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ORIGINAL RESEARCH ARTICLE

Genetic variability of Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L. Karst.) natural regeneration compared with their maternal stands

Justyna Anna Nowakowska*, Tadeusz Zachara, Agata Konecka

Forest Research Institute, Department of Silviculture and Forest Tree Genetics,
Sękocin Stary, ul. Braci Leśnej 3, 05–090 Raszyn, Poland.

Tel. +48 22 7150467, e-mail: j.nowakowska@ibles.waw.pl

Abstract: The genetic variability and biodiversity of tree populations ensure the stability and sustainability of forest ecosystems. New research tools based on molecular DNA markers enable precise characterisation of forest genetic resources, i.e. detection of different allele frequencies in mature trees and progeny populations. The paper describes the genetic structure of mature stands of Scots pine (*Pinus sylvestris* L.) in Oława Forest District and Norway spruce (*Picea abies* L. Karst.) in Jawor Forest District and stands of their respective progeny.

In the Scots pine stand, there was a slight increase (0.6%) in heterozygosity level and a larger increase (4.9%) in the inbreeding coefficient of progeny trees. In the Norway spruce stand, despite a small reduction (0.9%) in heterozygosity, a similar increase (4.6%) in the inbreeding coefficient of progeny was revealed. In both stands, allele richness and the partition probability of basic clustering were high. Both pine and spruce adults and progeny trees were characterised by high levels of genetic similarity (96% and 79%, respectively). Gene flow between the mature and progeny populations was high ($N_m > 1$) for both Scots pine and Norway spruce.

Conservation of the gene pool within forest tree stands requires an increase in the proportion of natural regeneration. To estimate the extent to which genes are transmitted between adult trees and their progeny, more studies are needed, especially taking into account the influence of silviculture measures, like selective tree cutting, on the genetic variability of the younger generation.

These results confirm that the gene pool was conserved when transmitted between the stands studied, as well as highlight the usefulness of such a study for silvicultural purposes.

Key words: nSSR markers, gene flow, genetic differentiation

1. Introduction

Knowledge about gene pool of forest tree species provides valuable information on the genetic structure of stands, including the genome sequence of an individual tree, as well as genetic variation at the population level. The richness of the gene pool of each population is determined on the basis of DNA allele occurrence in the genome. Despite the phenomenon of outbreeding depression, caused by a sudden drop in the mating of individuals from remote and isolated stands, it can be assumed that a more diverse gene pool of the species ensures a greater likelihood of a favourable combination of alleles, guaranteeing the survival and adaptation to changing environmental conditions (Reed, Frankham 2003).

Many studies have been devoted to the genetic structure analysis relying on nuclear, mitochondrial and chloroplast DNA markers, as well as to the transmission of favourable breeding and resistance traits from mature trees to their progeny (Hamrick, Nason 2000; Sperisen

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et al. 2001; Avise 2004; Kremer, Reviron 2004; Neale, Ingvarsson 2008). In Poland, the research concerning genetic structure of forest tree populations was carried out, for example, Scots pine and Norway spruce based on nuclear and mitochondrial DNA markers, including RAPD, STS, PCR-RFLP and SSR (Csaikl et al. 2002; Dering, Lewandowski 2009; Nowakowska 2009, 2010). Those studies revealed for example two main directions of recolonisation routes of Norway spruce migration in Poland (Dering, Lewandowski 2009).

The forest area deriving from natural regeneration in Poland increased from 3% to 10% in recent decades (Report on the State of Forests in 2012), but still remains below the average of 67% portion in Europe (State of Europe's Forests 2011). An important issue then arises: is there any advisability and possibility of increasing the share of the naturally regenerated trees, regarding the environmental, economic and social functions of the forest? The postulate of sustainable forest management implied increased interest in natural regeneration methods e.g. for pine (Dobrowolska 2010).

The present research topic stays in line with the current study. The assessment of the genetic structure of pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L. Karst.) mature stands and their progeny may help to understand the gene pool transmission between generations. The preliminary hypothesis was that the local genetic pool of pine and spruce stands is at a similar level, both in mature trees as well as in young generation resulting from natural regeneration (self-seeding). The study aimed to determine the gene pool level in both groups of trees of each species, defined on the basis of the level of genetic variability of nuclear DNA *loci*.

