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Genetic differentiation between generations of *Pinus sylvestris* natural population: a case study from the last European primeval forest

Genetische Differenzierung zwischen Generationen der *Pinus sylvestris* aus natürlicher Population: eine Fallstudie aus dem letzten europäischen Urwald

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Keywords: *native gene pool, age genetic differentiation, biodiversity conservation, isozyme, nSSR markers*

Abstract

Old stands of trees in the Białowieża Primeval Forest in north-eastern Poland represent a primeval gene pool, which has not been subject to human selection and which maintains attributes of the ancient virgin forests that once covered central Europe. We used isozyme and simple sequence repeat (SSR) markers to estimate the level of genetic variation in a naturally renewed population of *Pinus sylvestris*, with respect to its demographic structure. Our study revealed a high level of intra-population diversity, expressed by uneven allele distribution, observed and individual heterozygosity and a very high percentage of unique multi-locus genotypes (92% for isozymes

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and 100% for SSR). Significant temporal changes in allele frequencies were detected between particular age classes. The observed fixation index F_{IS} indicated an excess of homozygotes increasing with age class. The Bayesian clustering method detected two genetic clusters: the first containing the two youngest age classes and the second containing the remaining three age classes. Our results revealed temporal dynamics of the genetic structure of the Scots pine population, probably resulting from the interaction of many factors in the past such as fluctuating reproduction episodes, gene inflow, selection and adaptation processes, and fire regimes.

Zusammenfassung

Alte Baumbestände im Białowieża Urwald im nordöstlichen Polen repräsentieren einen Urgen-Pool, der nicht Subjekt menschlicher Auslese wurde und welcher weiterhin über Attribute des altertümlichen Urwaldes verfügt, der einst Zentraleuropa bedeckte. Wir verwendeten Isoenzym und einfache Sequenzwiederholungs-Marker (SSR), um das Niveau der genetischen Variation in der natürlich verjüngten Population des *Pinus sylvestris* zu schätzen, mit Hinblick auf seine demographische Struktur. Unsere Studie brachte ein hohes Niveau an Inner-Populations-Diversität zum Vorschein, welche durch eine ungleichmäßige Allelverteilung, beobachtete und individuelle Heterozygotität und einen sehr hohen Anteil einzigartiger Multi-Locus-Genotypen (92% für Isoenzyme und 100% für SSR) ausgedrückt wurde. Signifikante zeitliche Veränderungen in Allelhäufigkeit wurden zwischen bestimmten Altersklassen festgestellt. Der beobachtete Fixationsindex F_{IS} zeigte einen Überschuss an Homozygoten, die mit Altersklasse zunahm. Die Bayesianische Clustering-Methode zeigte zwei genetische Cluster: das erste bestehend aus den zwei jüngsten Altersklassen und das zweite bestehend aus den übrigen drei Altersklassen. Unsere Ergebnisse ergaben zeitliche Dynamiken der genetischen Struktur der Waldkieferpopulation, wahrscheinlich resultierend aus der Interaktion von vielen Faktoren in der Vergangenheit, wie zum Beispiel fluktuierende Reproduktionsepisoden, Genzufluss, Selektion und Anpassungsprozesse sowie Feuerregime.

1. Introduction

As long-lived plants, forest trees are exposed to a sequence of different environmental conditions that vary during their lifespan and create a heterogeneous environment. Their adaptive potential of forest trees is determined by the amount and pattern of their genetic variation, which is considered a measure of the stability of a complex forest ecosystem and its population sustainability (Müller-Starck 1997; Nkongolo et al. 2012). Populations of a long-lived tree species, composed of cohorts established at different times, can be genetically differentiated in spatial and temporal aspects

(Linhart et al. 1981; Chung et al. 2003). As underlined by Chen and Song (1997), the demographic genetic structure can reveal important factors influencing the population genetic structure in the past (adult trees) and the present (juvenile trees). While the spatial genetic structure has been investigated in many plant species (Epperson and Chung 2001; De Lucas et al. 2009), there are still few studies of genetic variation in the age classes of forest trees. Differences in the level of genetic diversity between the parental and progeny populations of *Pinus strobus* presented studies of Rajora et al. (2002) and Nijensohn et al. (2005) while an opposite tendency showed Chhatre and Rajora (2014).

