenzymatic variability in progeny of *Pinus mugo* Turra x *Pinus sylvestris* L. hybrids from Bór na Czerwonem, in experimental culture. *Acta Soc. Bot. Pol.* **2000**, *69*, 137–144. [CrossRef]
90. Kremer, A.; Dupouey, J.-L.; Deans, J.; Cottrell, J.; Csaikl, U.; Finkeldey, R.; Espinel, S.; Jensen, J.; Kleinschmit, J.; Dam, B.C.; et al. Leaf morphological differentiation between *Quercus robur* and *Quercus petraea* is stable across western European mixed oak stands. *Ann. For. Sci.* **2002**, *59*, 777–787. [CrossRef]
91. Petit, R.J.; Bodénès, C.; Ducousso, A.; Roussel, G.; Kremer, A. Hybridization as a mechanism of invasion in oaks. *New Phytol.* **2004**, *161*, 151–164. [CrossRef]

92. Boratyński, A.; Marcysiak, K.; Jasińska, A.K.; Iszkuło, G. Interrelations among con-generic and co-occurring tree species: Asymmetric hybridization and the high success of *Quercus petraea* (Matt.) Liebl. regeneration in mixed *Q. petraea Q. robur* L. stands. *Pol. J. Ecol.* **2010**, *58*, 273–283.
93. Petit, R.J. Biological invasions at the gene level. *Divers. Distrib.* **2004**, *10*, 159–165. [CrossRef]
94. Petit, R.; Bialozyt, R.; Garnier-Géré, P.; Hampe, A. Ecology and genetics of tree invasions: From recent introductions to Quaternary migrations. *Ecol. Manag.* **2004**, *197*, 117–137. [CrossRef]
95. Martinsen, G.D.; Whitham, T.G.; Turek, R.J.; Keim, P. Hybrid populations selectively filter gene introgression between species. *Evolution* **2001**, *55*, 1325–1335. [CrossRef]
96. Fay, M.F.; Cowan, R.S.; Simpson, D.A. Hybridisation between *Schoenoplectus tabernaemontani* and *S. triqueter* (*Cyperaceae*) in the British Isles. *Watsonia* **2003**, *24*, 433–442.
97. Álvarez, I.; Wendel, J.F. Cryptic Interspecific Introgression and Genetic Differentiation within *Gossypium Aridum* (*Malvaceae*) and Its Relatives. *Evolution* **2006**, *60*, 505–517. [CrossRef]
98. Wachowiak, W.; Trivedi, U.; Perry, A.; Cavers, S. Comparative transcriptomics of a complex of four European pine species. *BMC Genom.* **2015**, *16*, 234. [CrossRef]
99. Dzialuk, A.; Muchewicz, E.; Boratyński, A.; Montserrat, J.M.; Boratyńska, K.; Burczyk, J. Genetic variation of *Pinus uncinata* (*Pinaceae*) in the Pyrenees determined with cpSSR markers. *Plant Syst. Evol.* **2009**, *277*, 197–205. [CrossRef]
100. Dzialuk, A.; Boratyński, A.; Boratyńska, K.; Burczyk, J. Geographic patterns of genetic diversity of *Pinus mugo* (*Pinaceae*) in Central European mountains. *Dendrobiology* **2012**, *68*, 31–41.