2. Materials and methods

Plant material (needles) was collected from 60 trees located in the following areas:

– Scots pine population from Oława Forest District, Karwiniec Forest Division 98c, 114-year old stand, growing on fresh mixed forest habitat, in a class of renewal, with 10% of the 7-year-old progeny coverage;

– Norway spruce population from Jawor Forest District, Muchów Forest Division 155h, 105-year-old stand, a moderate habitat of upland fresh mixed forest, with 30% of 7-year-old of natural regeneration coverage.

Genomic DNA was isolated from 30–35 wood samples of each adult tree, randomly located within the stand, separated by a 10-metre distance, as well as from 30 needles from young generation plants. Extraction was performed with DNeasy 250 Plant Mini Kit (QIAGEN), and yielded DNA was analysed spectrophotometrically in NanoDrop ND-1000 (TK-Biotech). In order to characterise the genetic structure of Scots pine samples, three nuclear micro satellite *loci* were used: SPAG 7.14, SPAC 12.5 and SsrPt-ctg-4363 (Soranzo et al. 1998; Chagné et al. 2004). In the case of Norway spruce samples, the following three nuclear microsatellite *loci* were applied: SpA-G2, SpAC1-H8 and SpAG-D1 according to Pfeiffer et al. (1997). Genotyping of investigated trees was performed with CEQ™ 8000 Series Genetic Analysis System software v 9.0 in CEQ 8000 sequencer (Beckman Coulter®).

Genetic variation parameters for adult and progeny trees were estimated by effective (n_a) and expected (n_e) allele number per *locus*, as well as frequencies of null allele occurrence in GenePop v.4.0.10 software (Rousset 2008). Polymorphic informative content (PIC) was calculated with MolKin v.3.0 software (Gutiérrez et al. 2005). Observed (H_O) and expected heterozygosity (H_E) (Nei 1978) were evaluated with GenAlEx 6.5 software (Peakall, Smouse 2012). FSTAT v. 2.9.3.2 software (Gudet 2002) was used to calculate allelic richness (A_R), taking into account unequal number of individuals in the experiment, as well as the inbreed coefficient (F_{IS}) and the differentiation coefficient (F_{ST}) according to Weir and Cockerham (1984). Genetic similarity between adult and progeny trees was estimated by partition probability (P_t), resulting from basic clustering Markov Chain Monte Carlo algorithm (MCMC), $p = 0.02$ with BAPS 2.0 software (Corander et al. 2003). Gene flow between groups of trees was given by N_m parameter (Nei 1987).

3. Results

Genetic analysis of Scots pine stand

In SPAG 7.14 *locus*, 27 different allele variants were obtained, ranging from 176 base pairs (bp) to 248 bp (Fig. 1). Two alleles (192 and 211 bp) were predominantly present with 11% of frequency within adult pines, whereas in progeny trees allele 192 bp was predominant (14% of frequency). Six alleles were missing in progeny trees (178, 184, 186, 197, 211 and 235 bp) but six new ones appeared (176, 188, 190, 195, 230 and 248 bp). In SsrPt-ctg-4363 *locus*, 12 allele variants were found ranging from 92 to 123 bp (Fig. 1). In this locus, allele 97 bp was predominant both in adult and progeny trees, with frequencies of 44% and 47%, respectively. Next generation of trees had two new alleles (101 and 123 bp) not found in mature stand, but had missed five

alleles (92, 95, 107, 111 and 119 bp). Twenty-four different alleles were observed in *locus* SPAC 12.5 from 123 to 178 bp. Adult trees had eight unique alleles, not present in progeny. Conversely, next generation of trees had three new alleles, i.e. 137, 141 and 171 bp (Fig. 1). All three SSR *loci* had a high level of PIC = 84.1%.