It should be emphasised that research into genetic diversity in several age classes is difficult because of the longevity of the trees, and also the fact that most of the managed tree stands have no complex age structure. A unique possibility is offered by the Białowieża Primeval Forest (BPF), located in northeastern Poland and western Belarus. This includes forest stands which show characteristics of primeval forest, a unique fragment of lowland natural forest in this part of Europe. BPF is an irreplaceable area for biodiversity conservation, due in particular to its size, protection status, and substantially undisturbed nature. These forest areas have been subject to protection since the beginning of the 15th century, when they were the hunting grounds of the kings of Poland. This Polish royal protection strictly limited the exploitation of forest resources, and thereby helped to maintain the natural values of the forests better than in other European temperate-zone forest regions (Więcko 1984). Up until 1888, the forest regenerated any losses strictly through natural renewal; therefore, all trees above 100 years old are relics of the native population and have resulted from the process of natural selection (Korczyk 2008). Due to its unique character, the most valuable section of the BPF was officially covered by a protection order in 1932, at which time it became the Białowieża National Park (Okołów et al. 2009). This is one of only seven transboundary biosphere reserves worldwide (and one of three in Europe) to be included in the UNESCO World Heritage List. Old tree stands in the BPF are valuable to science and forest management, as they represent the primeval gene pool of a native wild population which has not been subject to human selection (Korczyk 1994; 1995).

Scots pine (*Pinus sylvestris*) the most ecologically and economically important tree in Poland and Europe, is one of the main forest forming species in the BPF. In the Polish part of the BPF, Scots pine and Norway spruce (*Picea abies*) cover 52% of the forest area.

Scots pine is a light-demanding species, which in recent years has significantly reduced its contribution to the creation of tree stands in the BPF. Its life strategy is renewal on open, lighted areas, which appear very rare in BPF. Currently, pine is not self-renewed in an effective way and is a species displaced of their habitats by shade tolerant Norway spruce. *Picea abies* is a major competitor for Scots pine in the forest community of BPF. Although in some areas arise numerous renewals, they usually die

back within a few years. The mechanism of the dieback is not completely known. In the BPF one of the factors responsible for the dying of trees (not only the old ones) is a considerable shortage of groundwater as a result of decreased precipitation levels and the climate warming (Paluch 2014).

Pinus sylvestris has been intensively managed for over 200 years in Europe. Therefore, the naturally renewed native populations within the BPF, with their complex age structure, are unexampled and valuable to more specific studies. The BPF offers a unique opportunity for monitoring processes involved in the natural regeneration of tree population in a completely developed forest ecosystem which has only been minimally affected by human activity. Populations of keystone tree species, such as Scots pine, are descendants of the native wild populations, which have developed in the process of natural selection and their genotypes represent primeval gene pool (Korczyk 1994). Selection processes may act differently upon the following ontogenetic stages of self-renewed natural tree population. It would be interesting to compare the degree of local adaptation of different life stages.

Taking the above into account, we were particularly interested in the following questions: (1) Are there differences of genetic structure and diversity among age classes? (2) Does natural selection contribute to fluctuations of genetic diversity of complex age structure population?

2. Material and methods

2.1 Study site and sampling

This research was conducted in the naturally regenerated tree stands of Scots pines (*Pinus sylvestris*) located in the BPF in northeastern Poland (in the 'Sitki' reserve, compartment 667B and 668A of the Hajnówka Forest District) (Fig. 1). The reserve was created in 1979 (34.09 ha) 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 only treatment recently applied in this compartment, is the removal of trees that had toppled over and been infested by bark beetles (Korczyk 1994).

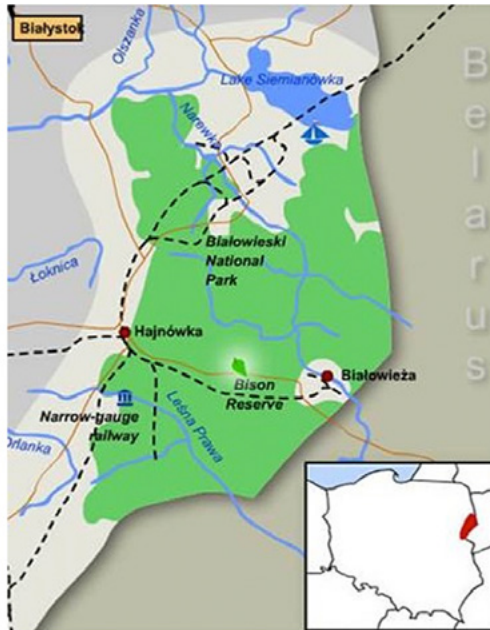


Figure 1: Location of the Białowieża Primeval Forest in Poland (<http://holidays.staypoland.com/bialowieza-forest.aspx>; modified)

