Genetic Diversity among Age Classes of a *Pinus sylvestris* (L.) Population from the Białowieża Primeval Forest, Poland

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Abstract: The Białowieża Primeval Forest (BPF) is Europe's last primeval forest and an irreplaceable area for biodiversity conservation due to its size, protection status, and substantially undisturbed nature. There is no other forest in Europe with such a large surface representing highly-advanced natural succession. This article reports on the first analysis of the genetic variability and demographic structure of a self-renewed *Pinus sylvestris* population located in BPF, using both chloroplast and mitochondrial DNA markers. The analysis of molecular variance (AMOVA) for chloroplast simple sequence repeats (cpSSRs) revealed a significant genetic differentiation among age classes that accounted for about 2% of the total variance, comparable to those reported among different populations of Scots pine. None of the 117 detected chloroplast haplotypes were common to all age classes. Haplotype diversity ranged from 0.370 to 0.415 for cpSSRs and from 0.320 to 0.455 for mitochondrial markers. The genetic variation of the studied age classes—represented by mitochondrial markers—strongly depicts the maternal genetic structure, indicating limited seed dispersal. Temporal genetic substructuring is maintained within a self-renewed population of Scots pine from the BPF.

Keywords: demographic structure; genetic structure; cpSSR; mitochondrial genome; natural renewal

1. Introduction

The Białowieża Primeval Forest (BPF; 1.470 km^2, 52°30/−53° N, 23°30/−24°15 E) is located on the border between Poland and Belarus, and is Europe's last remaining lowland broad-leaved and mixed forest that has not been exploited by forestry or influenced by other human activities for nearly one hundred years [1]. The forest was protected by successive Polish monarchs and Russian Tsars as a hunting reserve. The strictly protected part of the forest on the Polish side—the Białowieża National Park (BNP), established in 1921—is the oldest park in Poland and among the oldest parks throughout Europe, and comprises exceptional stands of primeval forest [2]. The BNP was added to the World Heritage List in 1979. It is estimated that over 4500 plant species, about 3000 fungal species, and nearly 10,000 animal species have been preserved in the Białowieża Primeval Forest [2]. There is no other forest in Europe with such a large surface representing well-advanced natural succession [3]. The BPF is considered as a "flagship ecosystem in European nature conservation" [3]. The BFP is an invaluable reference area for scientists studying the natural characteristics of European forests.

Nowadays, most central and northern European forests are intensely managed, and almost no natural primary forests remain in Europe [4]. It is difficult to study the dynamics of natural temperate zone forests in Europe due to the necessity of operating over a suitable timescale, and also because the suitable stands are so few in number and of too small a size [5]. The genetic diversity of tree species is

a key component for the functioning of a forest ecosystem [6]. Regeneration is the basic process that maintains the dynamics of a forest ecosystem, and as such, is a key aspect of any sustainable forest management system [7]. A self-renewed natural population involves individuals that often belong to genetically different age classes along with a differentiated sensitivity of these classes for selection, which may cause fluctuations in genetic variation [8].

Selection occurring upon germination and the following stages may act differently than during the adult stage. For example, early-stage shade tolerance for seedlings may be favored in dense populations, whereas light tolerance may prove to be important at later stages for the same tree [9]. At the population level, selection for light will favor fast-growing and vigorous seedlings in dense stands. The age-dependent climate sensitivity of French oaks [10], *Picea glauca* [11], and *Pinus sylvestris* [12] was reported to possibly have been caused by differences in the physiological processes of differently-aged trees. Chen et al. [13] observed temporal trends in the growth and net biomass change in the boreal forests of Canada. The relationships between the genetic diversity and demographic structure of a population is of fundamental importance for understanding the adaptive strategies of forest trees [14]. Studies of genetic variation in the naturally regenerated population of *P. sylvestris* from the BPF (performed using nuclear microsatellites and isoenzyme markers) revealed a high level of intra-population diversity between generations. The significant subdivision of genetic variation (F_{ST}) detected across the age classes was comparable to those found between different populations of this species [15].