Mean observed number of alleles per locus was higher in adult trees (n_a = 16.333) than in natural regeneration (n_a = 14.333, Table 1). Expected mean allele numbers per locus were at the same level in both groups of trees: n_e = 10.521 and n_e = 10.566, respectively (Table 1). Allelic richness was higher in mature stand (A_R = 15.290) than in the progeny (A_R = 14.150). Null allele share (8%) was high in adult trees compared with 2% in progeny (not illustrated).

The observed heterozygosity (H_O = 0.884) in progeny was higher than in the parental trees (H_O = 0.841). The expected heterozygosity (H_E = 0.859) in progeny was about 0.6% higher than the parental trees (H_E = 0.853). The level of inbreeding coefficient in adult trees was lower (F_{IS} = 0.011) than in progeny (F_{IS} = 0.049), but both values were statistically insignificant (Table 1). The average allele fixation index between parental and regenerated stand was low (F_{ST} = 0.082).

Small genetic distance D_N = 0.041 separated both groups of pines, confirmed by high (96%) genetic similarity between the studied adult and young trees (Pt = 1, p = 0.02).

It is generally considered that a value of $N_m > 1$ characterises large gene flow within a given population (Slatkin, Barton 1989). Thus, our study value of N_m = 30.297 obtained indicates a high gene flow between investigated groups of trees. It can therefore be assumed that mainly mature trees of the Oława stand had taken a major role in shaping the gene pool of the offspring.

Genetic analysis of Norway spruce stand

For both groups of trees – adult and progeny spruces from Jawor Forest District – 16 different allelic variants ranging from 83 to 125 bp were obtained in *locus* SpA-G2 (Fig. 2). In this locus, 101 bp allele was the most frequent (22%) in adult trees, whilst 97 and 99 bp alleles were predominant (18%) within the progeny. Two rare alleles – 85 and 125 bp – from a gene-pool of adult population were missing in renewal trees. In *locus* SpAC1 H8, 33 allelic variants were reported, including dominant alleles of size 99, 101 and 105 bp (frequency c.a. 8%) in adult trees and

Table 1. Genetic variation parameters for adult and progeny Scots pine trees

Locus	Type of trees	A_0	n_a	n_e	A_R	H_O	H_E	$F_{IS}{}^a$
SPAG 7.14	D	50	20.000	15.077	18.922	0.893	0.934	0.022^{ns}
	O	56	21.000	17.606	19.801	0.960	0.943	0.089*
SsrPt-ctg-4363	D	60	10.000	3.352	8.344	0.667	0.702	-0.062^{ns}
	O	48	7.000	3.704	6.642	0.792	0.730	0.067^{ns}
SPAC 12.5	D	54	19.000	13.1351	18.597	0.963	0.924	0.058^{ns}
	O	40	15.000	10.390	16.000	0.900	0.904	-0.006^{ns}
Mean	D	57	16.333±5.508	10.521±6.284	15.290	0.841±0.155	0.853±0.131	0.011^{ns}
	O	46	14.333±7.024	10.566±6.952	14.150	0.884±0.085	0.859±0.113	0.049^{ns}

D – adult trees
O – natural regeneration
A_0 – mean allele number
n_a, n_e – effective and expected allele number
A_R – allelic richness
H_O – observed heterozygosity
H_E – expected heterozygosity
F_{IS} – inbreeding coefficient: Fisher exact test P values for heterozygote deficiency, ns – not significant, * for $p < 0.05$