Abbildung 1: Lage des Białowieża Urwaldes in Polen (<http://holidays.staypoland.com/bialowieza-forest.aspx>; modified)

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 and 100 m. Dormant buds were collected for genetic analyses from 327 individuals, representing four age classes of Scots pines (Table 1). Additionally, cones were collected from 50 adult fruiting trees (age 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 80 individuals (Table 1).

2.2 Isoenzyme analysis

Enzyme extracts were prepared from 1–2 winter buds taken from a single tree (or one seedling germinated under laboratory conditions). The bud tissues were ground with an extraction buffer (0.5 M Tris-HCl pH 7.5 containing EDTA, KCl, MgCl₂, PVP and

Triton). For electrophoresis, two buffer systems were used: A) Tris-citrate pH 7.0 (electrode buffer)/Tris-histidine pH 7.0 (gel buffer); and B) LiOH-borate pH 8.3 (electrode buffer)/Tris-citrate-LiOH-borate pH 8.3 (gel buffer) (Conkle et al. 1982; Cheliak and Pitel 1984).

The following 11 enzyme systems, encoded by 14 loci, were used for the analyses: fluorescent esterase (EC 3.1.1.1. FEST), glutamate dehydrogenase (EC 1.4.1.2. GDH), glutamate-oxaloacetate transaminase (EC 2.6.1.1. GOT), isocitrate dehydrogenase (EC 1.1.1.42 IDH), malate dehydrogenase (EC 1.1.1.37. MDH), shikimate dehydrogenase (EC 1.1.1.25 SHDH), phosphoglucosmutase (EC 2.7.5.1. PGM), 6-phosphogluconate dehydrogenase (EC 1.1.1.44 6PGD), NADH-dependent dehydrogenase (EC 1.6.5.3 NDH), phosphoglucose isomerase (EC 5.3.1.9. PGI) and diaphorase (EC 1.6.4.3. DIA).

2.3 DNA extraction and SSR amplification

DNA was extracted from needles (100 mg of fresh material) using the modified (without 2-mercaptoethanol, incubation in 70° C) cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1990). The quality and quantity of extracted DNA was measured using a Nanodrop™ ND-1000 spectrophotometer (ThermoScientific).

The 8 nuclear microsatellite loci that were variable and provided a high-quality amplification product were used for the analysis of all samples. These were: SPAC 11.4 and SPAC 11.6 (Soranzo et al., 1998), and PtTX 4001, PtTX 4011, PtTX 3016, PtTX 3032, PtTX 3107 and PtTX 3116 (Auckland et al. 2002). These loci were variable and provided a high quality amplification product. PCR amplification was carried out in a total volume of 25 µl, containing about 20 ng of template DNA, 2.5 mM MgCl₂, 100 µM of each dNTP, 0.2 µM of each primer and 1U HiFiTaq Polymerase (Novazym, Poznań, Poland), with the respective 1 × PCR buffer, according to the protocol described by Celiński et al. (2013). 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. Amplification products were separated using the 3130xl Genetic Analyzer (Applied Biosystems) capillary electrophoresis system with GeneScan™ 600 LIZ™ as an internal size standard. The reverse primer of each primer pair was labelled with fluorescent 6FAM, PET, NED and VIC dyes. Individuals were analysed and genotyped using GeneMapper version 3.7 software (Applied Biosystems).

2.4 Data analysis

Each type of marker was analysed separately, and then jointly as combined data (isozyme and SSR loci).

Data were analysed using GenePop v.4.0 (Raymond and Rousset 1995a; Rousset, 2008) and GenAlEx v.6.5 population genetic software (Peakall and Smouse 2012). Micro-

Checker software (v.2.2.3) was used to examine null alleles at microsatellite loci using the Oosterhout algorithm (Oosterhout et al. 2004). Genetic variability was described by the total number of alleles, the mean number of alleles per locus, the number of private alleles, the percentage of polymorphic loci, the frequency of alleles, and the expected (H_e) and observed (H_o) heterozygosity. We calculated the Wright's fixation index (F_{IS}) for all polymorphic loci in each age group, as a relative measure of deviations from the Hardy–Weinberg equilibrium (HWE) expected for random mating (Jain and Workman 1967; Nei and Roychoudhury 1974). Differences between age classes were estimated using the pairwise P values for allele distribution between generations, performed using Fisher's exact test and the log-likelihood G statistics over all loci (Raymond and Rousset 1995b), calculated by GenePop v.4.0. The pairwise genetic distance according to Nei (1972) was calculated between particular age classes.

