Evaluation of Pollen Contamination in an Advanced Scots Pine Seed Orchard

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Abstract

The pollination pattern in a Scots pine (*Pinus sylvestris* L.) seed orchard consisting of 28 clones was studied using nine microsatellite (SSR) loci. The nine SSR loci produced unique multilocus genotypes for each of the orchard's 28 clones and allowed paternal assignment of the studied 305 seed using paternity exclusion probability of 99.9%. Fifty two percent of the studied seeds were sired by outside the orchard pollen sources (i.e., pollen contamination) and as expected, low selfing (2.3%) was detected. These results are valuable for the evaluation of the seed orchard function and the impact of contamination on the expected genetic gain.

Key words: Paternity analysis, *Pinus sylvestris*, pollination, SSR marker.

Introduction

Genetically advanced and well-functioning seed orchards that incorporate selections from the ongoing long-term tree breeding program seem by far the most important and realistic means to increasing sustainable harvest from Swedish forest land during the next century (LINDGREN et al., 2008). Seed orchards have also become more important quantitatively. Currently, seed orchards supply 60% of Sweden seedlings for reforestation – 75% for Scots pine (*Pinus sylvestris* L.) and 50% for Norway spruce (*Picea abies* (L.) H. Karst) (LINDGREN et al., 2008), and new seed orchards are expected to completely satisfy this need. Selections used in the newly established orchards are genetically more advanced than some decades ago, with potential for a higher gain.

Pollen contamination is a serious problem in most wind-pollinated forest seed orchards (LINDGREN, 1991). The loss measured in forest production caused by pollen contamination becomes higher when the genetic value of the seed orchard clones increases over time. Thus, accurate estimation of pollen contamination is necessary for evaluating seed orchards' function, the prediction of gain in the subsequent forest plantations as well as the development of management practices aiming at reducing pollen contamination.

Studies on pollen contamination in Scots pine seed orchards were conducted in the late 80s and early 90s using allozyme markers (e.g. WANG et al., 1991; YAZDANI and LINDGREN, 1991). High rates of pollen contamination (>50%) were sometimes reported. However, the lower discrimination power of the markers sometimes can lead to a considerable uncertainty in the estimates, e.g. in WANG et al. (1991) the unidentified contamination can be as high as 20–25%. Since then, both marker techniques and statistical methods have improved considerably, making the parentage assignment and contamination estimate more accurate. The seed orchard establishment, design and management have improved over time, and the awareness of the severity of contamination has resulted in actions to reduce its impact. However, the perception on the magnitude of the problem usually rely on data from the first batches of the low-gain seed orchards, the function of the advanced orchards await individually and empirically examined.

The purpose of this study was to develop the analytical procedure for paternity analysis in Scots pine using high resolution microsatellite (SSR) DNA markers and investigate the pollination pattern in an advanced Scots pine seed orchard. Through the achievement of high paternity exclusion power, we estimated the rate of pollen contamination in and evaluate the function of the orchard. This information is valuable for seed orchard management and the evaluation of the impact of contamination on the expected gain from the seed orchard crops.

Material and Methods

Seed orchard

The Scots pine seed orchard "Västerhus" (63°18'N, 18°32'E) close to Örnsköldsvik in Sweden was selected for the study. More details about this seed orchard are given in FRIES et al. (2008). It is 13.7 ha in size and consists of 4,640 grafted trees and was established in 1991. The seed orchard has 28 clones selected for their breeding values among 208 clones, which were progeny-tested in field trials. It is the genetically most advanced Scots pine seed orchard in Sweden that is in commercial seed production. At the age 15 it contributed a good seed production and a satisfactory pollen production (20 kg/ha) and has reached the stage of full commercial production by 2005. It is the first existing seed orchard in Sweden that applied "linear deployment" (LINDGREN and MATHESON, 1986) in its design, meaning the ramet number is intentionally variable among clones (ranging from 1–9% of the total ramets composition), with higher representation of the better clones. This concept is commonly adapted in the establishment of new generation seed orchard in Sweden.

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The trees were planted in a semi-randomized design, i.e. the grafts were packed in boxes for each orchard block of 6 x 10 plants to get clones spread out over the orchard, but within block assignment was designed at plantation. The idea was to maximize the distance between ramets of the same clone. The seed orchard is situated on a southwest slope towards a river and is surrounded by a 30 m corridor of broad leave trees and open fields. The closest Scots pine trees to the seed orchard are more than 500 m. The 2007 seed orchard inventory indicated 82% survival (3811 of the initially 4,640 planted grafts).

Cone and needle collection

In June 2007 needles were collected from three ramets of each of the 28 clones for genotyping (for one clone only two grafts were collected). The selected ramets were distributed over the seed orchard for a better representative. The needles were stored at –20 °C till DNA analysis. Seed-cone collection was done on the 1st of October 2007 and seeds were extracted in the following three weeks. During the whole process, the seed-cones and seedlots from each ramet were kept separate. Seeds were stored at –20 °C till analysis.