The Scots pine (*P. sylvestris*) dominates the boreal forests of Northern and Eastern Europe, but is also an important species in Europe's managed temperate forests. In the Białowieża Primeval Forest, it occupies more than 27% of the total area [16].

We combine genetic data (chloroplast and mitochondrial DNA markers) to analyze the consequences of demographic events on genetic diversity and structuring in a self-renewed population of Scots pine. The combination of data from multiple polymorphic sites in the non-recombining, uniparentally-inherited haploid chloroplast and mitochondrial genome offers a robust and highly-informative assay for the analysis of diversity in natural populations of plant species.

2. Materials and Methods

2.1. Study Site and Sampling

We collected samples for genetic analysis in the naturally regenerated tree stands of *P. sylvestris* in the "Sitki" reserve of the Białowieża Primeval Forest (compartment 667B and 668A of the Hajnówka Forest District). The "Sitki" reserve (34.09 ha) was created in 1979 to preserve forms of oligotrophic pine forests in their natural state, which do not occur in any of the existing reserves of the BPF. The removal of trees that had toppled over and had been infested by bark beetles was the only recently applied treatment in this reserve [17]. The age of each individual tree was estimated by measuring its diameter at breast height and by counting its growth rings (using cores). The distance between sampled trees was between 10 m and 100 m. Fresh needles were collected from 125 individuals, representing four age classes of Scots pines (Table 1). Additionally, cones were collected from 40 adult fruiting trees (age class M and O)—one cone per tree. Seeds from these cones were mixed together in equal proportions, as a pooled sample, which was then germinated in laboratory conditions to form the youngest age class E, comprising 31 individuals (Table 1).

Table 1. Number of alleles and haplotype genetic diversity (H) of the analyzed cp and mt loci in age classes of *Pinus sylvestris* population.

Locus	Range	Number of Alleles							Total H
		Total	E	S	Y	M	O	Mean	
Number of individuals		156	31	32	32	29	32		
cpSSR									
Total		48	31	36	36	36	34	9.6	0.393
Pt 15169	123–130	8	4	7 (1)	6	6 (1)	5	5.6	0.734
Pt 26081	105–108	4	3 (1)	3	3	3	3	3.0	0.405
Pt 30204	139–146	8	5	6	7 (1)	6 (1)	6	6.0	0.719
Pt 36480	141–151	3	1	2	2	3 (1)	2	2.0	0.130
Pt 45002	165–170	4	3	4 (1)	3	3	3	3.2	0.472
Pt 71936	143–147	5	4	4	5	5	5	4.6	0.666
PCP 1289	102–106	5	3	3	4 (1)	4 (1)	3	3.4	0.415
PCP 41131	134–139	4	3	3	2	1	3	2.4	0.096
PCP 87314	111–114	4	4 (1)	3	3	3	2	3.0	0.271
PCP 102652	113–115	3	1	1	1	2 (1)	2 (1)	1.4	0.025
mt markers									
Total		3	3	3	3	3	3	3	0.188
nad 1	217 1170	1	1	1	1	1	1	1	0.000
nad 7	and 1175	2	2	2	2	2	2	2	0.375
cp and mt	-	51	34	39	39	39	37	10.2	0.358

cpSSR—chloroplast simple sequence repeat; E—embryos germinated from seeds; S—seedlings 1–3 years old; Y—young trees 10–20 years old; M—middle-aged trees 40–80 years old; O—old trees above 100 years. Number of private alleles is given in parentheses.

2.2. DNA Extraction and Amplification

DNA was extracted from needles and two-week-old seedlings (100 mg of fresh material) by means of the modified cetyltrimethylammonium bromide (CTAB) protocol [18]. The quality and quantity of the extracted the DNA was measured using a Nanodrop™ ND-1000 spectrophotometer (ThermoScientific, Waltham, MA, USA).