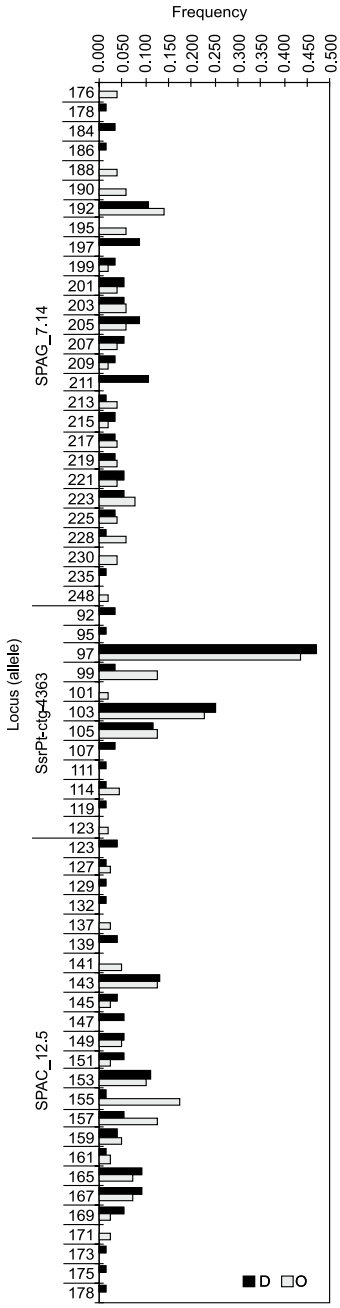

Figure 1. Frequency of three microsatellite DNA *loci* within adult (D) and progeny (O) trees in Scots pine stand

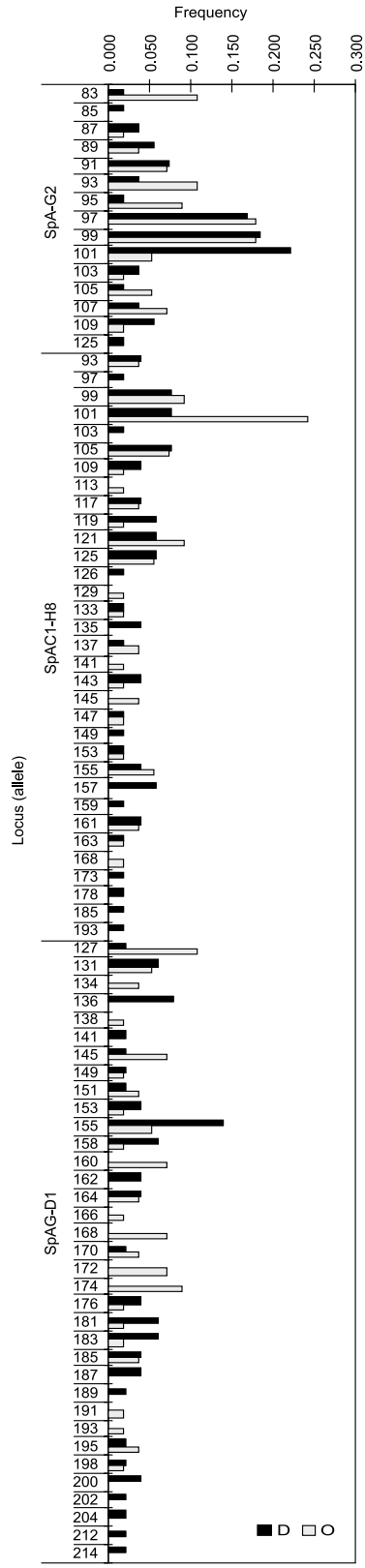

Figure 2. Frequency of three microsatellite DNA *loci* within adult (D) and progeny (O) trees in Norway spruce stand

one dominant 101 bp allele (frequency 24%) in progeny trees. Eleven allelic variants present in the progeny population (ranging from 97 to 193 bp) were missing in adult population (Fig. 2). Conversely, the later population was enriched by five other rare alleles, i.e. occurring with c.a. 2% in the investigated trees.

The *locus* SPAG-D1 had 35 different allelic variants, ranging in size from 127 to 214 bp. In adult tree population, 10 unique alleles (i.e. not present in the progeny population) were revealed, while the progeny population had nine new allelic variants present in this locus (Fig. 2). High level of polymorphism (PIC = 93.9%) characterised all investigated microsatellite loci.

The observed and expected average numbers of alleles per locus, as well as allelic richness (A_R), were higher (n_a = 22.667, n_e = 15.404, A_R = 22.690) in adult trees compared with the progeny (n_a = 20.000, n_e = 12.178, A_R = 19.200; Table 2). The average share of null alleles in all loci was estimated for 10% for both groups of spruce.

In progeny population, the observed and expected heterozygosity (H_O = 0.723 and H_E = 0.913, respectively) was lower than in the adult population (H_O = 0.756 and H_E = 0.922). Compared with the adult population, a 0.9% loss of heterozygotes was observed in progeny population.