DNA extraction and microsatellite genotyping

Genomic DNA was extracted from approximately 100 mg of needles per tree using an E.Z.N.A.™ SP plant DNA mini kit (OMEGA Bio-tek). Eight to 12 seeds from each tree were germinated in Petri dishes on moist filter paper at room temperature till the root tips grown to a length of 1 cm. Megagametophyte and embryo of each seed were separated and DNAs were isolated from both tissues using the plant DNA mini kit (OMEGA Bio-tek). A total of 83 trees and 305 seeds were genotyped.

Guided by the preliminary results of polymorphism testing in Scots pine (Rosario García Gil and Jian-Feng Mao, personal communication), nine SSR loci were selected in this study, which included loci LOP1 (Liewlaksaneeyanawin et al., 2004), PtTX2146, PtTX3025, PtTX3107, PtTX3116, PtTX4001 (Auckland et al., 2002), SsrPt_ctg1376 and SsrPt_ctg4363 (Chagne et al., 2004) that are developed for *P. taeda*, and SPAC12.5 for *P. sylvestris* (Soranzo et al., 1998). All loci, except PtTX2146, were amplified using fluorescently labeled forward primers with a unlabeled reverse primer in a reaction volume of 16-µl containing 1.6 µl of 10x PCR buffer (QIAGEN), 2.0 mM of $MgCl_2$, 0.16 mM of each dNTP, 0.13 µM of each primer, 0.25 unit of Top-Taq polymerase (QIAGEN), and 5–50 ng of template DNA. Locus PtTX2146 was amplified using a tailed universal M13 forward primer approach (Boutin-Ganache et al., 2001), which employs three primers in a PCR amplification: a locus-specific forward primer with an M13 universal tail (18 bp, 5'-TGTAAAACGACG-GCCAGT-3'), a locus-specific reverse primer and a fluorescently labeled M13 universal primer. PCR reaction was performed using 0.25 µM of each fluorescently labeled M13 universal primer and ordinary reverse primer and 0.06 µM of M13-tailed forward primer. Other PCR mixture components were the same as for the other loci. The amplifications were performed using either a PTC-100, PTC-200 Thermal Cycler (MJ Research), or iCycler (BIO-RAD). The amplification protocol for PtTX3107 and PtTX4001 consisted of an initial denaturation for 3 min at 94 °C followed by 30 s at 94 °C and 30 s of annealing with touch down from 55 °C to 45 °C at 0.6 °C decrement per cycle and 30 s at 72 °C, then 25 cycles of 30 s at 94 °C, 30 s at 45 °C, 30 s at 72 °C, and a final extension of 10 min at 72 °C. Loci PtTX2146, PtTX3025, PtTX3116, and SPAC12.5, SsrPt_ctg1376 were amplified using the same protocol but with a touch down from 60 °C to 50 °C, and LOP1 and SsrPt_ctg4363 with a touch down from 64 °C to 54 °C. PCR amplification products of 0.7–4.2 µl (depending on the fluorescent dye used) were mixed with 30 µl sample loading buffer and fluorescently labeled size marker (size standard-400, Beckman-Coulter), and resolved on a CEQ8000 capillary sequencer (Beckman-Coulter). Allele identification and genotyping were performed using the CEQ8000 Fragment Analysis software (Beckman-Coulter).

Data analysis

After genotyping three ramets per clone, trees with identical multilocus genotypes were considered to be members of the same clone. Null alleles at SSR loci, caused by mutations at the primer binding sites, can cause false exclusions in paternity analysis. We investigated the presence of null alleles at the nine SSR loci selected for this study by first checking the maternal-offspring genotypic relationships (Dakin and Avise, 2004). If the maternal tree appeared as homozygous at a SSR locus was documented to produce seed with a different homozygous genotype, we interpret that both the maternal tree and the seed were heterozygous with a null allele at the locus in question. After comparison of maternal tree genotype with that of the offspring, clones appeared to contain null alleles at different locus were further analyzed for segregation using eight megagametophytes.

Clones identified to contain null allele at a locus were assigned as heterozygote with a null allele. The observed allele frequencies, including null allele, were calculated from the 28 clone genotypes. Observed heterozygosity and the effective number of alleles (by reconstructing visible allele frequencies after excluding null alleles at the null-identified loci) were calculated for each locus from the 28 genotypes. The paternity exclusion probability at each locus (Q_l) was calculated following Jamieson and Taylor (1997) as:

$$Q_l = \sum_{i=1}^{n} p_i (1-p_i)^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} p_i^2 p_j^2 (4 - 3p_i - 3p_j)$$

where p_i and p_j represent the allele frequencies at locus l, respectively. For locus that contains null allele, the probability of false exclusion of a true parent due to a null allele is

$$\sum_{i=1}^{n} p_i p_k (1-p_i),$$

where p_i and p_k represent the visible and null allele frequencies, respectively (Dakin and Avise, 2004). The corrected paternity exclusion probability can be calculated

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by subtracting this probability from the paternity exclusion probability assuming every allele is visible (see above Q_l). The combined exclusion probability (JAMIESON and TAYLOR, 1997) was calculated over the nine loci.

