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Are we narrowing genetic variability in seed orchards? An attempt to answer, based on the analysis of microsatellite DNA of grafts growing in Scots pine (*Pinus sylvestris* L.) seed orchard in the Forest District Susz

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Abstract. Scots pine (*Pinus sylvestris* L.) is the most common species in Poland's forest stands. The mode of pine stands renovation requires that silviculture practitioners have continuous access to seed banks. Orchard-grown seeds are predicted to constitute an increasingly larger part of the average demand for pine seeds in Poland. Seed orchards, due to a limited number of maternal trees as well as the irregularity of their blooming and pollination, enhance the risk of genetic diversity reduction in planted forest stands. This is of particular importance in the context of dynamic climate change. Markers based on microsatellite DNA fragments are effective tools for monitoring genetic variability. In the present study, three different microsatellite DNA fragments were used: SPAC 12.5, SPAG 7.14 and SPAC 11.4. The main objective of this research was to study genetic variability in one of the biggest seed orchards in Poland, located in the Forest District Susz. The obtained results indicated heterozygosity loss within the orchard, proving the existence of specimen selection effects on genetic variability. Hence, it seems quite important to take account of molecular genetic variability of maternal trees in future breeding strategies.

Keywords: microsatellite DNA, tree breeding, seed orchard

1. Introduction

Scots pine (*Pinus sylvestris* L.) is one of the essential forest-forming species in Poland and occupies 59.9% of Poland's total forest area, regardless of the ownership type (Forests in Poland 2011; CILP 2012). Based on up to date knowledge on pine ecology and breeding, it is predicted that in the coming decades, Scots pine will be planted using seedlings from forest nurseries. In view of forest management practice, there will have to be ensured regular access to seed collecting areas, which now include in Poland: source-identified tree stands (previously: managed seed stands) - 88%, selected tree stands (previously: excluded seed stands) - 6% and seed orchards with generative or vegetative origin - 6% (DGLP unpublished data).Advanced seed production in Poland will involve greater than before utilization of seed orchard production, i.e. up to 40% of overall seed demand (Chałupka et al. 2010). Among others, this tendency results from market indicators forecasting the growth of demand for wood raw materials. However, the latter can be contrary to ecological forest management aspects, including the protection of forest habitats. Hence, it is vital to achieve greater wood production efficiency on the reduced forest areas under management. The requirement for greater stand production on smaller areas can only be fulfilled if, through selection, stand progeny inherits economically desirable traits. Genetically improved seeds are produced in seed orchards, and genetic gain is the measure of selection process success. For example, in Sweden, 25% genetic gain was achieved in forest stands derived from third generation seed orchards (Almqvist 2013).

In addition to the achievement of genetic gain (a positive effect from the silviculturist's perspective), the selection process carried out in seed orchards also brings about negative silvicultural effects. The main important problem in this context is narrowing down the number of maternal

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generation specimens, which may in turn result in lead to decreasing genetic variability genetic erosion in the next generation. The authors such as Koski (1980, 1982), Lindgre and, Matheson (1986), Burczyk (1998) as well as Kang (2001) believe that the insufficient number of genotypes and their unequal participation in forming progeny generation are the main reasons behind decreasing genetic variability in seed orchards. Besides, phenotypic traits have so far been key criteria used in the selection of maternal generation specimens. On the other hand, there has been available no data on individual intensity of Scots pine flowering and pollination as well as on individual tree genetic value (White et al. 1993, Gomory et al. 2000). Lack of this data may ultimately lead to failure in obtaining seed orchard panmixia described, among others, by Friedman and Adams (1981).

In order to preserve genetic diversity, seed orchards in Poland are established with at least 40 clones of pine or fir each, or else 30 clones of other species. The growers theoretically, presume that all planted trees take part in generative reproduction. This postulate, however, is not realistic, therefore, all over the world, wide-ranging studies are carried out on determination of the minimum number of seed orchard specimens allowing for retention of genetic variability in future generations. The University of North Carolina (2001) recommends establishing new seed orchards based on 20–30 clones. McKeand et al. (2003) refer to orchards made of no more than 5–10 clones. The issue was also studied in British Columbia (Canada), where it was evaluated by means of the effective clone number method that the clone number higher 7 was sufficient to retain acceptable genetic diversity (Stoehr, El-Kassaby 1997). El-Kassaby and Benowicz (2000) showed that the effective number of alleles in Douglas fir seed orchards was higher than that in natural populations (Table 1). Similar results were obtained for Scots pine, when comparing genetic variability in seed orchards with that in natural stands (Matheson, Lindgren 1985, Kosińska et al. 2007).