The level of genetic differentiation between and within age groups was estimated by a hierarchical analysis of molecular variance AMOVA (Excoffier et al. 1992) using GenAlEx 6.5 (Peakall and Smouse 2012), for which new AMOVA routines enable the estimation of standardized F'_{ST} following Meirmans (2006). A standardised measure of population genetic differentiation (F'_{ST}) allows the comparison of results based on the two different types of markers.

Therefore, we performed an outlier test using the LOSITAN software (Antao et al. 2008) to detect loci under selection at a 95% confidence level. An initial run of 100,000 simulations was conducted, and was followed by computing the distribution of neutral F_{ST} . Markers with unusually high or low F_{ST} violating these thresholds were identified as candidates for selection (Beaumont et al. 1996; Antao et al. 2008).

The genetic population structure was analysed using a Bayesian clustering approach with the STRUCTURE v. 2.2.3 program (Evanno et al. 2005). The data were explored without considering prior classification within the sample, by performing 20 replicates of each simulation with a burn-in of 50 000 steps, followed by 10 000 Markov chain Monte Carlo iterations under the admixture model. Additionally, we used the STRUCTURE HARVESTER v.0.6.92 program (Earl and van Holdt 2012) that applies the Evanno method to assess the optimal level of K , number of assumed clusters were from 1 to 5 (Evanno et al. 2005).

3. Results

3.1 Allelic heterogeneity

The percentage of polymorphic isozyme loci was 93%, and only the IDH locus was monomorphic in all age classes. The eight microsatellite loci analysed were polymorphic over five age classes. Micro-Checker revealed the occurrence of null alleles in locus PtTX 3107, which was excluded from further calculations.

Table 1: Genetic characteristics of the analysed Scots pine age classes

Tabelle 1: Genetische Charakteristiken der analysierten Waldkiefer-Altersklassen

Age class	<i>N</i>	<i>PA</i>	<i>N_a</i>	<i>A_e</i>	<i>H_o</i>	<i>H_e</i>	<i>F_{IS}</i>
Isozyme loci							
E (embryos germinated from seeds)	80	3	3.0	1.6	0.301	0.313	0.046
S (seedlings 1–3 years old)	87	1	3.2	1.6	0.296	0.318	0.096
Y (young trees 10–20 years old)	85	3	3.3	1.6	0.278	0.316	0.100
M (middle-aged trees 40–80)	80	0	3.1	1.6	0.300	0.319	0.069
O (old trees above 100 years)	75	0	3.1	1.6	0.295	0.320	0.075
Total	407		3.1	1.6	0.294	0.317	0.077
SSR loci							
E (embryos germinated from seeds)	40	13	10.7	3.4	0.764	0.642	-0.218
S (seedlings 1–3 years old)	40	15	11.4	4.3	0.632	0.571	-0.066
Y (young trees 10–20 years old)	38	3	7.7	3.0	0.563	0.569	0.073
M (middle-aged trees 40–80)	42	6	7.9	3.2	0.507	0.511	0.093
O (old trees above 100 years)	42	6	10.4	3.3	0.627	0.639	0.050
Total	202		9.6	3.4	0.619	0.586	-0.014
Isozyme and SSR loci							
E (embryos germinated from seeds)	40		5.3	2.2	0.453	0.420	-0.052
S (seedlings 1–3 years old)	40	13	5.8	2.5	0.429	0.405	0.005
Y (young trees 10–20 years old)	38	16	4.5	2.1	0.385	0.403	0.070
M (middle-aged trees 40–80)	42	5	4.6	2.2	0.376	0.383	0.073
O (old trees above 100 years)	42	6	5.5	2.2	0.377	0.410	0.092
Total	202	6	5.1	2.2	0.404	0.404	0.038

N – number of examined individuals, *PA* – number of private alleles, *N_a* – mean number of alleles per locus, *N_e* – effective number of alleles, *H_o* – observed heterozygosity, *H_e* – expected heterozygosity, *F_{IS}* – fixation index