Paternity of each seed was assigned based on a modification to the null-assuming simple exclusion method of MORIGUCHI et al. (2004). In the present study, when both the maternal and seeds genotypes appeared as homozygous at a null-identified locus, they were treated as heterozygous possessing one null allele. In such cases, we could not exclude paternal candidates with homozygous genotypes different from the seed due to the null allele. If the genotype of a seed does not match any of the 28 clones, it was considered as sired by a father outside the seed orchard, thus indicating pollen contamination. When all but one candidate clone genotype were excluded (an exact match), we designated the clone as the father. The confidence of the paternity assignment of each of the exact matched seeds were evaluated based on a likelihood ratio of two competing hypotheses, known as the paternity index (*PI*) in human paternity testing (PENA and CHAKRABORTY, 1994). The two competing hypotheses are:

H_1: the candidate father is the true father

H_2: the candidate father is an unrelated random tree outside the seed orchard.

The likelihood ratio $PI_l = H_1/H_2$ for locus l is calculated using the method developed for cases where the mothers' genotypes are known and null alleles are considered (BRENNER, 1997). The posterior likelihood (W; i.e., the probability of paternity) of H_1 is calculated using Bayes' theorem (EVETT and WEIR, 1998):

$$W = p_{prior}\prod_{l=1}^{9} PI_l \Big/ \left[p_{prior}\prod_{l=1}^{9} PI_l + \left(1 - p_{prior}\right)\right]$$

where p_{prior} is the probability of H_1 prior to considering the genetic evidence. In the present study, p_{prior} was calculated as the proportions of grafts of each clone relative to the seed orchard population of 3,811 trees.

In paternity analysis using simple exclusion, bias can be introduced by cryptic gene flow (NAKANISHI et al., 2005; WESTNEAT and WEBSTER, 1994), i.e., clones matching genotypes outside seed orchard by chance. This factor would under bias the true contamination rate of the seed orchard. To estimate the cryptic gene flow, we first calculated the probability P_{nl} that the n^{th} clone possessing alleles i and j (A_iA_j) at locus l cannot be excluded from the candidate pollen parents of a seed given the genotype of a mother:

$$P_{nl}\left(A_iA_j\right) = \begin{cases} p_i + \delta_l p_k, & i = j \\ p_i + p_j, & i \neq j \end{cases}$$

where δ_l is 1 when the l^{th} locus possesses the null allele with the frequency of p_k, and otherwise 0.

The probability of cryptic gene flow from outside seed orchard is:

$$P_{cryptic} = 1 - \prod_{n=1}^{28}\left(1 - \prod_{l=1}^{9} P_{nl}\right)$$

The probability was estimated based on the allele frequencies derived from all ramets (i.e. 3,811 multilocus genotypes) to take into account of the impact of different numbers of ramets per clone on the fertilizing pollen cloud. Thus, the number of seeds that match unrelated fathers in the orchard by chance can be estimated as:

$P_{cryptic}$ x (the number of seeds with paternal parent within the seed orchard).

This correction factor was incorporated to derive a corrected contamination rate of the seed orchard.

Results

Using the nine SSR loci, each of the 28 clones in the orchard was assigned to a unique multi-locus genotype. The number of visible alleles at the nine loci ranged from 5 to 21 in the 28 clones, with an average of 9.3 (*Table 1*). The observed heterozygosity ranged from 0.500 to 0.929 among loci, with an average of 0.69. Except for locus SsrPt_ctg1376, alleles unique to a single clone in the seed orchard were found, ranging from 1 to 10 alleles per locus (*Table 1*). These unique alleles were distributed among 18 clones.

After genotyping 305 seeds from the 28 clones, we found mismatches of the maternal-offspring genotypic relationships at locus PtTX3107 in clone AC1006, AC3015, X4203, Y3012, and Z4022, at SsrPt_ctg4363 in AC1075 and Z3029, and at both loci in Y4103, indicating presence of null alleles. For further confirmation, we conducted segregation test using eight megagametophytes of each AC1006, Y4103, Z3029 and Z4022. Segregation ratio of 3:5 and 4:4 were observed at locus PtTX3107 and SsrPt_ctg4363 in the tested clones. These results confirm the presence of null alleles at the two loci. No null allele was observed at any other loci.

The paternity exclusion probability, assuming that the contaminating pollen cloud had the same allele frequency (including null alleles) as the orchard clones, ranged from 0.370 to 0.861 among loci (*Table 1*), and the combined exclusion probability over the nine loci was 0.999. The alleles unique to the pollen pool, which must have originated outside the seed orchard as contaminating pollen, were identified and the numbers ranged from 0 to 7 among loci, with an average of 2.3 per locus (*Table 1*).

By using the method that considers the presence of null alleles at the locus PtTX3107 and SsrPt_ctg4363, paternity analysis showed that 147 out of 305 seeds (48.20 %) had at least one candidate pollen parent within the seed orchard. These 147 seeds were, therefore, considered as the products of mating among the orchard's clones. The remaining 158 seed did not match any of the orchard's clones and were likely sired by pollen from outside the orchard, resulting in contamination rate of 51.80 % (S.E. = 3.89). The probability of cryptic gene flow from outside the seed orchard ($P_{cryptic}$) was very low (less than 0.008) and, thus, the correction factor (i.e., number of seeds that match unrelated fathers in the orchard by chance) was less than 0.1 for each clone, leading to 52.19 % (S.E. = 3.86) of the average corrected contamination rate in Västerhus seed orchard. Some of the registered contamination may originate from rootstocks within the seed orchard. Since there

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Table 1. – Allele frequencies among 28 diploid clones in the Västerhus seed orchard and the genetic variation at the nine SSR loci. n_e, effective number of alleles (not including null alleles); H_O, observed heterozygosity; Q, paternity exclusion probability given that the maternal genotype is known; *Null*, null allele. Note that Q was calculated consider the presence of null alleles at locus PtTX3107 and SsrPt_ctg4363. **Bold** and *italic* indicate the alleles unique to a single clone in the seed orchard and the pollen pool, respectively.

