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Genetic resources of the oldest trees of *Pinus sylvestris* L. from the last natural forest in Europe

Abstract: The last fragments of primeval forests in the European lowlands have survived in the Białowieża Forest (BF). A characteristic feature of its forest stands is a complex and multi-age structure. From a genetic perspective, old trees represent a genetic pool that is a product of the selection forces acting over centuries. Therefore, it is extremely important to make a genetic inventory of such old trees and preserve their genetic resources for future generations. The aims of this study were: 1) genotyping the oldest trees of *Pinus sylvestris* in the Sitki Reserve of the Białowieża Forest in Poland; and 2) characterisation of the genetic structure of this old Scots pine tree stand. In total, 98 old trees aged above 100 years were analysed using eight nuclear microsatellite markers. The first study on the genotyping of the oldest Scots pine trees in the BF, indicated that each of the trees has a unique genotype, so it can be unambiguously identified by a genetic profile. Overall, 85% of trees showed an individual heterozygosity of 0.5 or higher. We demonstrated, that eight polymorphic microsatellite loci of the nuclear genome are sufficient to create an individual genetic profile of each tree. Our results will provide necessary background information for the conservation of native genetic resources of *Pinus sylvestris*.

Keywords: genotyping, nSSR markers, genetic profile, gene pool

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Introduction

The Białowieża Forest (BF) in north-eastern Poland (23°31'–24°21'E; 52°29'–52°57'N; Fig. 1) is unique in Europe in terms of its natural science and education opportunities, with over a five-century tradition of dedicated protection. Many of the best-preserved forest stands in the Białowieża Forest, can be considered as the natural forests (there are no originally primeval forests in Europe that would not have been affected by human influence), having many features of the primeval forest in the European lowlands. The BF has been subject to various forms of human use for centuries, however the special status

Fig. 1 Location of the Białowieża Forest and Sitki Reserve

of a royal forest limited its exploitation and agricultural expansion from the 15th to the beginning of the 20th century (Samojlik et al., 2013). Exploitation of forest wood resources on a large scale began during World War I. In the years 1924–1929, on the basis of an agreement signed with the Polish government, the English company "The Century European Timber Corporation" cut down 2.5 million m³ of wood using clear-cut harvest method. Only seed trees and trees monuments of nature were left on these areas where no afforestation was carried out (Więcko, 1984). The clear-cuts areas have been naturally regenerated mainly by light-seed species (aspen, birch, alder, hornbeam) and are called post-Century stands. A large share of these species is the result of natural, spontaneous regeneration processes, which were only slightly affected by the care and protection treatments of forest stands (Borecki & Brzeziecki, 2001). Compared to other Polish and European forests, BF is characterised by a pretty good preservation of the natural forest habitats, largely because of the regeneration of forest trees based on the natural process of pollen and seed dispersion. It is the last forest in Europe where preserved complex and multi-species ecosystems can be observed in their natural state (Drozdowski et al., 2012). The BF was inscribed on the United Nations Educational, Scientific and Cultural Organisation (UNESCO) World Heritage List, as the first transboundary site (Polish–Belarusian) in Europe. The extremely valuable resources in the Białowieża Forest include quite numerous fragments of natural tree stands, with a significant number of old trees preserved within them (Korczyk, 2008).

These old trees are often over 200 years old, and can therefore be considered the remains of genetically 'wild' forms that in the process of evolution developed specific natural mechanisms for adapting to the changing environmental conditions. Their value for both science and forest management cannot be overestimated (Matras, 2013).

Pinus sylvestris is the main species forming the forests in Poland and in Europe, with enormous economic importance. However, its natural (primeval) gene pool has been significantly modified as a result of extensive forestry, cultivation and selection activities, as well as due to seed transfers (Puumalainen et al., 2003).The Scots pine is also the most important forest-forming species in the Białowieża Forest, covering 27% of the forest area (Sokołowski, 2004). Long-term studies (since 1936) on the development dynamics of the natural tree stands in Białowieża National Park (which is part of the Białowieża Forest) under strict protection conditions have indicated a decrease in pine numbers and a regression of the forest's natural renewal in the 20th century (Bernadzki et al., 1998; Samojlik, 2006). As a light-demanding species, the pine requires open, sunny stands for its restoration, and such stands are quite rare in the forest. Currently, the pine is not being renewed effectively in the Białowieża Forest and is gradually being replaced by shadow-tolerant spruce trees. Studies in recent years have also shown that *P. sylvestris* trees are dying out within the already existing, restored areas. The mechanisms of this phenomenon are not fully known, but the decreasing levels of ground water as a consequence of global warming are indicated as one of the reasons (Paluch, 2014).