In addition, in pine seed orchards, similarly to mature forest stands, there is often observed an increase of heterozygosity. This tendency is explained by gradual elimination of homozygous specimens derived from self-pollination or pollination by closely related specimens. Burczyk (1998) found that the progeny population grown from seeds produced in the seed orchard indicated negative values of the inbreeding coefficient. These results suggest the advantage of crossbreeding over inbreeding, which is perceived by Burczyk (1998) as the effect of specific distribution of clones within the seed orchard area as well as variability in flowering phenology.

Given the aforesaid, the question arises as to whether genetic variability is indeed being narrowed in seed orchards. The answer to this research hypothesis can be obtained using molecular marker data. However, in subject literature, there is available only scarce information on genetic diversity in Poland's seed orchards with reference to DNA markers. There have been accessible, to the author's knowledge, no more than the results of PhD dissertations by Trojankiewicz (2006) and Cieślewicz (2009). Thus, the present study can constitute value added to the knowledge on the potential reduction of genetic diversity as a result of phenotypic selection.

The goal of the present study was to determine a level of genetic variability change in Scots pine maternal trees growing in the Forest District Susz seed orchard The results obtained will, among others, allow answering the question: what is the effect of the selection of limited number of maternal specimens on seed orchard gene pool and genetic variability?

2. Materials and methodology

Plant material

The plant material used in the study consisted of Scots pine needles collected in the early spring of 2011, in the seed orchard located in the Forest District Susz (the Baltic Forest and Nature Region), The orchard (32.25 ha) was established in 1977-1985 and named after Prof. S. Kocięcki.

Table 1. Comparison of genetic variability parameters between Douglas fir natural population and breeding population (1st and 2nd generation) (El-Kassaby, Ritland 1995)

Population	Percent of polymorphic loci	Number of alleles per locus	Expected heterozygosity
Natural stand	53%	2.14	0.171
Breeding population	65%	2.50	0.176
Breeding population 1st generation	63%	2.28	0.172
Breeding population 2nd generation	56%	2.25	0.163

It encompasses in total 30 quarters with 175 clones. The examined plant material allowed to depict genetic variability in 117 clones of maternal trees. Genetic analyses were carried out only on the genotypes positively verified with those of maternal trees growing in the field. The maternal trees originated from five Forest Districts: Jagiełek, Susz, Stare Jabłonki, Miłomłyn and Kudypy (Table 2).

Microsatellite DNA markers

Three polymorphic systems of microsatellite DNA: SPAC 12.5, SPAG 7.14 and SPAC 11.4 were used in the analyses (Sorenzo et al. 1998). Total DNA was extracted from the collected plant material. The extraction was performed using A&A Biotechnology commercial isolation kit. The extraction efficiency was checked in 1% agarose gel using the GelDoc^{tm}Xr+ scanner and ImageLab 4.0 software (BioRad). To confirm genetic material quality, 260 and 280 nm light wavelength absorbance was measured using the NanoDrop ND-1000 spectrophotometer.

In the next stage, the chosen microsatellite DNA markers were amplified using the polymerase chain reaction (PCR). PCR procedure was performed with the use of 12.5µl of ready-to-use mixture 2xPCR MixPlus High GC (Taq DNA polymerase 0.1 U/µl, MgCl2 4mM, dNTPs 0,5mM, reagents increasing reaction specificity) (A&A Biotechnology) along with fluorescently labeled primers at the concentration of 5µM: SPAG 7.14 (1 µl), SPAC 12.5 (1 µl), SPAC 11.4 (1 µl) (SigmaAldricht), and template DNA at the final concentration of 20 ng/µl (1 µl). The mixture was transferred to the Eppendorf Mastercykler eGradientS thermocycler, programmed for 40 cycles (Soranzo et al. 1998, modified). Each cycle comprised three steps: denaturation at 92^{o}C (60 s), anneling at 56^{o}C (60 s), and elongation at 72^{o}C (60 s). Initial denaturation was observed at 94^{o}C (5 min), and final elongation - at 70^{o}C (7 min). PCR efficiency was checked in 2% agarose gel, using the GelDoc^{tm}Xr+ scanner with ImageLab 4.0 software (BioRad).