N – Anzahl der untersuchten Allele, *PA* – Anzahl der privaten Allele, *N_a* – Anzahl der Allele pro Ort, *N_e* – effektive Anzahl der Allele, *H_o* – beobachtete Heterozygotität, *H_e* – erwartete Heterozygotität, *F_{IS}* – Fixierungsindex

The mean number of alleles per isozyme locus ranged from 3.0 to 3.3 (overall 3.1), for SSR loci from 7.7 to 11.4 (overall 9.6) and for combined markers from 4.5 to 5.8 (overall 5.1). The mean effective number of alleles was 1.6 (allozymes), 3.0–4.3 (SSR) and 2.1–2.5 (combined markers) in particular age classes, which means that only a few alleles had high frequencies in each locus (Table 1). Private alleles for isozyme markers were identified in the embryo class E (3 private alleles), the seedling class S (1 private allele) and the young trees class Y (3 private alleles). For SSR markers, as well as for combined markers, private alleles were detected in all the age classes (Table 1). Allelic differentiation was significant (by the G test) for all SSR loci and combined markers, and with respect to the following isozyme loci: DIA C, GOT A, GOT B, GOT C, PGI B and PGM.

3.2 Genetic diversity

A multi-locus match analysis revealed that 92% of individuals had a unique multi-locus genotype with respect to isozyme markers, and 100% with respect to SSR and combined markers. The average observed heterozygosity varied across age classes: for isozyme loci, 0.278–0.301 (overall 0.294); for microsatellite loci, 0.507–0.764 (overall 0.619); and for combined markers, 0.376–0.453 (overall 0.404) (Table 1). The highest H_o value occurred in the youngest class E (embryos from germinated seeds), regardless of the set of markers. Class M (middle-aged trees) was characterised by the lowest observed heterozygosity in respect of SSR and combined loci (0.507 and 0.376, respectively) (Table 1).

The mean fixation index (F_{IS}) for the five age classes was estimated at 0.129 and differed significantly from Hardy–Weinberg (H–W) expectations ($P < 0.001$) for isozyme loci. The F_{IS} across isozyme loci indicated positive values, from 0.04 (DIA C) to 0.413 (PGM), and was significant for 8 out of 14 loci ($P < 0.01$). Negative values (but in accordance with H–W expectations) of F_{IS} were found for the SSR loci (from -0.034 to -0.170, mean -0.04), with the exception of locus PtTX 4011, which showed significant deficiency of heterozygotes ($F_{IS} = 0.174$) as well as combined markers ($F_{IS} = 0.038$).

3.3 Genetic differentiation between age classes

AMOVA revealed high and significant rate of genetic differentiation among age classes ($F_{ST} = 0.072$, $F'_{ST} = 0.123$) (Table 2). Significant differentiation was observed in loci GOT A, GOT C, PGI B and in the all SSR loci. The greatest genetic differentiation was found between the seedlings class (S) and the middle-aged trees (M) ($F_{ST} = 0.129$), while the lowest was between class Y and class M ($F_{ST} = 0.017$ and $F_{ST} = 0.007$, respectively) (Table 2). The AMOVA analysis showed that, depending on the set of loci, 87%–99% of the total variation occurred within the age classes (Table 3).

Table 2: Pairwise F_{ST} (below diagonal) and genetic distances (above diagonal) between age classes - Isozyme and SSR markers

Tabelle 2: Paarweise F_{ST} (unten diagonal) und genetische Distanzen (oben diagonal) zwischen Altersklassen - Isoenzyme und SSR-Marker

	E	S	Y	M	O
E		0.029	0.088	0.089	0.065
S	0.027***		0.111	0.111	0.082
Y	0.095***	0.121***		0.013	0.031
M	0.102***	0.129***	0.007*		0.034
O	0.070***	0.091***	0.029***	0.037***	

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; E, S, Y, M, O – symbols of the age classes, denotations as in Table 1

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; E, S, Y, M, O – Symbole der Altersklassen, Bedeutung wie in Tabelle 1

Table 3: Results from analysis of molecular variance (AMOVA) for Scots pine age classes

Tabelle 3: Ergebnisse aus Analyse der molekularen Varianz (AMOVA) für Waldkiefer-Altersklassen