The ten variable chloroplast microsatellite loci provided a high-quality amplification product and were used for the analysis of all samples. These loci consisted of: PCP102652, PCP1289, PCP41131, PCP87314 [19], Pt15169, Pt26081, Pt30204, Pt36480, Pt45002, and Pt71936 [20]. PCR amplification was carried out in a total volume of 25 μL, containing about 20 ng of template DNA, 2.5 mM MgCl$_2$, 100 μM of each dNTP, 0.2 μM of each primer, and 1U HiFiTaq Polymerase (Novazym, Poznań, Poland), with the respective 1 × PCR buffer in accordance with Celiński et al. [21]. The PCR conditions were as follows: initial denaturation for 3 min at 94 °C, followed by 35 cycles of 15 s denaturation at 95 °C, 1 min annealing at 59 °C, 1 min incubation at 72 °C, and final extension at 72 °C for 10 min. The amplification products were separated using the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) capillary electrophoresis system with GeneScan™ 600 LIZ™ as an internal size standard. The reverse primer of each primer pair was labeled with fluorescent 6FAM, PET, and VIC dyes. Individuals were analyzed and genotyped using GeneMapper version 3.7 software (Applied Biosystems). In order to amplify a known polymorphic indel 1 in *nad1* intron (exon B/C), the diagnostic primers *nad1* H and *nad1* I were used according to Soranzo et al. [22]. For the *nad7* intron 1, sequences from the GeneBank [23] were used to design primers flanking the deletion of 5 bp and 32 bp (DQ665913–DQ665915). Thus, the PCR products would result in the following fragments: 300 bp (*a* mitotype), 295 (*b* mitotype), and 268 (*c* mitotype). The total volume of PCR reaction for *nad1* and *nad7* was 25 μL, containing 20 ng of template DNA, 2.5 mM MgCl$_2$, 100 μM of each dNTP, 0.2 μM of each primer, and 1U HiFiTaq Polymerase (Novazym, Poznań, Poland), with the respective 1 × PCR buffer. The indel of 31-bp in *nad1* intron (exon B/C) was scored by an electrophoresis of the PCR product on 1.5% agarose gel containing ethidium bromide. The amplification protocol for *nad1* was 4 min at 94 °C, followed by 35 cycles of 40 s at 94 °C, annealing for 1 min at 53 °C, 1 min at 72 °C, and finally 10 min at 72 °C. The PCR conditions for *nad7* consisted of 2 min at 95 °C, followed by 25 cycles of 30 s at 94 °C, annealing for 45 s at 60 °C, 1 min at 72 °C, and finally 10 min at 72 °C.

The PCR products were separated by capillary electrophoresis in a Genetic Analyser (Applied Biosystems). Genotyping of individuals was made with GeneMapper version 3.7 software (Applied Biosystems).

2.3. Data Analysis

The level of polymorphism of both the entire population and within each age class was estimated using the percentage of polymorphic loci (P%), allele frequencies at each locus, the number of alleles per locus (A), effective number of alleles (Ne), number of haplotypes (H), and haplotype diversity [24] using GenAlex 6.5 [25]. Fisher's exact test [26] calculated by GenePop v.4.0 [27,28] was used to estimate the pairwise *p* values for allele distribution between generations.

The level of genetic differentiation between generations was estimated using PhiPT-statistics for haploid data (analogous to F-statistics for diploid data) calculated via the analysis of molecular variance (AMOVA) option following Merimans [29]. The genetic distance between pairs of subpopulations was computed according to Nei [30]. Genetic relationships between populations were evaluated via principal coordinate analysis (PCoA) based on the genetic distance between each age classes.

3. Results

3.1. Chloroplast simple sequence repeats (cpSSRs)

The cpSSR loci were highly polymorphic among the Scots pine age classes: from 80% (class E) to 100% (class O). Loci Pt15169 and Pt 30204 (eight alleles) appeared to be the most polymorphic, whereas loci Pt36480 and PCP102652 (three alleles) appeared to be the least polymorphic (Table S1). A total of 48 alleles from the ten chloroplast microsatellite loci were found, ranging from 31 (class E) to 36 (classes S, Y, M) (Table 1).