The longevity of trees is associated with the actions of a variety of selective pressures, which are conducive to maintaining a high level of genetic variation and a resulting adaptability to changing environmental conditions (Petit et al., 2004; Savolainen et al., 2007). In the case of intensively cultivated species such as *P. sylvestris*, genetic diversity is also very important from an economic point of view, because it determines the durability of the economic traits and is the basis for selection activities focused on improving selected traits of the tree stands and ensuring resistance to the negative effects of biotic and abiotic factors (Matras, 2006). The old pine trees in the Białowieża Forest represent the genotypes that have been growing in this area since before intensive forest management began (Sokołowski, 2004). Therefore, it is extremely important to determine their original gene pool and the level of genetic variation. Research questions we want to address are the following: 1) establishing a genetic inventory of the old trees; and 2) determining the characteristics of the genetic structure of the old *Pinus sylvestris* stand in the Sitki Reserve of the Białowieża Forest.

Material and methods

Study site and sample collection

The material for the genetic analysis (fresh needles) was collected from 98 individual Scots pines, aged 124–220 (Table S1), in the Sitki Reserve (52°37'27"N, 23°40'10"E) of the Białowieża Forest (Fig. 1). A collection of fresh needles for genetic analyzes was made after obtaining the formal consent of the Regional Director for Environmental Protection, in which the Sitki reserve is located. The harvesting of plant material was carried out in accordance with the regulations provided for such activities in the forest reserve.

The reserve with an area of 34.09 ha was established in 1979 to protect the oligotrophic forms of a pine forest in their natural habitat, which is not found in any other area of the Białowieża Forest. The reserve is located in the Leśna river catchment area. The groundwater levels are deep, usually below 3 m, and deeper on dune elevations. Only in the north-eastern part of the reserve, groundwater occurs from 0.5 to 1 m. One of the main forest complexes in the reserve is the *Vaccinio vitis-idaeae – Pinetum*. It occupies dune elevations. The stand is composed of pine with a loose density, reaching a height of 26–28 m. A small single admixture is *Betula pendula,* and also *Picea abies* (Sokołowski, 2004). According to reports by Korczyk (1994), the only maintenance carried out in this reserve was the removal of overturned trees infected by bark beetles.

The age of the individual trees was determined by measuring their diameter at breast height (so-called "dbh age" of the tree determined from the age table) and for verification by counting annual growth rings using cores of randomly selected individuals. The studied trees were randomly distributed and the distance between them was from 10 to 100 m, on average.

SSR genotyping

Nuclear DNA was isolated from the pine needles (100 mg from each individual) using a modified CTAB method (Doyle & Doyle, 1990). The quantitative and qualitative measurement of the isolated DNA was done with a Nanodrop™ ND-1000 spectrophotometer (ThermoScientific).

The usefulness of the microsatellite markers was proven in studying genomic differentiation, inter- and intrapopulational diversity, determination of genetic structure and gene flow of forest tree species (Li et al., 2002; Zhang & Hewitt, 2003). For the genetic analyses, 10 loci of the microsatellite markers (SSR) of the nuclear genome were selected for the study with polymorphism and high-quality amplification products: SPAG 7.14, SPAC 11.4, SPAC 11.6 and SPAC 12.5 (Soranzo et al., 1998), PtTX 4001, PtTX 4011, PtTX 3016, PtTX 3032, PtTX 3107 and PtTX 3116 (Auckland et al., 2002). A DNA amplification reaction (PCR) was carried out on a target volume of 25 μl for each sample, and included: 20 ng of isolated DNA, 2.5 mM of MgCl2, 100 μM of each dNTP nucleotide, 0.2 μM of each primer, 1U of HiFiTaqPolymerase (Novazym, Poznań, Poland), and the appropriate amount of $1 \times$ PCR buffer, according to the protocol developed by Celiński et al. (2013). The thermal profile of the PCR reaction was as follows: initial denaturation for 3 min at 94 ℃, then 35 cycles of 15s denaturation at 95 ℃, annealing for 1 min at 59 ℃, 1 min incubation at 72 ℃, and a final extension at 72 ℃ for 10 min. The amplification products were separated by capillary electrophoresis on a genetic analyser (Applied Biosystems). The reverse primer of each pair was fluorescently labelled with 6FAM, PET, NED and VIC labels. The individuals were then analysed and genotyped using the GeneMapper v.3.7 program (Applied Biosystems). All the questionable cases were repeated and trees re-genotyped to obtain unambiguous results of the analyzes.