Locus	LOP1		PtTX2146		PtTX3025		PtTX3107		PtTX3116		PtTX4001	
n_e	3.5		3.9		2.7		4.2		2.7		4.1	
H_O	0.643		0.750		0.571		0.571		0.679		0.857	
Q	0.485		0.549		0.370		0.524		0.445		0.564	
	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
	153	0.321	176	0.036	**211**	0.018	150	0.125	115	0.107	**198**	0.018
	155	0.089	197	0.125	270	0.518	**153**	0.018	**127**	0.018	200	0.036
	156	0.036	209	0.214	277	0.143	156	0.268	139	0.036	204	0.054
	158	0.411	218	0.054	283	0.286	159	0.054	142	0.054	**208**	0.018
	160	0.071	221	0.036	305	0.036	162	0.304	145	0.036	212	0.054
	165	0.018	**224**	0.018			165	0.089	151	0.589	215	0.411
	167	0.018	227	0.054			168	0.036	157	0.054	217	0.232
	175	0.018	236	0.429			Null	0.107	163	0.054	219	0.071
	189	0.018	**251**	0.018			*141*		166	0.036	227	0.089
	151		**264**	0.018			*171*		**169**	0.018	**229**	0.018
			172				*174*		*121*		*202*	
			182						*133*		*223*	
			194						*154*			
			200									
			242									

Table 1 continued.

SPAC12.5		SsrPt_ctg1376		SsrPt_ctg4363	
14.5		3.9		3.0	
0.929		0.714		0.500	
0.861		0.522		0.426	
Allele	Frequency	Allele	Frequency	Allele	Frequency
118	0.054	88	0.071	95	0.321
126	0.018	118	0.107	97	0.089
134	0.018	120	0.071	98	0.036
136	0.018	122	0.071	100	0.429
138	0.018	123	0.357	102	0.054
140	0.054	124	0.321	**106**	0.018
142	0.089			Null	0.054
144	0.071				
146	0.071				
148	0.054				
150	0.018				
152	0.089				
154	0.018				
156	0.071				
160	0.125				
162	0.071				
164	0.071				
169	0.018				
174	0.018				
176	0.018				
184	0.018				
122					
128					
158					
166					
168					
172					
178					

were 46 rootstocks registered, and if each rootstock gives the same contribution to the seed orchard pollen as the clones, which is a high estimate as rootstock usually has a reduced pollen production, 1.2% (46/3811) of the seed orchard pollen originates from rootstock and the external contamination is consequently reduced to 51% or less.

Among the 147 seeds that were determined as sired by seed orchard clones, one was compatible with two candidate pollen parents (Y2005 and Z4019). To assign its pollen parent into one of the two candidates, we calculated the probability of paternity (i.e., W in the Material and Methods) given the genotype of the mother (i.e., AC3033). The probability was higher for Y2005 than for Z4019, thus paternity of the seed was assigned to Y2005. For 138 out of the 146 exact matched seeds, the probabilities of paternity (W) exceeded 0.95, indicating high confidence of the assigned fathers. For the remaining 8 seeds, the W ranged from 0.733 to 0.895.

Discussion

SSR marker property

SSR markers are powerful genetic tools for parentage analysis, and most of the recent advances in parentage analysis have been aimed at studies employing SSRs (JONES and ARDREN, 2003). An undesirable feature with SSR is the presence of null alleles which can cause an apparent excess of homozygotes and result in erroneous estimates of allele frequencies and false parentage exclusion. Null alleles have been identified frequently in SSR-based analyses (DAKIN and AVISE, 2004). Our present study identified null alleles at two of the nine (22.2%) investigated loci, which is lower than those reported for other conifers such as *Cryptomeria japonica* (35%, MORIGUCHI et al., 2004), *Pinus radiata* (35%, FISHER et al., 1998), and *Pinus thunbergii* (40%, GOTO et al., 2005). In a literature survey by DAKIN and AVISE (2004), the null allele frequencies are reported to be usually less than 0.20. In this study, the frequencies of null alleles at locus PtTX3107 and SsrPt_ctg4363 were 0.107 and 0.054, respectively, among the 28 clones in the seed orchard. Verification of the null alleles is necessary for accurate parentage analysis. In conifers, segregation test using haploid megagametophytes should be performed for loci that show discrepancy in maternal-offspring genotypic relationship.

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In addition to a rigorous selection of SSR loci and control of genotyping errors, the method of parentage analysis should be chosen carefully to reduce the false exclusions caused by null alleles. In this study, when we ignored the null alleles, the estimates of pollen contamination increased from 52.19% to 56.39%, indicating that null alleles apparently caused false exclusions of candidate fathers. This is in accordance with the result of MORIGUCHI et al. (2004), in which they indicate that method incorporating the presence of null alleles minimizes the risk of overestimating pollen contamination.