The samples were separated using the Beckman Coulter capillary sequencer, and the reaction products were archived using CEQ^{TM}800 software. Capillary electrophoresis was performed using LPA I polymer and Frag I length standard. The values obtained were grouped into allele classes with an accuracy of a single base pair (bp).

Table 2. List of examined maternal trees

FRI Ref. No.	Year of confirmation	Forest District	Forest Division	Compartment
335	1970	Miłomłyn	Sarni Dół	96g
336	1970	Miłomłyn	Sarni Dół	100b
337	1970	Miłomłyn	Sarni Dół	100a
339	1970	Miłomłyn	Sarni Dół	100b
341	1970	Stare Jabłonki	Perkunicha	57g
342	1970	Stare Jabłonki	Perkunicha	57g
344	1970	Stare Jabłonki	Perkunicha	57g
345	1970	Stare Jabłonki	Perkunicha	78c
347	1970	Stare Jabłonki	Perkunicha	77d
348	1970	Stare Jabłonki	Perkunicha	77d
349	1970	Stare Jabłonki	Perkunicha	77d
350	1970	Stare Jabłonki	Perkunicha	101d
351	1970	Miłomłyn	Tabórz	94c
352	1970	Miłomłyn	Przylądek	132b
353	1970	Miłomłyn	Przylądek	132b
354	1970	Miłomłyn	Przylądek	132b
359	1970	Miłomłyn	Perskie	7d
360	1970	Miłomłyn	Perskie	8b

FRI Ref. No.	Year of confirmation	Forest District	Forest Division	Compartment
361	1970	Miłomłyn	Perskie	8b
362	1970	Miłomłyn	Zakątek	105c
364	1970	Miłomłyn	Zakątek	106c
365	1970	Miłomłyn	Zakątek	106c
366	1970	Miłomłyn	Zakątek	106c
367	1970	Stare Jabłonki	Białe Błota	254b
1311	1970	Stare Jabłonki	Białe Błota	254b
1316	1974	Miłomłyn	Bagińsko	132a
1317	1974	Miłomłyn	Jeziory	85n
1318	1974	Miłomłyn	Jeziory	85n
1319	1974	Miłomłyn	Jeziory	85n
1320	1974	Miłomłyn	Zakątek	146k
1321	1974	Miłomłyn	Zakątek	146k
1322	1974	Miłomłyn	Zakątek	146k
1323	1974	Miłomłyn	Zakątek	106c
1324	1974	Miłomłyn	Sarni Dół	100b
1325	1974	Miłomłyn	Tabórz	94c
1326	1974	Miłomłyn	Tabórz	94c
1695	1975	Miłomłyn	Tabórz	70d
1697	1975	Stare Jabłonki	Perkunicha	101d
1698	1975	Stare Jabłonki	Perkunicha	100f
1699	1975	Stare Jabłonki	Perkunicha	80i
1705	1975	Stare Jabłonki	Gąsiory	243a
1708	1975	Kudypy	Stary Dwór	293c
1713	1975	Kudypy	Żelazowice	468d
1714	1975	Kudypy	Żelazowice	468d
1715	1975	Kudypy	Żelazowice	468g
1716	1975	Kudypy	Żelazowice	468g
1717	1975	Kudypy	Żelazowice	468g
1718	1975	Kudypy	Żelazowice	480a
1719	1975	Kudypy	Żelazowice	480a
1720	1975	Kudypy	Żelazowice	488h
1721	1975	Kudypy	Żelazowice	488h
1725	1975	Kudypy	Żelazowice	468d
2118	1976	Susz	Michałowo	18d
2127	1975	Kudypy	Stara Góra	287h
2128	1976	Kudypy	Stary Dwór	293c
2130	1976	Stare Jabłonki	Perkunicha	78c