Statistical analysis

All trees were analyzed using ten nuclear SSR loci. The software Micro-Checker (Van Oosterhout et al., 2004) was used to test the analyzed microsatellite loci for null alleles. We excluded from further analysis two problematical loci (SPAG 7.14 and SPAC 12.5) with high null allele frequency.

Based on the obtained results (for the 8 selected loci), standard population genetic diversity indices were calculated using the GenAlEx v.6.5 program (Peakall & Smouse, 2012) according to the following: percentage of polymorphic loci (P%), total number of alleles (A), average number of alleles per locus (A / L), effective number of alleles (Ae), allele frequencies, expected (H_e) and observed heterozygosity (H_o), individual heterozygosity (H_I), fixation index (inbreeding coefficient, $F_{IS} = 1 - H_o/H_e$) and deviation from the statistically tested Hardy-Weinberg equilibrium (Jain & Workman, 1967; Nei & Roychoudhury, 1974). The individual genotype profile (based on 8 nSSR loci) of each tree was determined . The genetic structure of the population was visualized using principal components analysis PCA based on a pairwise genetic distance matrix between particular trees (GenAlEx v.6.5).

Results

The studied trees were each given an identification number, to which the defined genotype was

assigned. All the analysed loci turned out to be polymorphic ($P\% = 100\%$), and a total of 95 alleles (from 4 to 21) were found in the 8 analysed microsatellite loci. The average number of alleles per locus (Na) was 11.9, while the average effective number of alleles per locus (Ne) was 3.5 (Table 1). The average number of alleles with a frequency greater than 5% was 4.125. The distribution of the frequency of individual alleles in the analysed SSR loci indicates that, in most cases, there is one allele with a visibly higher frequency and several alleles which are less common. The multi-locus match analysis showed that each of the studied trees has a unique genotype, so it can be unambiguously identified (see Supplementary Table S1 online).

The average observed heterozygosity was high (0.596) and ranged from 0.194 (SPAC 11.6) to 0.949 (PtTX 4001). The distribution of the individual heterozygosity values revealed that one tree was completely heterozygous, while 85% of the studied trees had a heterozygosity of 0.5 and higher (Fig. 2).

The average value of the fixation index (0.065) for the analysed loci indicated that the studied population of old pine trees presents an excess of homozygotes, in relation to the values expected in the Hardy-Weinberg equilibrium (in 6 loci). The analysis of the individual loci showed that the locus PtTX 3107 population was in the H-W equilibrium (-0.058),

Fig. 3 The Principal Coordinates Analysis based on the genetic distances between trees of *P. sylvestris*

and a statistically significant excess of heterozygotes (-0.254) was found in the locus PtTX 4001 (Table 1).

Principle Coordinate Analysis (PCoA), generated using genetic distances between the trees in a system of two coordinates (describing 22% and 19% of the total genetic variability, respectively), indicated a relatively random distribution of particular individuals (Fig. 3). No grouping due to genetic distance was found among the examined trees. The exception was outlier tree number 65. For this tree, a unique combination of alleles was found in three loci: SPAC 114 (150 bp), PTtX 3032 (256 bp), PTtX 3116 (148 bp), which was not present in the other trees.

Discussion

Monitoring the level of genetic variation is important, both from the aspect of protecting and preserving genetic resources, as well as for the purpose of breeding forest-forming species of trees. A polymorphism analysis of microsatellite sequences carried out on 98 old *P. sylvestris* trees from the Sitki Reserve allowed for the genotyping these individuals, making it possible to determine a "genetic profile" for each tree. All the examined trees had a unique genotype, which indicates the richness of the gene pool within this stand. The obtained results (100% polymorphic loci, number of alleles, individual heterozygosity of particular trees) support the statement that the