The nine SSR loci selected for this study were highly polymorphic among the 28 clones and produced a high combined exclusion probability (99.9%). Although null alleles were detected at two loci, the development of method that take into account of their presence made the paternity assignments highly accurate; 94.5% of exact matched seeds were assigned with high paternity probability (*W*) exceeding 0.95.

Pollen contamination in Scots pine seed orchards

A number of studies on pollen contamination have been conducted in conifer seed orchards. Initially the technique used was based on allozyme markers, but recently DNA markers have been used with increasing frequency (SLAVOV et al., 2005; HANSEN and KJÆR, 2006; FUNDA et al., 2008). Studies of contamination in Scots pine seed orchards are compiled in *Table 2*, including a few examples of contamination in other pine species. For Scots pine the contamination rates mostly fall in the interval 40–70%. Scots pine seed orchards in Scandinavia are generally located in regions where Scots pine is a common species and are exposed to an extensive natural pollen background. For other pine species the contamination rates are more variable, and may be low even when pollen sources of the same species are in the vicinity (GOTO et al., 2002; STOEHR and NEWTON, 2002). Estimates of identified contamination need adjustment as some alien pollen may fit to a genotype of seed orchard clone. In the allozyme-based studies, the adjusted contamination can be double as high as the observed values. The contamination rate (52%) obtained in this study is compatible with other reports on Scots pine in Nordic countries. The pollen production in Västerhus was measured to 20 kg/ha, which is regarded as satisfactory for seed crop production. Pollen production is likely to rise when the seed orchard becomes older, and this may lead to a slight reduction of contamination; however, contamination in old seed orchards is still at the magnitude of 50% or even higher (*Table 2*).

The Västerhus seed orchard is on a SW slope in a locally mild site which accelerates reproductive phenology development. At northern latitudes close to the arctic circle where the sun never raises very high, the phenology in seed orchards, and particularly young seed orchards, can be expected to be earlier than in local

Table 2. – Estimates of pollen contamination in pine seed orchards. The ages are given in years from first graft plantation to seed harvesting for analysis. Some estimates are adjusted for undetected alien pollen by the authors and some are unadjusted.

Species	Seed orchard	Age (years)	Type of marker	Contamination (%)	Reference
Scots pine	Västerhus, Sweden. lat. 63°18'N	18	SSR, 9 loci	52, adjusted	Present
	Robertsfors, Sweden. lat. 64°12'N	25	Allozyme, 18 loci	37, unadjusted	NAGASAKA and SZMIDT (1985)
	Skaholma, Sweden. lat. 63°50'N	29	Allozyme, 21 loci	28-43, unadjusted	YAZDANI et al. (1995)
	Bogrundet, Sweden. lat. 62°30'N	17	Allozyme, 21 loci	21, unadjusted	EL-KASSABY et al. (1989)
	Klocke, Sweden. lat. 62°54'N	14	Allozyme, 21 loci	36, unadjusted	
	Askerud, Sweden. lat. 59°53'N	22	Allozyme, 21 loci	34-55, unadjusted	PAULE (1991)
	Skaholma, Sweden. lat. 63°50'N	27	Allozyme, 18 loci	51-55, adjusted	WANG et al. (1991)
	Bogrundet, Sweden. lat. 62°30'N	18	Allozyme, 21 loci	59, adjusted	YAZDANI and LINDGREN (1991)
	Askerud, Sweden. lat. 59°53'N	27	Allozyme, 21 loci	30-38, unadjusted	ALMQVIST et al. (1995)
	Askerud, Sweden. lat. 59°53'N	36	SSR, 5 loci	54-74	ALMQVIST and PULKKINEN (2005)
	Parkkola, Finland. lat. 62°12'N	15	Allozyme, 10 loci	48, adjusted	HARJU and NIKKANEN (1996)
	Viitaselkä, Finland. lat. 62°15'N	33	Allozyme, 13 loci	17-39, adjusted	HARJU and MUONA (1989)
	Vilhelminmäki, Finland. lat. 62°05'N	27	Allozyme, 13 loci	33, adjusted	
	Heinäsuo, southern Finland, with northern clones. lat. 62°03'N	34	Allozyme, 12 loci	62-76, unadjusted	PAKKANEN and PULKKINEN (1991)
	Parkkola, southern Finland, with northern clones. lat. 62°12'N	23	Allozyme, 11 loci	45-52, unadjusted	
Pinus thunbergii	Fukuoka, Japan.	12	RAPD, 28 loci	2, unadjusted	GOTO et al. (2002)
Pinus contorta	Vernon, Canada.	13	SSR and cpDNA SSR, 6 loci	8, adjusted	STOEHR and NEWTON (2002)
Pinus brutia	Antalya, Turkey.	11	Allozyme, 14 loci.	86	KAYA et al. (2006)

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stands because of less ground shadow and more open canopy. This is likely to decrease local contamination, but may render the orchard vulnerable to contamination from southern pollen sources. If seed orchards were situated closer to the Baltic the phenology would be delayed due to the ambient cold weather, thus decreasing the influx of southern pollen. The Västerhus seed orchard is surrounded by open fields that do not permit the presence of any natural barriers to pollen flow. The establishment of barriers such as "hedges" would likely filter some of the contamination pollen and may reduce wind speed and increase turbulence inside the seed orchard, thus improving its efficiency. The grafts at the border of the seed orchard could be allowed to grow taller to function as pollen production trees releasing pollen at a higher level. Clones that tend to have higher pollen production and bigger success in pollination could be used for this application.