FRI Ref. No.	Year of confirmation	Forest District	Forest Division	Compartment
2131	1976	Stare Jabłonki	Perkunicha	78c
2133	1976	Stare Jabłonki	Perkunicha	78c
2135	1976	Stare Jabłonki	Perkunicha	77d
2136	1976	Stare Jabłonki	Perkunicha	77d
2137	1976	Stare Jabłonki	Perkunicha	57i
2138	1976	Stare Jabłonki	Draby	196a
2139	1976	Stare Jabłonki	Draby	196a
2140	1976	Stare Jabłonki	Draby	196a
2141	1976	Stare Jabłonki	Draby	196a
2142	1976	Stare Jabłonki	Draby	196a
2143	1976	Stare Jabłonki	Draby	196a
2144	1976	Stare Jabłonki	Draby	150h
2145	1976	Stare Jabłonki	Draby	159c
2147	1976	Stare Jabłonki	Draby	159c
2148	1976	Stare Jabłonki	Draby	159m
2149	1976	Stare Jabłonki	Draby	159c
2151	1976	Stare Jabłonki	Draby	159c
2152	1976	Stare Jabłonki	Draby	159c
2153	1976	Stare Jabłonki	Draby	159c
2154	1976	Stare Jabłonki	Draby	160c
2155	1976	Stare Jabłonki	Draby	160c
2156	1976	Stare Jabłonki	Draby	160c
2157	1976	Stare Jabłonki	Draby	187a
2158	1976	Kudypy	Żelazowice	480c
2159	1976	Kudypy	Stara Góra	310d
2161	1976	Miłomłyn	Przemysławów	138f
2165	1976	Miłomłyn	Zakątek	106h
2166	1976	Miłomłyn	Zakątek	106h
2167	1976	Miłomłyn	Zakątek	106h
2168	1976	Miłomłyn	Zakątek	108d
2169	1976	Miłomłyn	Perskie	7a
2170	1976	Miłomłyn	Perskie	27c
2171	1976	Miłomłyn	Perskie	7a
2172	1976	Miłomłyn	Perskie	28a
2173	1976	Miłomłyn	Perskie	28a
2174	1976	Miłomłyn	Jeziory	85n
2201	1976	Kudypy	Stary Dwór	360a
2202	1976	Kudypy	Stary Dwór	346b

FRI Ref. No.	Year of confirmation	Forest District	Forest Division	Compartment
2204	1976	Stare Jabłonki	Draby	150a
2205	1976	Stare Jabłonki	Draby	150a
2207	1976	Stare Jabłonki	Laski	140d
2208	1976	Stare Jabłonki	Laski	140d
2210	1976	Stare Jabłonki	Laski	140d
2211	1976	Stare Jabłonki	Laski	140d
2212	1976	Stare Jabłonki	Laski	140d
2213	1976	Stare Jabłonki	Laski	140d
2214	1976	Stare Jabłonki	Laski	140d
2215	1976	Stare Jabłonki	Laski	140d
2222	1976	Jagiełek	Jagiełek	358c
2223	1976	Jagiełek	Jagiełek	358c
2224	1976	Jagiełek	Jagiełek	358c
2225	1976	Jagiełek	Jagiełek	358c
2226	1976	Jagiełek	Jagiełek	358c
2227	1976	Stare Jabłonki	Ostrowin	296g
2228	1976	Stare Jabłonki	Gąsiory	281i
2229	1976	Stare Jabłonki	Gąsiory	281f
2230	1976	Stare Jabłonki	Gąsiory	281f
2232	1976	Stare Jabłonki	Gąsiory	243a
2233	1976	Stare Jabłonki	Gąsiory	243a
2234	1976	Stare Jabłonki	Gąsiory	243a
2235	1976	Stare Jabłonki	Perkunicha	77d

Statistical analysis

PopGen 32 (Yeh et al. 2000) and GeneAlEx 6.1 (Peakall & Smouse 2006) software products were used for calculations concerning genetic variability parameters in the seed orchard tested. Data analysis allowed for computing such parameters as: observed and expected heterozygosity, the effective number of alleles, the number of polymorphic loci,, the Hardy–Weinberg equilibrium and the Wright's Inbreeding Coefficient (F). The effect of null alleles on genetic parameters was evaluated by means Cervus 3.0.7 (Marshal 2014) and Micro Checker (van Oosterhout et. al 2005) software products.