In the case of adult spruces, inbreeding coefficients for SpAC1-H8 and SPAG-D1 *loci* were statistically significantly lower than in next generation of trees (Table 2). Similar to the pine stand, adult spruce population have lower coefficient of inbreeding (F_{IS} = 0.190) compared with progeny (F_{IS} = 0.236). The average value of the coefficient of fixation, determining a decrease of heterozygosity in natural regeneration population, was low (F_{ST} = 0.032).

Small genetic distance between the generation of mature and progeny trees was observed (D_N = 0.241), confirmed by high level of 79% genetic similarity between the studied groups of trees (Pt = 1, p = 0.02).

The migration coefficient N_m = 18.678 indicates high gene flow between the studied generations of Norway spruce trees.

4. Discussion

The nuclear microsatellite DNA *loci* are nowadays considered to be the most precise tools to determine the genotypes of living organisms. These markers are

Table 2. Genetic variation parameters for adult and progeny Norway spruce trees

Locus	Type of trees	A_0	n_a	n_e	A_R	H_O	H_E	F_{IS}[a]
SpAC1-H8	D	54	15.000	7.677	14.613	0.778	0.870	0.255***
	O	56	13.000	8.859	12.667	0.821	0.887	0.264***
SpA-G2	D	52	26.000	19.882	27.456	0.731	0.950	0.126[ns]
	O	54	23.000	12.150	21.164	0.704	0.918	0.092*
SpAG-D1	D	50	27.000	18.657	26.000	0.760	0.946	0.182***
	O	56	24.000	15.525	23.761	0.643	0.936	0.341***
Mean	D	52	22.667 ± 6.658	15.404 ± 6.723	22.690	0.756 ± 0.024	0.922 ± 0.045	0.190**
	O	55	20.000 ± 6.083	12.178 ± 3.333	19.200	0.723 ± 0.091	0.913 ± 0.024	0.236**

D – adult trees
O – natural regeneration
A_0 – mean allele number
n_a, n_e – effective and expected allele number
A_R – allelic richness
H_O – observed heterozygosity
H_E – expected heterozygosity
F_{IS} – inbreeding coefficient: Fisher exact test P values for heterozygote deficiency; ns – not significant, * for $p < 0.05$, ** for $p < 0.01$;*** for $p < 0.001$

characterised by high polymorphism and repeatability. The high PIC, average percentage of null alleles and high probability of identity calculated with MCMC algorithm prevailed the small number of three microsatellite markers used in the present analysis of approximately 30 individuals from each generation. Similar studies carried out on a smaller number of individuals allowed conducting a similar genetic study on isolated populations of wild cherry, bat and the falcon (Oddou-Muratorio et al. 2003; Rivers et al. 2005; Rutkowski et al. 2010).

The present study revealed c.a. 0.6% greater variety of gene pool of young pines and 0.9% poorer gene pool of natural regeneration of spruce compared with the population of adult trees. The observed small changes in the gene pool of both generations are most likely due to differences in allele frequencies caused by the natural selection during adaptation of the stand to changing environmental conditions (Gömöry 1992). High genetic similarity between populations of parental and progeny was also observed by Chomicz (2013) in few spruce stands from Silesian and Żywiecki Beskid, where a slight enrichment of the gene pool of the offspring in relation to parental generation was observed.

Both in the case of pine and spruce stands, the average expected number of alleles *per locus* was almost the same in adults and progeny trees. Among the studied populations, non-significant heterozygote deficiency, determined by the coefficient of inbreeding F_{IS} = 0.011 and F_{IS} = 0.049 respectively, was reported (Table 1).

A heterozygote deficiency at the population level may occur in nature. Sometimes, selection agents may favour homozygous allele in a single *locus* of the genome, and sometimes may favour heterozygous alleles – in another *locus* – by choosing alleles responsible for the advantageous adaptive features of a population (Whitlock, 2002). The positive correlation between the level of heterozygosity and the adaptation of population during the evolution of many plants and animals species has been observed. The opposite (negative) trend may also occur, when the elimination of harmful alleles and inbreeding process leads to the impoverishment of a gene pool (Reed, Frankham 2003).