Pollen contamination is a problem not only because it reduces gain, but it also makes the genetic quality of seed orchard crop more variable among years. Not only the quantity but also the source and genetic property of the contaminating pollen are likely to vary among years depending on e.g. wind directions, temperatures and rainfall during the pollination period (NILSSON and LINDGREN, 2005). In the absence of pollen contamination, volume genetic gain is expected to reach 22% at the Västerhus seed orchard, of which 18% is expected to be a selection effect, and the additional 4% in the gain is a "heterosis-effect" as parents come from unrelated stands, and an "epigenic" effect caused by the favourable seed orchard environment (ROSVALL et al., 2001; 2003). As the contamination gives no selection gain, the loss of gain by contamination is only attributable to the paternal side and can thus be estimated to 4.7% (=0.5 x 0.52 x 18%) volume production. Thus, the volume production of the Västerhus seed crop could probably be raised by 4–5% if contamination could be eliminated. Even with 100% contamination, seed crop from the advanced seed orchard should still be a superior alternative to those from 1st cycle orchards, unless the seed orchard is located far south of the clone origin. With moderate south transfer the added genetic gain on the maternal side, due to physiologically better seeds and epigenic effects, often outweighs the disadvantages brought by contamination (NILSSON and LINDGREN, 2005). Not harvesting the phenologically earliest developing clones may reduce the contamination marginally, but not to an extent justifying this application in Scots pine seed orchards (YAZDANI et al., 1995).

Another factor that adds to the observed contamination is the occurrence of alien genotypes in the orchard. For example in three Slovak Scots pine seed orchards, GÖMÖRY et al. (2000) found that 12–27% of the ramets represent alien genotypes. At the Västerhus seed orchard inventory in 2007, there were 46 rootstocks (1.2% of all living seed orchard trees) where the grafts had died and the rootstock taken over (FRIES et al., 2008). These trees do not belong to any of the orchard clones and could contribute 1.2% of the observed pollen contamination. It is recommendable to have rootstock of a similar origin as the clones, so that their pollen would fit reasonable into the genetic set up of the orchard if they "take over" the grafts. In Västerhus, the rootstocks are plants from one of the first generation orchards. The "genetic gain" in the rootstock at Västerhus due to plus tree selection effect is expected to be 4.5% (ROSVALL et al., 2001). Although this gain is less compared to the Västerhus clones, where a selection gain of 18% is expected (ROSVALL et al., 2001), rootstocks contribute with pollen and seeds that are clearly superior to wild stands. The reduction in the genetic gain of the Västerhus clones from pollination with the 1.2% rootstocks should be negligible. Removing them at the early stage of orchard production may increase contamination rate and thus decrease gain. Whether or not to collect cones from rootstocks depends on shortage or surplus in the seed storage. Cones from rootstocks are inferior to cones from grafts but superior to cones from wild stands. Thus, the immediate disadvantage of rootstock of acceptable genotypes at the early phase of seed orchard production seems small, but will increase with time. Removing rootstocks will then be advantageous since it leaves more space to the genetically better clones to increase their seed set.

The contamination problem can be eliminated by using material derived from controlled crosses, but this seems forbiddingly expensive for Scots pine in the foreseeable future. Different studies and approaches have been attempted to reduce the contamination problem, but most were unsuccessful (ALMQVIST and PULKKINEN, 2005; LINDGREN, 1991). In the previous decades, Scots pine seed orchards were often localized at more southern latitudes than the origin of the clones to enhance seed production by placing the orchards in a more favorable seed maturation climate (NIKKANEN, 2008), but nowadays new seed orchards for northern areas are localized at more northern latitudes to limit southern genetic contamination, thus reducing the negative effect of contamination.

The effective number of clones in Västerhus, based on their representation by ramet number, is 19.7 (FRIES et al., 2008), which is low compared to many other older Swedish Scots pine seed orchards (LINDGREN et al., 2008). Low effective number of clones is expected to lead to more selfing, but it is suggested by this study that the problem is marginal as the selfing rate was found as low as 2.3%. A high pollen contamination is not just negative, it reduces selfing and increases the gene diversity of the seed orchard crop (NILSSON and LINDGREN, 2005; EL-KASSABY and RITLAND, 1986). In addition, a rather low fraction of the self-pollinated seedlings will survive to the mature stand (YAZDANI et al., 1985), thus selfing in Scots pine seed orchards is a small problem from forest production point of view.

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References

ALMQVIST, C., U. ERIKSSON and R. YAZDANI (1995): Effektivare fröplantageutnyttjande. Tallfröplantage 493 Askerud. Arbetsrapport 298.