3. Results

The seed orchard was examined in consideration of the presence of alleles located at the three studied loci. There were obtained 29 alleles (DNA length from 117 bp to 205 bp) at SPAC 12.5 locus, 26 alleles (DNA length from 173 bp to 237 bp) at SPAG 7.14 and 14 alleles (DNA length from 132 bp to 159 bp) at SPAC 11.4.

Among all the examined specimens, the most numerous alleles were: at SPAC 12.5 – an allele with DNA length 149 bp, at SPAG 7.14 – an allele with DNA length 207 bp, and at SPAC 11.4. – an allele with DNA length 139 bp, .There were also determined the least frequent (less than 1% frequency) alleles at the three loci: 5 alleles at SPAC 12.5 locus, i.e. 117 bp, 121 bp, 125 bp, 195 bp and 205 bp; 1 allele at SPAG 7.14, i.e. 178 bp, and 2 alleles at SPAC 11.4, i.e. 132 bp and 134 bp.(Fig. 1).

All the loci studied were polymorphic, therefore, the values of probability of identicality (P_{ID} = 6.59E-07) as well as those obtained for related specimens (P_{IDsib} = 0.024) allowed to associate a given graft genotype with the genotype of the mother tree.

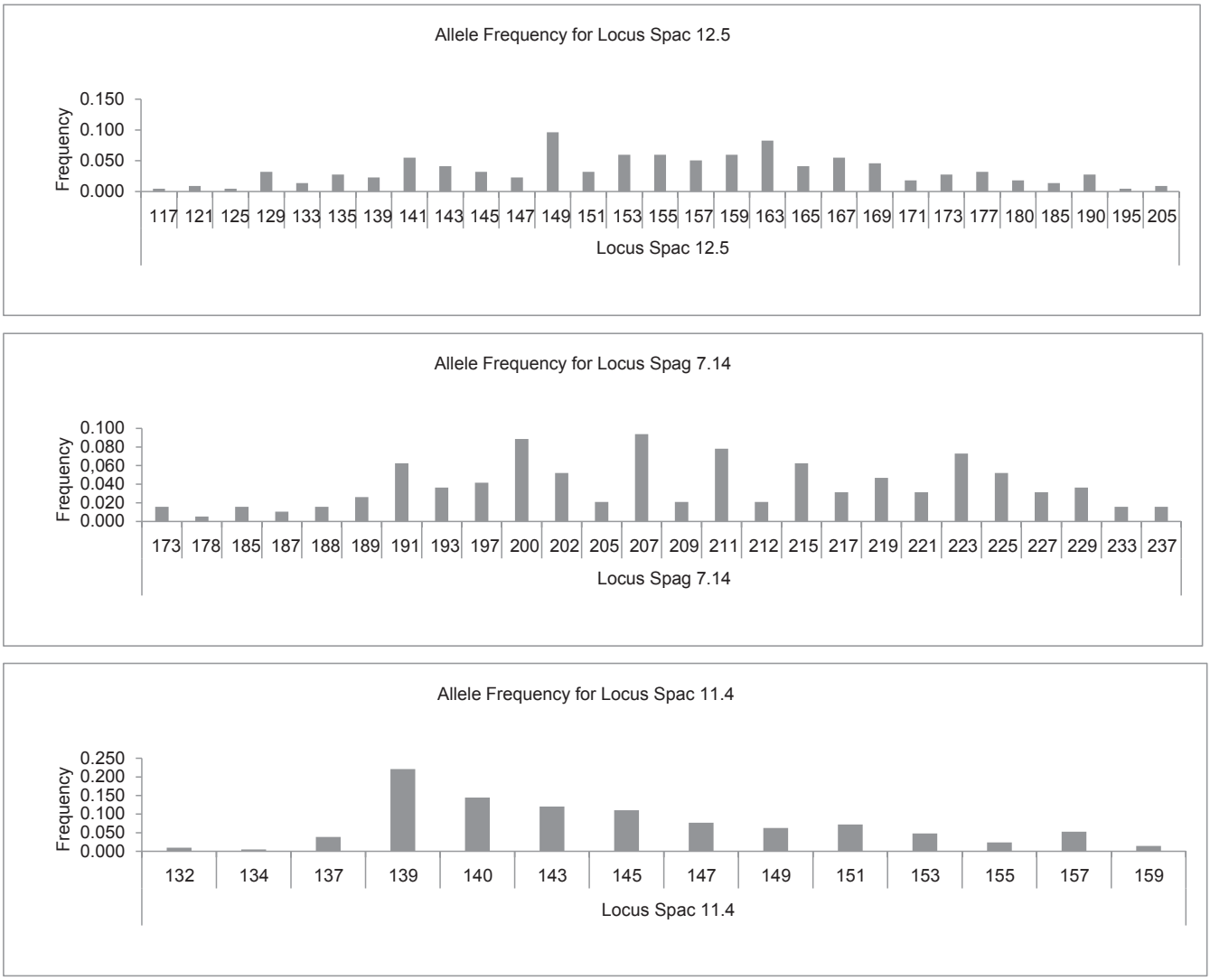

Figure 1. The allele frequency in the Forest District Susz seed orchard at 3 loci examined

Based on the chi square test results ($P < 0.001$), it was found that the markers in 2 of 3 analyzed loci (SPAC 12.5 and SPAG 7.14) showed a deviation from the Hardy-Weinberg equilibrium. In these loci, inbreeding coefficient values (*F*) were positive, (0.054 and 0.196 for SPAC 12.5 and SPAC 7.14, respectively). At the same time, average, *F* value for the whole population, was 0.097.

The effect of null alleles on the results obtained was tested for all the loci analyzed. Null alleles had no significant effects on SPAC 12.5 and SPAG 7.14 loci, however, they showed a significant effect ($P < 0.001$) on SPAC 11.4

The mean effective number of alleles in the examined seed orchard was 15.67, with the following values obtained for the loci observed: SPAC 12.5 – 20.15, SPAG 7.14 – 18.46, and SPAC 11.4 - 8.41.The highest percentage difference (0.4) between obtained and effective allele numbers was observed at SPAC 11.4.

All the loci examined, showed lower values of observed heterozygosity (H_o) when compared to expected heterozygosity (H_e), i.e.: for SPAC 12.5 – $H_o = 0.89$ and $H_e = 0.95$; for SPAG 7.14 – $H_o = 0.76$ and $H_e = 0.94$; for SPAC 11.4, $H_o = 0.84$ and $H_e = 0.88$.