The lowest mean number of alleles per locus (Na), 3.1, was noted in the class of Embryos, while the highest, 3.6, was observed in the classes of Seedlings, Young trees, and Middle-age trees (Table 2). The mean effective number of alleles per locus (Ne) was lower, and ranged from 2.0 to 2.2. A total of 12 private alleles were found: one in the Oldest trees, five in the Middle-age trees, and two alleles in the other classes. A considerable variance in the allele frequencies between particular age classes was observed, especially in respect to loci Pt15169, Pt30204, and Pt45002. Fisher's exact probability test showed that allele frequencies in locus Pt30204 of the Oldest trees class were significantly different from classes E, Y, and M (*p* values were 0.013, 0.035, and 0.011, respectively).

Table 2. Genetic characteristics of the analyzed Scots pine age classes.

Locus	E Embryos	S 1–3	Y 10–20	M 40–80	O >100	Mean
cpSSR						
Number of alleles per locus *Na*	3.1	3.6	3.6	3.6	3.4	3.5
Effective number of alleles per locus *Ne*	2.0	2.0	2.2	2.0	2.0	2.0
Number of haplotypes	29 (22)	30 (19)	31 (19)	23 (13)	32 (19)	29
Percentage of polymorphic loci	80%	90%	90%	90%	100%	90%
mt markers						
Number of alleles per locus *Na*	1.5 [2]	1.5 [2]	1.5 [2]	1.5 [2]	1.5 [2]	1.5 [2]
Effective number of alleles per locus *Ne*	1.4 [1.7]	1.4 [1.6]	1.2 [1.5]	1.2 [1.5]	1.5 [1.8]	1.3 [1.6]
Number of mitotypes	2	2	2	2	2	2
Frequency of mitotypes						
a	0.70	0.75	0.80	0.79	0.65	0.738
b	0.30	0.25	0.20	0.21	0.36	0.264
Percentage of polymorphic loci	50%	50%	50%	50%	50%	50%

cpSSR—chloroplast simple sequence repeat; E—embryos germinated from seeds; S—seedlings 1–3 years old; Y—young trees 10–20 years old; M—middle-aged trees 40–80 years old; O—old trees above 100 years. Number of private haplotypes in parentheses; number of alleles only for locus *nad7* in square parentheses.

A joint analysis of the alleles of the ten chloroplast loci identified 117 different haplotypes among the 156 individuals, from 23 (class M) to 32 (class O). The majority of these haplotypes (77%) were detected only once (unique haplotypes), and the rest were observed in two-to-six individuals. None of the detected haplotypes were common amongst all of the age classes of the studied population. The majority of specimens in an age class demonstrated a private haplotype: from 13 (M class) to 22 (E class) private haplotypes, with a mean of 79% (Table 2).

Haplotype diversity differed considerably between the chloroplast microsatellite loci, from 0.025 in PCP102652 to 0.734 in Pt15169, with the mean over the entire population amounting to 0.393 (Table 1). The lowest values were observed in the Embryos and the Oldest trees classes (0.381 and 0.370, respectively), while the highest value was observed amongst the Young trees (0.415) (Figure 1).
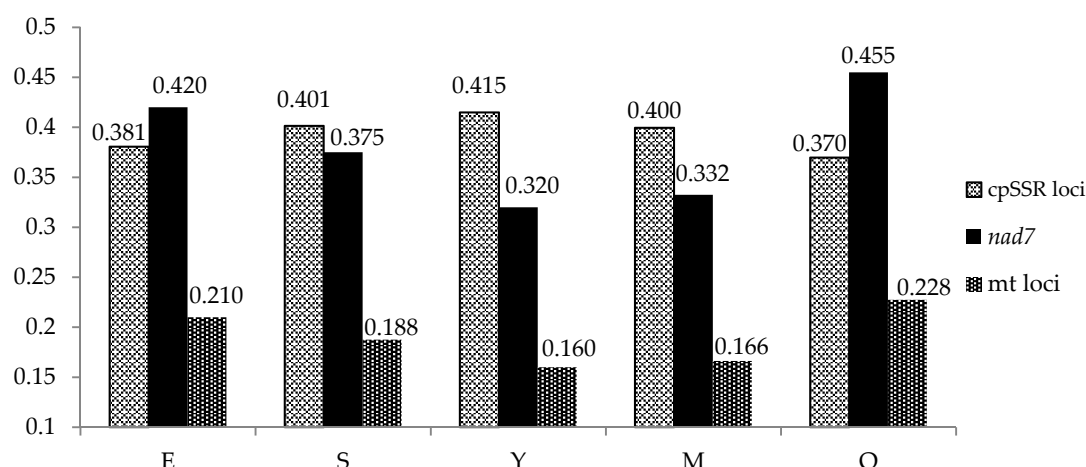