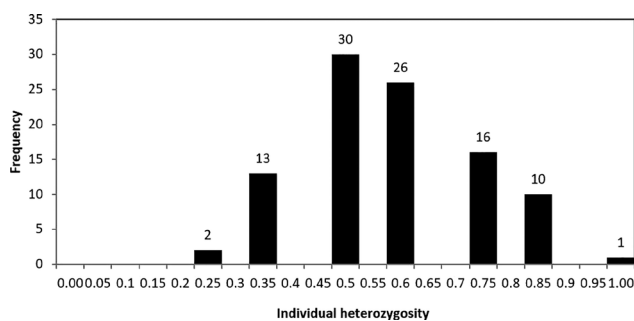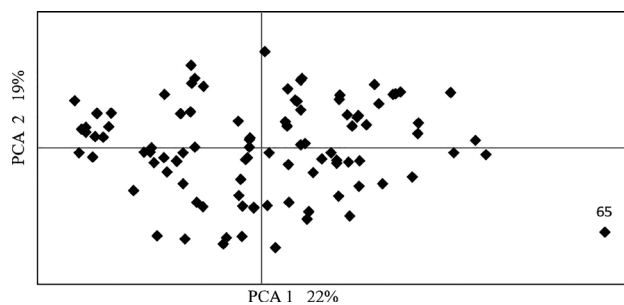Fig. 2 Individual heterozygosity of the studied Scots pine trees

Table 1. Genetic diversity estimates of the studied population of *P. sylvestris*: n – number of individuals; Na – number of alleles; Ne – effective number of alleles; Ho – observed heterozygosity; He – expected heterozygosity; F – fixation index; * – significant (p <0.001) deviation from Hardy-Weinberg equilibrium

Locus	n	N_e	N_e	H_o	H_e	F_{is}	p
PTtX 3016	98	4.0	1.6	0.276	0.367	0.249	*
PTtX 3032	98	21.0	2.3	0.541	0.560	0.034	*
PTtX 3107	98	7.0	4.0	0.796	0.752	−0.058	
PTtX 3116	98	12.0	6.4	0.796	0.843	0.056	*
PTtX 4001	98	13.0	4.1	0.949	0.757	−0.254	*
PTtX 4011	98	6.0	2.6	0.439	0.621	0.294	*
SPAC 11.4	98	19.0	5.5	0.776	0.819	0.054	*
SPAC 11.6	98	13.0	1.3	0.194	0.228	0.149	*
Overall	98	11.9	3.5	0.596	0.618	0.065	
Standard deviation	0	2.1	0.7	0.097	0.079	0.061	

examined old pine stand shows a level of genetic variation, which is characteristic of *P. sylvestris* in this part of natural range.

In order to estimate the level of genetic diversity of the studied trees from the Białowieża Forest against the background of the species within the central European range, the values of genetic parameters from previous reports on the populations of *P. sylvestis* were compared (Table 2). For such a comparison to be meaningful, we tried to select studies conducted on natural populations and those aged over 100 years (as far as it was possible). The nuclear microsatellite markers used in this study detected medium level of genetic variation in comparison to Scots pine populations from other regions of natural distribution of this species in Europe and Poland. Genetic diversity revealed in our study was very similar to that seen in some populations from Lithuania (Danusevicius et al., 2016), Carpathians (Sofletea et al., 2020) and two stands of Kampinos National Park in Poland (Przybylski et al., 2021). However, in comparison with other Scots pine populations from Poland (Przybylski et al., 2021; Nowakowska, 2016; Lesiczka et al., 2017), Sweden (García Gil et al., 2015), Estonia (Pazouki et al., 2015) it shows a lower level of genetic variation as measured by basic statistics (Table 2). On the other hand, genetic diversity estimates were higher compared to that found in the Finnish and Polish populations studied by Hebda et al. (2017) and Wójkiewicz et al. (2016) which presented lower values of mean number of alleles, effective number of alleles, observed and expected heterozygosity (Table 2). These noticeable differences in the parameters describing the within population genetic variation of the core distribution reflect the differences in the number of trees and analyzed loci, the age of the

trees, colonization of Poland and Europe by *P. sylvestris* from different postglacial refugia, intensive forest management and seed transfer.

The average value of the fixation index indicated a slight heterozygote deficit in the population of old pines. This value was the closest to the Polish Scots pine proveniences studied by Hebda et al. (2017) and Wójkiewicz et al. (2016) (0.062–0.07) as well as to one Finnish population (0.07) (Wójkiewicz et al., 2016) (Table 2). As can be seen from the comparison of several reports in Table 2, the fixation index usually indicates a much greater, than in our studies, deficit of heterozygotes in the populations from the core continuous species distribution.