ALMQVIST, C. and P. PULKKINEN (2005): Improved utilization of the internal pollen production in a *Pinus sylvestris* seed orchard by use of a mist blower, pp. 79-80. *In:* Proceedings of the Meeting of Nordic Forest Tree Breeders and Forest Geneticists, edited by A. FEDORKOV, Syktyvkar 2005.

AUCKLAND, L. D., T. BUI, Y. ZHOU, M. SHEPHERD and C. G. WILLIAMS (2002): Conifer microsatellite handbook. Corporate Press, Raleigh, N.C.

BOUTIN-GANACHE, I., M. RAPOSO, M. RAYMOND and C. F. DESCHEPPER (2001): M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. Biotechniques **31**: 24–28.

BRENNER, C. H. (1997): Symbolic kinship program. Genetics **145**: 535–542.

CHAGNE, D., P. CHAUMEIL, A. RAMBOER, C. COLLADA, A. GUEVARA, M. T. CERVERA, G. G. VENDRAMIN, V. GARCIA, J. M. M. FRIGERIO, C. ECHT, T. RICHARDSON and C. PLOMION (2004): Cross-species transferability and mapping of genomic and cDNA SSRs in pines. Theor. Appl. Genet. **109**: 1204–1214.

DAKIN, E. E., and J. C. AVISE (2004): Microsatellite null alleles in parentage analysis. Heredity **93**: 504–509.

EL-KASSABY, Y. A., and K. RITLAND (1986): The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas-fir seed orchard. Silvae Genet. **35**: 240–244.

EL-KASSABY, Y. A., D. RUDIN and R. YAZDANI (1989): Levels of outcrossing and contamination in two *Pinus sylverstris* L. seed orchards in northern Sweden. Scand. J. For. Res. **4**: 41–49.

EVETT, I. W. and B. S. WEIR (1998): Interpreting DNA evidence. Sinauer Associates, Sunderland, MA.

FISHER, P. J., T. E. RICHARDSON and R. C. GARDNER (1998): Characteristics of single- and multi-copy microsatellites from *Pinus radiata*. Theor. Appl. Genet. **96**: 969–979.

FRIES, A., D. LINDGREN and B. ANDERSSON (2008): The Swedish Scots pine seed orchard Västerhus, pp. 70–77. *In:* Proceedings of Seed Orchard Conference, edited by D. LINDGREN, Umeå, Sweden, 26–28 September 2007.

FUNDA, T., C. C. CHEN, C. LIEWLAKSANEEYANAWIN, A. M. A. KENAWY and Y. A. EL-KASSABY (2008): Pedigree and mating system analyses in a western larch (*Larix occidentalis* Nutt.) experimental population. Ann. For. Sci. **65**: DOI 10.1051/forest:2008055

GOTO, S., F. MIYAHARA and Y. IDE (2002): Identification of the male parents of half-sib progeny from Japanese black pine (*Pinus thunbergii* Parl.) clonal seed orchard using RAPD markers. Breed. Sci. **52**: 71–77.

GOTO, S., A. WATANABE, F. MIYAHARA and Y. MORI (2005): Reproductive success of pollen derived from selected and non-selected sources and its impact on the performance of crops in a nematode-resistant Japanese black pine seed orchard. Silvae Genet. **54**: 69–76.

GÖMÖRY, D., R. BRUCHANIK and L. PAULE (2000): Effective population number estimation in three Scots pine seed orchards based on an integrated assessment of flowering, floral phenology and seed orchard design. For. Genet. **7**: 65–75.

HANSEN, O. K., and E. D. KJÆR (2006): Paternity analysis with microsatellites in a Danish *Abies nordmanniana* clonal seed orchard reveals dysfunctions. Can. J. For. Res. **36**: 1054–1058.

HARJU, A. and O. MUONA (1989): Background pollination in *Pinus sylvestris* seed orchards. Scand. J. For. Res. **4**: 513–520.

HARJU, A. M. and T. NIKKANEN (1996): Reproductive success of orchard and nonorchard pollens during different stages of pollen shedding in a Scots pine seed orchard. Can. J. For. Res. **26**: 1096–1102.

JAMIESON, A. and S. S. TAYLOR (1997): Comparisons of three probability formulae for parentage exclusion. Anim. Genet. **28**: 397–400.

JONES, A. G. and W. R. ARDREN (2003): Methods of parentage analysis in natural populations. Mol. Ecol. **12**: 2511–2523.

KAYA, N., K. ISIK and W. T. ADAMS (2006): Mating system and pollen contamination in a *Pinus brutia* seed orchard. New For. **31**: 409–416.

LIEWLAKSANEEYANAWIN, C., C. E. RITLAND, Y. A. EL-KASSABY and K. RITLAND (2004): Single-copy, species-transferable microsatellite markers developed from loblolly pine ESTs. Theor. Appl. Genet. **109**: 361–369.

LINDGREN, D. (Ed.) (1991): Pollen contamination in seed orchards. Proceedings of the Meeting of the Nordic Group for Tree Breeding, Umeå, Sweden, 26–28 September 2007.

LINDGREN, D., B. KARLSSON, B. ANDERSSON and F. PRESCHER (2008): The Swedish seed orchard program for Scots pine and Norway spruce, pp. 142–154. *In:* Proceedings of Seed Orchard Conference, edited by D. LINDGREN, Umeå, Sweden, 26–28 September 2007.