Figure 1. Haplotype diversity among age classes for chloroplast and mitochondrial markers. E—embryos germinated from seeds; S—seedlings 1–3 years old; Y—young trees 10–20 years old; M—middle-aged trees 40–80 years old; O—old trees above 100 years.

An analysis of molecular variance (AMOVA) using chloroplast SSRs revealed a significant (probability 0.027) variation among the age classes, accounting for about 2% of the total variance (Table 3). The highest differentiation occurred in loci Pt30204 (6.8%, probability 0.009) and Pt45002 (4.4%, probability 0.035) (Table 4). The pairwise *PhiPT* value varied among the age classes, from 0 to 0.084 (Table 5).

Table 3. Analysis of molecular variance (AMOVA) in Scots pine age classes for cpSSRs markers.

Source	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among age classes	4	35.893	0.106	2% ($p = 0.027$)
Within age classes	196	877.589	5.519	98%
Total	200	913.482	5.625	100%

d.f.—degrees of freedom; *p*—probability based on 1000 permutations.

Table 4. Genetic differentiation (AMOVA *PhiPT*) across five age classes of Scots pine.

Locus	*PhiPT*	*p*
cpSSR		
Total	0.018	0.037
Pt 15169	−0.020	0.865
Pt 26081	−0.001	0.434
Pt 30204	0.068	0.009
Pt 36480	−0.004	0.630
Pt 45002	0.044	0.035
Pt 71936	0.014	0.216
PCP 1289	−0.024	0.944
PCP 41131	0.014	0.176
PCP 87314	−0.004	0.452
PCP 102652	0.004	0.186
mt markers		
Total	−0.005	0.560
*nad*1	-	-
*nad*7	−0.005	0.537

p—probability. Significant values are bolded.

Table 5. Pairwise age class *PhiPT* values (below diagonal) and Nei's genetic distance (above diagonal) for cpSSRs markers.

E	S	Y	M	O	
	0.013	0.011	0.016	0.021	E
0.022		0.018	0.017	0.019	S
0.000	0.008		0.023	0.029	Y
0.000	0.022	0.000		0.027	M
0.084 ***	0.002	0.058 **	0.051 **		O

Probability, *p*, based on 1000 permutations: *** $p < 0.001$; ** $p < 0.01$; E, S, Y, M, O—symbols of the age classes, denotations as in Table 1.

The lowest Nei's [30] genetic distance was observed between the class of Embryos and the Young trees (0.011), and the greatest distance was observed between the Oldest trees and the Young trees (0.029) (Table 5).

PCoA analysis based on the genetic distance between each age class revealed the genetic distinctiveness of the Oldest tree group (Figure 2). The first two factors of the PCoA analysis explained more than 84% of the variation found in the genetic distance matrix.
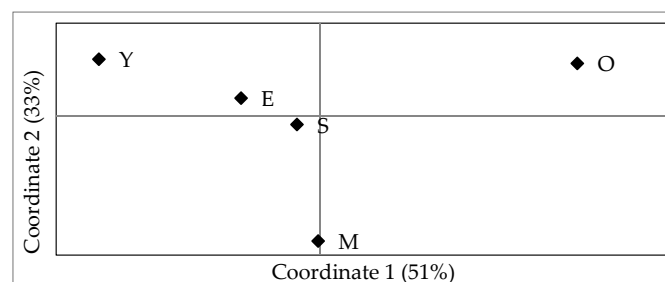
Figure 2. Principal coordinates analysis (PCoA) based on the genetic distances among the age classes of Scots pine.