In the case of Scots pine stand, the gene pool of young generation of trees has been enriched by 'new' alleles (as far as a slight increase in the value of H_E compared with adult trees was denoted), probably mediated via pollen migration from neighbouring stands. Low coefficients of fixation level (F_{ST} = 0.082 for pine, and F_{ST} = 0.032 for spruce) show little difference between the two generations of the investigated stands.

These findings endorse the hypothesis of high genetic similarity between the new generation and the adult trees, both for pine (96%) and spruce (79%) stands, suggesting genetic stability of those populations, well adapted to specific environmental conditions. The Scots pine from Oława Forest District revealed high gene flow (N_m = 30.297) between adult trees and offspring generation. Small enrichment of population with new genotypes indicates that adult generation took a major role in shaping the gene pool of the population, without excluding the participation of pollen deriving from neighbouring stands.

Similarly, high value migration level (N_m = 18.678) and a slight decrease of heterozygosity in the progeny trees in Norway spruce from the Jawor Forest District suggests that mainly trees growing within a stand took part in shaping the gene pool of the offspring, with little participation of the neighbouring stands. However, more studies are needed to elucidate this issue, e.g. the analysis of pollen flow under controlled conditions.

Breeding success largely depends on knowledge about the genetic value of forest tree stand. The awareness about genetic structure of the natural regeneration and mature trees producing pollen ensures the success of the silviculture measures. During artificial regeneration, there is a risk of reduction of genetic diversity (via selection) of plant material produced in forest nurseries, resulting sometimes in smaller tolerance of the young seedlings to the harmful environmental conditions.

According to the scope of this study, issues related to the forest management performed in mature stands have been omitted, although they may play a crucial role in gene pool of the offspring (Sabor 2003). In agreement with this author, thinning must allow free pollen flow between neighbouring populations. Another problem may occur in isolated populations where pollen exchange with neighbouring tree stands is limited, and often leads to inbreeding process (Tigerstedt et al. 1982). According to the observations made for fir and beech stands, a irregular shelterwood cutting and selection cutting are the most suitable for genetic stability of a stand (Konnert et al. 2007). Research conducted over the pines in Gubin Forest District showed great similarity of the genetic structure between natural regeneration and adult trees (Kosińska et al. 2007). Moreover, the results obtained in the pine population from Oława Forest District (present study) indicate that even the short-term regeneration period can get in the offspring a similar gene pool with the adult trees.

Another silvicultural measure, i.e. tending cuts (cleaning and thinning) has also an important effect on pop-

ulation structure. Thinning trials conducted in the past aimed at gaining valuable assortments of large size, and consequently led to less valuable trees remained in terms of phenotypic quality and health (Bernadzki et al. 1999). Research carried out in the stands where very strong cutting treatments were applied showed the risk of loss of rare alleles among next generation of trees (Konnert et al. 2007).

5. Conclusions

1. Genetic structure of the stand, defined by the heterozygosity coefficient of the population, can be useful in the gene pool assessment within progeny trees issued from natural regeneration.

2. There is no genetic variation between parental and naturally regenerated trees of Scots pine and Norway spruce in their respective stands; almost no changes in the presence and frequency of rare alleles were observed.

3. Minor changes in the genetic structure between two generations of pine and spruce trees suggest the maintenance of the same level of adaptability to changing environmental conditions in the progeny.

4. The conservation of the gene pool in the young generation of forest tree species requires an increase of natural regeneration as a reforestation method. To estimate the importance of natural regeneration scale in silviculture, more studies performed on a larger scale are needed, taking into account the influence of the different cutting methods on the genetic variability loss among the young generation of trees.

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Contributions

J.A.N. designed the study, conceived the experiments, analysed data and wrote the manuscript. T.Z. collected the plant material and wrote the manuscript. A.K. contributed to essential ideas in the discussion. A.M. collected the plant material and performed laboratory analysis.

Translated by: Justyna Anna Nowakowska