4. Discussion

Microsatellite DNA examined in the present study is one of the most common and precise research tools used to de-

termine forest tree genotypes (Nowakowska et al. 2014). Three variable loci were included in the study, hence it was possible to associate a given graft genotype with that of the maternal tree. This stage is an indispensable element of examination of genetic variability at the sites such as seed orchards. Indeed, relevant studies conducted in other European seed orchards showed average planting error that amounted to 27% (Gomory and Paule 1993, Gomory et al. 2000). This value is probably fairly close to that observed in Poland's seed orchards (Burczyk et al. 2000, Cieślewicz 2009, Przybylski 2012). Accordingly, alien genotypes are introduced into seed orchards and artificially boost a genetic variability level, thereby – negatively affect appropriate decision-making. In the present study, alien genotypes were excluded, and only a set of verified clones was analyzed.

The seed orchard examined is one of the largest in Poland and hosts 175 clones of maternal trees. This is much more than the number of clones recommended in regulations on seed orchards in Poland, which require minimum 40 different Scots pine genotypes in a given seed orchard. In the present paper, there are described genotypes of 117 maternal trees, and this allowed for evaluation of phenotypic selection effects on genetic variability. The obtained results confirmed a high level of polymorphism in examined satellite DNA markers. There were observed 29 alleles at SPAC 12.5 and 26 alleles at SPAC 7.14, and the results obtained were similar to those reported by Trojankiewicz (2006) and Cieślewicz (2009). Lower genetic variability was shown for the third of examined loci – SPAC 11.4 (14 alleles), nevertheless, polymorphism at this level seems sufficient enough for recommendation to use in verification procedures in other seed orchards. In Poland, currently applied verification systems most often use SPAC 12.5 and SPAG 7.14 as well as loci PtTx 3025, 3107, 3116 and 4001. The latter are DNA fragments with lower variability than SPAC 11.4 proposed in the present paper.