3.2. Mitochondrial Markers

All analyzed individuals had a single *a* mitotype (217 bp) for the locus *nad1*. At the locus *nad7*, two mitotypes were observed in all age classes: universal *a* of 300 bp (65–80%; overall 74%) and northern *b* of 295 bp (20–36%; overall 26%) (Tables 1 and 2). Haplotype diversity *H* (calculated for polymorphic

nad7 locus) ranged from 0.320 (class Y) to 0.455 (class O) and 0.420 (class E), indicating that the greatest values occurred in the oldest and the youngest age classes. The distribution of *H* values in different age classes showed the opposite tendency compared to the chloroplast loci (Figure 1). An AMOVA analysis conducted on the allele frequencies did not indicate any significant differentiation between the age classes (Table 4), and the entire genetic variation was found within the age classes. Nei's genetic distances among particular generations were very low: from 0 to 0.013.

4. Discussion

This article reports on the first analysis of the temporal genetic diversity across five age classes of a naturally renewing *P. sylvestris* population located in the BPF using both chloroplast and mitochondrial DNA markers. The ten chloroplast polymorphic microsatellite loci indicated a high genetic variation within the particular generations in terms of the number of alleles and haplotypes (31–36 and 23–32, respectively). Provan et al. [19] observed lower values for the number of haplotypes (from 10 to 18) by analyzing the loci of 17 cpSSRs in populations of the naturally-occurring range of Scots pine in Europe. A very high number of haplotypes (36–43) was found across three Estonian populations of this species [31]. In total, 152 haplotypes were identified of four cpSSR loci in 38 populations of *P. sylvestris* in Asia and Eastern Europe [32]. The alleles of six cpSSR loci were combined into 32 different haplotypes, all found in the set of 235 individuals from the 12 different populations of *P. pinaster* [33]. Parducci et al. [34] identified from 11 to 22 different haplotypes among the 169 individuals of four *Abies* species. The nine cpSSR loci were examined in *P. resinosa*, giving a total of 23 chloroplast haplotypes among the 159 individuals [35]. Schmidt et al. [36] observed 43 haplotypes in *A. sibirica*, 49 in *A. sachalinensis*, and 31 in *A. nephrolepis* in two chloroplast microsatellite loci. We detected a high percentage of unique haplotypes in every age class (from 56% to 76%), whereby 59% of the analyzed individuals could be individually genotyped. A similar percentage of unique chloroplast haplotypes (55%) was found in 13 Scots pine populations from the Iberian Peninsula [37]. A study by Naydenov et al. [38] revealed as much as 90% of unique haplotypes in a study of 12 populations of *P. sylvestris* in Bulgaria.

An analysis of molecular variance (AMOVA) using chloroplast SSRs revealed that the significant variation among age classes of *P. sylvestris* population from the BPF accounted for about 2% of the total variance. The *PhiPT* values for pairwise age classes were much higher, showing a significant genetic differentiation (0–6.2%). These values were comparable to those found between the different natural populations of this species through an analysis of chloroplast SSRs: 2.1% between populations from Asia and Eastern Europe [39], and 1.5% between three bog populations from Estonia [31]. Previous studies on *P. sylvestris* [40–42] have shown that inter-population variation accounted for between 1.7% and 7.5% of the total observed variation. Such pronounced genetic differentiation within a single population may be a consequence of a temporal variation in population reproductive episodes, such as the different participation of trees in pollen generation and pollination (wind and flowering period) or pollen inflow from the outside. Paternally-inherited cpDNA is dispersed by the movement of both seeds and pollen. In landscapes dominated by forests, the pollen source area may have a radius of 50–100 m [43]. In mixed woodlands, such as in the BPF, the presence of different tree species in the canopy may restrict pollen movement and cause partial reproductive isolation [44], and thus a higher genetic differentiation between age classes. These factors result in differences of allele frequencies among generations of seeds produced. The distribution of diversity at nuclear SSRs and allozyme loci appeared to follow a similar pattern, as revealed by our previous study on the same Scots pine population [15].