Marker	Source of variation	Percentage		P value
		d.f.	of variation	
Isozymes	Among age classes	4	1	0.001
	Among individuals	402	13	0.001
	Within individuals	402	87	0.001
SSR	Among age classes	4	13	0.001
	Among individuals	205	0	0.996
	Within individuals	210	87	0.001
Isozymes and SSR	Among age classes	4	7	0.001
	Among individuals	205	4	0.004
	Within individuals	210	89	0.001

d.f. – degrees of freedom, FG. - Freiheitsgrade

The F_{ST} outlier test violating the 95% confidence interval expectations under neutrality identified isozyme locus PGI B as a candidate for directional selection ($H_e = 0.470$, $F_{ST} = 0.098$, $P = 0.975$). Two microsatellite loci were candidates for directional selection: SPAC 11.4 ($H_e = 0.878$, $F_{ST} = 0.223$, $P = 0.988$) and PtTX 3032 ($H_e = 0.845$, $F_{ST} = 0.315$, $P = 0.998$), while locus PtTX 4001 was a candidate for balancing selection ($H_e = 0.816$, $F_{ST} = 0.021$, $P = 0.0003$).

Bayesian clustering of the information from the combined loci, which showed that the number of genetic groups (K value) best fitting our data was K=2. This indicates that the analysed age classes constitute two genetically differentiated groups (Figure 2 and 3).

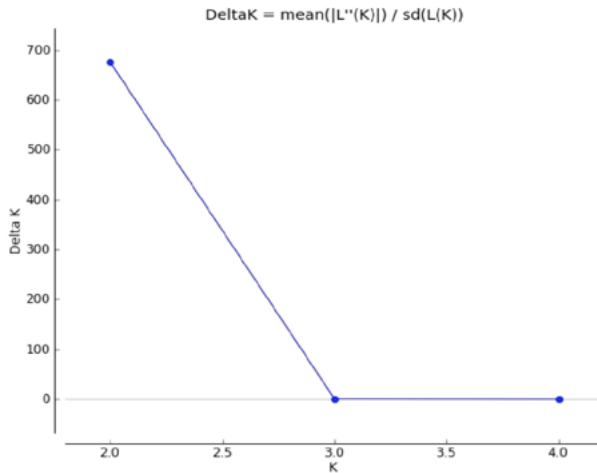


Figure 2: Values of the statistic "Delta K" for all calculated Ks. E, S, Y, M, O – symbols of the age classes, denotations as in Table 1

Abbildung 2: Werte der Statistik "Delta K" für alle berechneten Ks. E, S, Y, M, O – Symbole der Altersklassen, Bedeutung wie in Tabelle 1

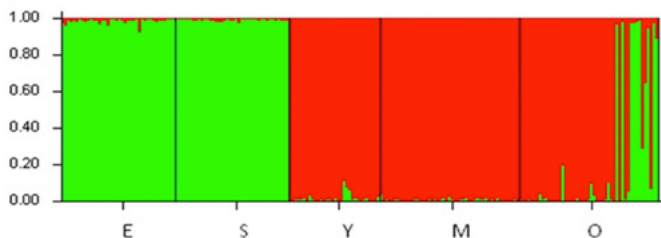


Figure 3: Bar plot from the Bayesian analysis of the age classes of Scots pine. Bar plot for a K = 2 model – combined markers

Abbildung 3: Balkendiagramm der Bayesianischen Analyse der Altersklassen der Waldkiefer. Balkendiagramm für ein K = 2 Modell – kombinierte Marker

4. Discussion

This study is the first to investigate the level of genetic variation in the naturally regenerated, unevenly aged population of *P. sylvestris* from the BPF, with respect to its demographic structure using biochemical and molecular markers. Characterising the native old-growth population of Scots pine is particularly difficult in European regions, where the anthropogenic effects have been of long duration and have interacted with natural factors (Barbati et al. 2012). Our study revealed a high level of intra-population diversity between generations expressed by the number of alleles, significant allele frequency differences (confirmed by G test), observed and expected heterozygosity, high values of F_{ST} and genetic distances. A very high percentage of unique multi-locus genotypes (92% for isozymes and 100% for SSR and combined loci) provides a large variation within a population, as indicated by the AMOVA results. Results of the exact G test confirmed that particular age classes differed significantly in allele frequency. As pointed out by Chhatre et al. (2013), such large allele frequency differences can be an indication that natural selection may have favored different alleles in different environments thereby driving frequencies of the selected alleles while filtering out the unfavorable alleles.