Statistical test results showed the lack of genetic equilibrium in the examined orchard for SPAC 12.5 and SPAG 7.14 loci. This phenomenon is rarely observed in forest tree populations, since the Hardy-Weinberg equilibrium is usually sustained as a result of open pollen transfer in between populations. The results obtained in this study apparently confirm the effect of phenotypic selection on population genetic structure. Comparable results were obtained by Cieślewicz (2009) in the Zdrojowa Góra seed orchard, where 2 loci showed significant deviations from the genetic equilibrium. In the present study, similarly to that of Cieślewicz (2009), high positive values of inbreeding coefficient were observed. Such result proves the domination of homozygotes in the population. Cieślewicz (2009) stated that this observable fact could be attributable to the presence of null alleles which prevented the distinction of homozygotes from heterozygotes (Callen et al. 1993). Null allele frequency in tree populations assessed by Scotti et al. (2000) was approximately 20%. Nonetheless, in the present study, null allele effects on the results obtained were excluded, based on negative values obtained in statistical tests. Therefore, it can be accepted as true that the reason behind increased homozygote frequency within the seed orchard examined is the selection process itself. In general, inbreeding is regularly observed in natural world, as this is how alleles with desirable genetic traits of adaptation are promoted within the population (Whitlock 2002). It is possible, however, that only and only increasing homozygote occurrence within seed orchards will have no effect on progeny generation variability, as evidenced by Burczyk's results (1998).

In Poland, Scots pine seed orchards, as the final link of selective breeding conducted by the State Forests National Forest Holding (Kowalczyk 2012) must comprise at least 40 unrelated plants. Up to date study results indicate that that this number should be sufficient to sustain genetic variability (Gulberg et al. 1985; Mejnartowicz, Bergmann 1985; Paule, Mrazikowa M 1990; Burczyk 1990; Burczyk et al. 2000). However, it must be borne in mind that Scots pine is characteristic of considerably high genetic variability (Wang et al. 1991; Prus-Głowacki, Bernard 1994; Dzialuk, Burczyk 2002; Kosińska et al. 2007; Nowakowska 2010), which comes in large part from its reproductive cycle with prevailing cross-pollination (Dzialuk 2004, Wasilewska et al. 2005) and gene flow between populations (Dzialuk, Burczyk 2005). The restrictions resulting from the rules on establishing seed orchards, e.g. separation from foreign pollen sources, stimulate scientific debates on a potential decrease in genetic variability. Genetic diversity loss is a serious threat in the context of current dynamic changes in climate conditions. The comparison of the results on the seed orchard studied with those obtained by Nowakowska (2007) in selected tree stands confirms increased inbreeding coefficient values observed in this study. In view of the presented results, it is supposed that phenotypic selection adds to a decrease of genetic variability in maternal generation, which is contrary to earlier studies (Paule, Mrazikova 1990). The differences between the conclusions drawn by the abovementioned authors and the author of the present study, are probably due including planting errors into genetic variability calculations in previous studies. In fact, a decrease of inbreeding coefficient values is expected in the seed orchard, as this directly results from the concept of selection works. In any case, it is vital to monitor molecular variability in progeny generations in subsequent breeding, stages in order to minimize the risk associated with breeding programs.

5. Conclusions

1. Genetic variability, expressed with observed heterozygosity, is narrowed in the seed orchard examined, which demonstrates the effectiveness of phenotypic selection.

2.With regard to planning and design of seed orchards it is essential to consider molecular variability in subsequent selection stages

3. Microsatellite DNA markers (SPAC 12.5, SPAG 7.14, SPAC 11.4) are useful tools for determination of genetic variability in seed orchards.

4. Planting errors significantly affect seed orchard allele pools.

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Conflict of interests

The author declares no competing interests.

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