A significant subdivision of genetic variation at the level of 5% was particularly evident in two loci: Pt 30204 and Pt 45002. The same loci revealed significant differences (Fisher's exact probability test) in allele frequencies for age class pairs. Locus Pt30204 is the intergenic space between the ATP-dependent protease proteolytic subunit (*clpP*) gene and ribosomal protein S 12 (*rpsl2*) gene [45]. SSR markers are assumed to be neutral, but there is some evidence that certain microsatellite loci show functional significance and may be closely linked to the target of natural selection or may even be directly under selection themselves [46,47]. Recent studies indicated that polymorphism in organelle

genomes may be important for adaptation processes in plants [48]. Rodriguez et al. [49] investigated the associations between cpSSR groups and phenotypes in *Phaseolus coccineus*. The existence of a cytonuclear disequilibrium between allozymes and cpDNA in a natural population of *P. ponderosa* was shown by Latta et al. [50].

The mitochondrial genome of the Pinaceae family does not recombinate and is passed through the maternal line over generations. In conifers, mtDNA shows a low level of variation through its exons and introns [51,52].

Among the age classes of the studied population from the BPF, only mitotype *a* (217 bp) of the *nad1* intron B/C was present and shared in common. It predominates in the Scots pine populations of Central and Northern Europe [22,23]. Locus *nad7* was represented by two mitotypes occurring in all of the analyzed generations, with mitotype *a* and northern mitotype *b* being more frequent. Both frequencies of alleles as well as haplotype diversity values did not differ between generations, which confirmed the AMOVA analysis. In conifers, variation in the mitochondrial genome represents the gene flow mediated by seeds, while variation in the chloroplast genome describes gene flow attributable to both pollen and seeds [53]. Because of the ease of movement, pollen dispersion is considered to be generally more important in the gene flow of forest trees than the dispersion of seeds, especially for wind-pollinated species [54,55]. Forest gaps and the surrounding crown canopies may locally influence the microenvironmental factors, thus affecting the seeds and pollen migration. As a result, genetic variation is limited by the low distance of seed dispersal, and the population structure of mtDNA variability within species remains conservative over the long term [56]. We can conclude that the genetic variation of the studied age classes—represented by mitochondrial markers—strongly depicts the maternal genetic structure of the overlying canopy, indicating limited seed dispersal. The same pattern of genetic variation was found in other conifer species; e.g., *P. ponderosa* [57] and *Picea abies* [58]. The results indicate that temporal genetic substructuring is more or less maintained within this self-renewed population of Scots pine from the Białowieża Primeval Forest.

5. Conclusions

The populations of long-lived forest tree species have a structure with overlapping generations, whose dynamic changes inevitably involve genetic differentiation at the spatial as well as temporal scales. The genetic study of successive generations allows for the inference of long-term demographic processes, and is a powerful tool for the analysis of processes that underlie the observed genetic structure [59]. The cpSSRs indicated that a natural population of Scots pine from the BPF appears to maintain a high level of genetic diversity between generations, while the mitochondrial genome markers represent a stable level of variation. The genetic structure of the studied self-renewed *P. sylvestris* population is naturally dynamic, calling for research into its fluctuations over time to better understand how they contribute to the adaptive potential of the population. Our study provides information on the genetic dynamics of a natural population of a keystone long-lived tree species, and is significant for the fields of conservation and adaptation. Finally, our analysis highlights the need to incorporate age profiles in analyses of the genetic variation of forest tree populations.

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