LINDGREN, D. and A. C. MATHESON (1986): An algorithm for increasing the genetic quality of seed from seed orchards by using the better clones in higher proportions. Silvae Genet. 3**5**: 173–177.

MORIGUCHI, Y., H. TAIRA, N. TANI and Y. TSUMURA (2004): Variation of paternal contribution in a seed orchard of *Cryptomeria japonica* determined using microsatellite markers. Can. J. For. Res. **34**: 1683–1690.

NAGASAKA, K. and A. SZMIDT (1985): Multilocus analyses of the external pollen contamination of a Scots pine (*Pinus sylvestris* L.) seed orchard. Lecture Notes in Biomathematics **60**: 134–138.

NAKANISHI, A., N. TOMARU, H. YOSHIMARU, T. MANABE and S. YAMAMOTO (2005): Interannual genetic heterogeneity of pollen pools accepted by *Quercus salicina* individuals. Mol. Ecol. **14**: 4469–4478.

NIKKANEN, T. (2008): A review of Scots pine and Norway spruce seed orchards, pp. 195–198. *In:* Proceedings of Seed Orchard Conference, edited by D. LINDGREN, Umeå, Sweden, 26–28 September 2007.

NILSSON, J.-E. and D. LINDGREN (2005): Using seed orchard seeds with unknown fathers, pp. 57–64. *In:* Proceedings of the meeting of Nordic forest tree breeders and forest geneticists, edited by A. FEDORKOV, Syktyvkar 2005.

PAKKANEN, A. and P. PULKKINEN (1991): Pollen production and background pollination levels in Scots pine seed orchards of northern Finnish origin, pp. 14–21. *In:* Proceedings of the meeting of the Nordic Group for Tree Breeding in Sweden, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences.

PAULE, L. (1991): Clone identity and contamination in a Scots pine seed orchard, pp. 22–32. *In:* Proceedings of

DOI:10.1515/sg-2009-0033
edited by Thünen Institute of Forest Genetics

the meeting of the Nordic Group for Tree Breeding in Sweden, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences.

PENA, S. D. J. and R. CHAKRABORTY (1994): Paternity testing in the DNA era. Trends Genet. **10**: 204–209.

RITLAND, K. (2002): Extensions of models for the estimation of mating systems using n independent loci. Heredity **88**: 221–228.

ROSVALL, O., G. JANSSON, B. ANDERSSON, T. ERICSSON, B. KARLSSON, J. SONESSON, and L.-G. STENER (2001): Genetiska vinster i nuvarande och framtida fröplantager och klonblandningar (Genetic gain from present and future seed orchards and clone mixes), Redogörelse nr 1 2001, SkogForsk, Uppsala. 41p.

ROSVALL, O., U. WENNSTRÖM, C. ALMQVIST, B. ANDERSSON, B. KARLSSON and J. SONESSON (2003): Underlag för operativ planering av tredje omgången fröplantager (TreO) i Sverige [Foundings for operational planning of the third cycle seed orchards (TreO) in Sweden]. Arbetsrapport 550.

SLAVOV, G. T., G. T. HOWE and W. T. ADAMS (2005): Pollen contamination and mating patterns in a Douglas-fir seed orchard as measured by simple sequence repeat markers. Can. J. For. Res. **35**: 1592–1603.

SORANZO, N., J. PROVAN and W. POWELL (1998): Characterization of microsatellite loci in *Pinus sylvestris* L. Mol. Ecol. **7**: 1260–1261.

STOEHR, M. U. and C. H. NEWTON (2002): Evaluation of mating dynamics in a lodgepole pine seed orchard using chloroplast DNA markers. Can. J. For. Res. **32**: 469–476.

WANG, X.-R., D. LINDGREN, A. E. SZMIDT and R. YAZDANI (1991): Pollen migration into a seed orchard of *Pinus sylvestris* L. and methods of its estimation using allozyme markers. Scand. J. For. Res. **6**: 379–385.

WESTNEAT, D. F. and M. S. WEBSTER (1994): Molecular analysis of kinship in birds: interesting questions and useful techniques, pp. 91–126. *In:* Molecular Ecology and Evolution: Approaches and Applications, edited by B. SCHIERWATER, B. STREIT, G. P. WAGNER and R. DESALLE. Birkhauser Verlag, Basel, Switzerland.

YAZDANI, R. and D. LINDGREN (1991): Variation of pollen contamination in a Scots pine seed orchard. Silvae Genet. **40**: 243–246.

YAZDANI, R., D. LINDGREN, F. SEYEDYAZDANI, L. PASCUAL and U. ERIKSSON (1995): Flowering, phenology and pollen contamination in a clonal seed orchard of *Pinus sylvestris* in Northern Sweden, pp. 309–319. *In:* Population genetics and genetic conservation of forest trees, edited by P. BARADAT, W. T. ADAMS and G. MÜLLER-STARCK. SPB academic Publishing, Amsterdam, Netherlands.

YAZDANI, R., O. MUONA, D. RUDIN and A. E. SZMIDT (1985): Genetic structure of a *Pinus sylvestris* L. seed-tree stand and naturally regenerated understory. For. Sci. **31**: 430–436.

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