The significant subdivision of genetic variation ($F_{ST}=0.005$ for isozyme loci; $F_{ST}=0.114$ for SSR loci; $F_{ST}=0.072$ for combined loci) detected across age classes is comparable (or higher) to those found between different populations of this species: 0.02 (Karhu et al. 1996), 0.032 (Maaten and Kurm 2007) and 0.007 (Pazouki et al. 2015). The standardised genetic differentiation values (F'_{ST}) were much higher, especially for SSR and combined loci, than the unstandardized values (F_{ST}), emphasising the genetic heterogeneity of the age classes. This tendency was also reported in the studies of Krutovsky et al. (2009) and Jordan and Snell (2008).

We found private alleles (isozyme loci) only in the youngest age classes (the embryos E, the seedlings S and the young trees Y). In microsatellite and combined loci, private alleles were present in all the age classes, but in E and S their number were more than two-fold higher, these can provide the inflow of genes from the outside of population. Gene flow is one of the most important factors shaping the genetic structure of population. The heterogeneity of genetic structure among age classes may reflect differentiation of pollen/seeds immigration from surrounding populations occurring in particular reproductive seasons. Effective pollen immigration into natural populations of wind-pollinated species is substantial, especially within larger continuous forest stands (Burczyk et al. 2004) such as Białowieża Primeval Forest. Studies of Adams (1992) and Ellstrand (1992) indicate that in widespread tree populations, the collective pollen contribution from a large number of distant trees may account for an important portion of a progeny. Genetic distinctiveness of the embryos and seedling age classes is, in our opinion, a complex phenomenon that depends on a number of variables: temporal fluctuations of reproductive episodes (wind, different pollinators, flowering periods etc.), pollen inflow from the outside and selection pro-

cesses that shaped genetic structure of the older generations adapting them to the local environment. As show Savolainen et al. (2007) the extent of local adaptation is determined by the balance between gene flow and selection. Endler (1973) indicated that selection is often so strong that the 'swamping out' effects of gene flow is negligible. Obviously, the impact of selection versus gene flow will vary across loci, depending on whether or not they experience selection.

Isozyme markers, originating from coding sequences, have been described as playing a role in generating adaptive genetic variation, while SSR loci can be closely linked to genes, being under selection processes (Hamrick and Nason 1996; Kashi et al. 1997, 1999; Pritchard et al. 2000; Achere et al. 2005; Wang et al. 2006; Bilela et al. 2012). The outlier test identified loci PGI B, SPAC 11.4 and PtTX 3032 as candidates for directional selection, and locus PtTX 4001 for balancing selection, which accounted for 19% of the 21 loci studied in the *P. sylvestris* population from the BPF. The F_{ST} value in these loci indicated significant genetic differentiation among the age classes; 2% (PGI B), 19% (SPAC 11.4) and 28% (PtTX 3032). Phosphoglucose isomerase (PGI) is a dimeric enzyme which catalyses the reversible isomerisation of glucose-6-phosphate and fructose-6-phosphate, and plays an essential role in energy production in both glycolysis and gluconeogenesis. These metabolic pathways are particularly important for the first ontogenetic stages of trees such as seed germination and embryo development. Bergmann and Mejnartowicz (2001) revealed a reciprocal relationship between hexokinase (HEK A) and phosphoglucose isomerase (PGI B) loci in several conifers indicating a metabolically-based model of selective advantage/disadvantage of two-locus genotypes at HEK A/PGI B. Molecular studies of the genes coding for PGI in *Festuca ovina* revealed evidence for positive selection within the PGI C1 locus (Li et al. 2015). As pointed out by Lind-Riehl et al. (2014), loci significantly deviating from neutral expectations of differentiation, or outlier loci, may be closely linked to the target of natural selection or even be directly under selection themselves.