In our studies F_{IS} values were heterogeneous across loci indicating that inbreeding may not be the only factor causing homozygous excess. Fageria and Rajora (2013) did not attribute an excess of homozygotes in *Picea glauca* to the inbreeding, considering that F_{IS} values were heterogeneous among the nuclear microsatellite loci. It can be the result of selection against heterozygotes, gene flow, assortative mating or the Wahlund effect. As reported by Spiess and Franklin (1996) this is quite a common phenomenon in long-lived species that undergo cross pollination. On the other hand, it may also be the effect of long-term selection processes leading to a maximum population adaptation to the local habitat conditions. Selection processes promote alleles responsible for the advantageous adaptive features of a population favoring homozygous allele in one locus of the genome and/or heterozygous alleles in another locus (Whitlock, 2002).

The genetic variability of trees is the basis for maintaining diversity in the population gene pool, which determines the resistance to biotic and abiotic

Table 2. Comparison of genetic diversity estimates of *P. sylvestris* populations: *N/population* – number of individuals per population; *nSSR* – number of analyzed nuclear microsatellite loci; *Na* – number of alleles; *Ne* – effective number of alleles; *Ho* – observed heterozygosity; *He* – expected heterozygosity; *F* – fixation index; * – significant (p < 0.001) deviation from Hardy-Weinberg equilibrium

N_o population/location	*N/population*	nSSR	N_a	N_e	H_o	H_e	F_{is}	References
4, Finland	24	13	5.8–6.4	3.1–3.7	0.452–0.491	0.470–0.500	0.02*–0.11*	20
2 provenances, Finland	24	10	5.2	2.49	0.484	0.448	−0.049	19
3 natural, Sweden	195	5	14.8–16.4	4.6–5.3	0.680–0.740	0.790–0.800	0.223*	17
North-eastern Lithuania	395	12	13.2	5.5	0.528	0.552	0.052	12
3 natural, bog, Estonia	60	5	16.8–21	7.4–9.1	0.940–0.953	0.770–0.800	−0.21–0.27	18
8 provenances, North-eastern Poland	21–24	10	4.4–5.5	2.3–2.8	0.390–0.456	0.425–0.501	0.01–0.077	19
4, Poland	22–45	13	5.5–6.5	3.2–3.8	0.409–0.467	0.475–0.536	0.07*–0.14*	20
Tabórz Pine Reserve, Poland	17	7	10	7.3	0.697	0.799	0.133	16
8 stands under strict protection, Poland	50	5	13.2–18	7.8–9.9	0.579–0.779	–	0.101–0.324*	14
14 management, North-eastern Poland	48–50	4	16.5–19.2	8.6–11.5	0.710–0.832	0.782–0.849	–	15
8, native to Eastern and Southern Carpathians	96	8	8.7–11.7	3.5–6.2	–	0.635–0.733	−0.046–0.187*	13
This study	98	8	11.9	3.5	0.596	0.618	0.065	

factors as well as the adaptability of the species to changing environmental conditions, and therefore is important for the long-term stability of a forest ecosystem (Spiess, 1989). Genetic pool of a population of old trees, is a consequence of reproductive episodes, long-term selection processes and competition for life resources (Spiess & Franklin, 1996).The oldest pines from the Białowieża Forest represent the natural, native gene pool of the species that has not been transformed by humans. It should therefore be 'genetically inventoried' as soon as possible in order to protect, as well as to preserve, its genetic potential. Conservation activities consist of both the protection and the sustained preservation of the genetic resources of an individual or a population even after their death, e.g. by creating a clone archive of the oldest trees (Korczyk, 1994). The preservation of genetic resources is considered one of the most important forms of biodiversity protection (Aravanopoulos, 2016; Geburek & Konrad, 2008; Rajora & Mosseler, 2001). Forest trees, along with their genetic diversity, are the most important component of forest ecosystems, shaping the ecological niches for other species of flora and fauna. Therefore, the preservation of the genetic resources of the main forest-forming species in the face of increasing anthropopression (forest management, seed transfer, industrial pollution) and expected climate change is of particular importance (Holliday et al., 2017; Vinceti et al., 2020).

This is the first study on the genotyping of the oldest Scots pine trees in the Białowieża Forest, which will provide necessary background information for the conservation of native genetic resources in this species. We demonstrate that eight polymorphic microsatellite loci of the nuclear genome are sufficient to create an individual genetic profile of each tree. As *P. sylvestris* is one of the most commercially important tree species under forest management in Europe, the native stands in the Białowieża Forest can become "genetic standards" when compared with man-made forests.

Acknowledgments

Author's contributions

A.W-P. conceived the work and planned the research design, supervised fieldwork, analyzed the data, wrote the manuscript; K.C. genotyped samples and performed all analyses; E.Ch. wrote the manuscript and critically revised it, reviewed the literature. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict interest.

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