Studies on natural recruitment in *P. sylvestris* revealed an excess of homozygotes in the embryo stage, which decreased in the adult trees (Muona et al. 1987; Yazdani et al. 1985). Similar results have been found in *P. radiata* D. Don (Plessas and Strauss 1986) *Abies alba* Mill (Hussendoerfer 1998), *Pseudotsuga menziesii* (Mirb.) Franco (Shaw and Allard 1982) and *Fraxinus excelsior* (Morand et al. 2002). In general, an excess of homozygosity is expected in the progeny of trees, whereas an excess of heterozygosity observed in adults may be attributed to selection against homozygotes during their lifetime. Selection against inbred progeny is considered the main factor responsible for differences in inbreeding levels between adults and the early stages of trees in the life cycle. Our results indicate an opposite tendency, i.e. low deviations from the expected HW proportions (isozymes) or pronounced excess of heterozygotes (SSR and combined loci) in the youngest class, and higher homozygosity in the older age classes. F_{IS} values close to zero for juvenile stages might be a consequence of a recent increase in gene flow (Kelly et al. 2004). A homozygote excess may be the result of a temporal variation in mating system dynamics, which is typical of conifer species

(Awad et al. 2014). To explain the positive value of the inbreeding coefficient, it is also possible to assume the presence of a Wahlund effect, which may also arise for temporal reasons (Plutchak et al. 2006). A temporal Wahlund effect may be generated by a temporal variation in population reproductive rates, caused by: differences in the allelic frequencies among generations; a difference in the within-year flowering phenology; or variations in the flowering expression of individuals between years (Gaffney et al. 1990; Tonsor et al. 1993; Morand et al. 2002; Chung et al. 2003; Kelly et al. 2004). It may be also caused by the differential movement of seeds and pollen. The Wahlund effect has been noted in a number of wind-pollinated tree species; Bacilieri et al. (1994) observed marked homozygote excess in a mixed stand of *Quercus robur* and *Quercus petraea*, and Finkeldey (2001) reported a modest but significant homozygote excess in the same species. However, although a Wahlund effect is expected to be locus independent and could explain the observed deficiencies of heterozygotes (Karlsson and Mork 2005), apparently this is not the case. Our results show that out of the analysed loci, the majority showed an excess of homozygotes in one age class, while in other age classes, there was an excess of heterozygotes.

The phenomenon of heterozygote deficiency may be due to selective forces acting over several generations in the site-specific environmental conditions of the BPF, favouring highly specialised homozygous genotypes that are the best-adapted to the particular micro-environment. As proved by Whitlock (2002), sometimes selection may favour the homozygous allele in a single locus of the genome and/or heterozygous alleles in another locus by choosing alleles responsible for the advantageous adaptive features of a population. Intense selection against heterozygotes would produce large heterozygote deficiencies (Gaffney et al. 1990). According to Gregorius and Bergmann (1995), the higher frequency of some alleles, as well as homozygosity, could be explained by adaptation to peculiar local environmental conditions. Tigersted et al. (1982) studied a very old population of *P. sylvestris* and found that a 300–400-year-old stand was at HWE, but the 100-year-old generation still contained an excess of homozygotes. In the studies of Morgante et al. (1993), fixation indices of approximately 5-year-old *P. leucodermis* grown in the wild were not significantly different to those of adult trees. Similar observations have been reported for *Picea omorika*, in which the onset of inbreeding depression was weak in the early development of the zygote, but strong in later growth (Kuittinen et al. 1991).

It should be taken into account that the initial population, that gave rise to the oldest Scots pine trees in the studied population, was generated more than 200 years ago. Up until 1874, frequent (18-year intervals) but low-intensity fires occurred in the BPF, promoting *P. sylvestris* dominance. As shown by Niklasson et al. (2010), fires were a major component in the past dynamics of conifer-dominated forests in the BPF and, among other factors, influenced the genetic structure of the studied population, especially the oldest generations.

Optimal adaptation to environmental homogeneity can lead to homozygosity at par-

ticular loci (Clark and Koehn 1992). It should also be taken into account that the particular form of selection in the BPF includes strong competition with spruce which, in contrast to the Scots pine, is a shade-tolerant species of tree, especially in the juvenile periods.

5. Conclusions

In conclusion, the complex genetic architectures that we have described for the *P. sylvestris* population from the BPF is worthy of interest for several reasons. Our data demonstrate that a self-renewed population, growing in continuous stands, can be genetically heterogeneous over time on a very small geographic scale. The presence of significant differences in allelic frequencies shows uneven distribution of alleles in particular age classes resulting in genetic subdivision. There is some evidence that certain loci show functional significance and could be subject to natural selection (or are located close to such outlier loci). This genetic differentiation between age classes is the result of the interaction of many factors, such as fluctuating reproduction episodes, gene inflow, selection and adaptation processes, and fire regimes in the past. Knowledge of the genetic structure dynamics of the forest trees has a fundamental importance for management practices and conservation strategies.

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