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# Microsatellites used to establish full pedigree in a half-sib trial and correlation between number of male strobili and paternal success

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## Abstract

- Paternity was established in a field trial of *Abies nordmanniana* with open-pollinated (OP) offspring from a clonal seed orchard (CSO) comprising 23 clones.
- The purposes were to: (1) investigate the violation of the assumption of true half-sibs in OP progeny trials; (2) assess the value of male strobili scorings to predict paternal contributions in CSOs; and (3) study the bias in breeding values and heritabilities obtained in breeding with OP family trials due to unknown paternity.
- The paternal contribution to the offspring varied a lot among the clones, but the resulting violation of the assumption of true half-sibs in the progeny trial was only modest.
- On average 84% of the relationships among the offspring were true half-sib, resulting in an average genetic correlation of 0.29 (range 0.26 to 0.33).
- Male strobili scorings from the seed harvest year did well in forecasting the paternal contribution to the offspring. The linear regression of sired progeny on the estimated clonal proportion of male strobili explained 76% of the variation.
- The large variation in siring success and existence of other types of relationships than true half-sibs among the offspring only gave minor bias in estimated genetic parameters.

## 1. INTRODUCTION

Selection based on open-pollinated (OP) family field testing is predominant in tree breeding – especially in the first part of breeding programmes aimed at backwards selection for seed orchard establishment or at forward selection for selecting 2nd generation plus trees (e.g. Wright, 1976). The inheritable variation in a metric character, which all breeding relies upon, is usually quantified by the genetic variance, which can be partitioned into additive and non-additive components (Falconer, 1989). The breeding value of an individual is the sum of the additive effects of its genes, and is equivalent to twice the expected deviation of its offspring mean phenotype from the population mean (Lynch and Walsh, 1998). Only additive genetic variance, which is the variance of the breeding values (Falconer, 1989), has been used in most conifer breeding programmes worldwide (Wu and Matheson, 2004). This is also the case for the Danish breeding programme of *Abies nordmanniana*, which employs clonal seed orchards (CSOs) and testing of OP families. In Denmark, *A. nordmanniana* is used for the production of Christmas trees, which are grown in intensively managed cultures with a fairly short rotation – 10–15 y from seed. The breeding programme aims to improve characters important in relation to production of Christmas trees – e.g. height, post-harvest quality and resistance to adelgids.

The rate of genetic gain by selection in a breeding programme depends in part on obtaining precise and unbiased estimates of genetic variances (Namkoong, 1966). As highlighted by Namkoong (1966), estimates of genetic additive variance obtained from offspring of OP trees tend to be biased upwards, due to the presence of natural inbreeding. This is the case because theoretically the genetic correlation among OP offspring is 0.25, which means that the additive genetic variance can be estimated by multiplying the among-family variance component by 4. However, the half-sibs are only truly half-sibs if none of them resulted from selfing and if all of them were sired by different males. If these conditions are not fulfilled, the average genetic correlation will be higher than 0.25 and estimates of additive genetic variance, resulting heritabilities and final genetic gains will be biased (Squillace, 1974).

In a first generation CSO of *A. nordmanniana*, the risk of having related individuals included is rather small. Nevertheless, even if the seed parents in a clonal seed orchard are unrelated, a paucity of effective pollen donors increases the genetic covariance within tree seed families and causes one to overestimate the additive genetic variance (Namkoong, 1966). This issue is highly relevant for Danish CSOs of *A. nordmanniana* which often have a fairly small number of clones, since genetic diversity is of little or no concern due to the short rotation and “crop” nature of Christmas trees.

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Thus, estimates of genetic gain from tested OP families, whether these originate from natural stands or CSOs, are based on the assumption of either true half-sibs or a mix of half- and full-sibs; the latter implies an average genetic correlation between 0.25 and 0.5. In concordance with these considerations, some studies have chosen to use an average genetic correlation between these two outer points – e.g. Sorensen and White (1988) assumed an average genetic correlation of 0.30 while Nielsen (2007) assumed it to be 0.33. Such assumptions, however, often lack data-based information.

New efficient and highly polymorphic DNA markers, commonly named microsatellites, have recently been developed in several *Abies* species (e.g. Cremer et al., 2006; Hansen et al., 2005; Saito et al., 2005). Paternity studies of *A. nordmanniana* (Hansen and Kjær, 2006) and *Abies alba* (Hansen, 2008) CSOs using these markers have revealed examples of highly skewed paternal contributions, where a few clones sired a large majority of the offspring. This skewness will increase the fraction of full-sibs even more than the fraction already induced by the limited number of potential pollen donors in a CSO, and thereby also the average genetic correlation. Microsatellites are excellent tools for investigating the basic assumptions of paternity. Therefore, in the present study we made a detailed paternity analysis with microsatellite markers in a half-sib progeny trial. Based on these data we evaluated the benefits of applying full pedigree information to estimate breeding values more exactly, and the effect of specific combining ability (SCA) compared to general combining ability (GCA). In a similar way Doerksen and Herbing (2008) recently presented results from a paternity analysis in an OP field trial of red spruce (*Picea rubens*). Their goal was to investigate unequal male reproductive success, selfing and pedigree errors – and to discuss how these factors may impact quantitative genetic parameters. Here we also assess the unequal male reproductive success and selfing, but furthermore directly calculate their impact on heritabilities and breeding values. Additionally, we study the relationship between the clonal proportions of male strobili in the CSO and actual paternal reproductive success in the progeny trial. This potential link is interesting, because despite the arrival of DNA markers, the assessment of paternal contribution by visual scoring of male strobili amounts may still be a more feasible approach, due to a much lower requirement for resources. How well such strobili scoring reflects the actual paternal contribution, though, is not usually known. Seeds for the half-sib trial in this study were harvested in a CSO, and in the spring before seed harvest visual male strobili scoring from the CSO was carried out (based on counting of male strobili), and these strobili scores were used as a predictor of the paternal contribution.

## Objectives

By making a detailed paternity analysis in a half-sib trial using microsatellite markers, combined with the use of contemporary male strobili scoring and quantitative genetic analyses, we attempted to do the following: (1) investigate the violation of the assumption of true half-sibs in progeny trials; (2) assess

the value of strobili scoring to predict paternal contributions in CSOs by comparing clonal scores with actual siring success based on microsatellite studies; and (3) study the bias in breeding values and heritabilities due to unknown paternity, as obtained in breeding with OP family trials for a variety of characters.

## 2. MATERIALS AND METHODS

### 2.1. Half-sib trial and clonal seed orchard

Experiment No. 1415 (EX1415) at the Arboretum in Hørsholm comprises a total of 63 OP families of Nordmann fir planted in single-tree plots that are distributed in seven blocks. Forty of the families originate from two Danish approved seed stands – approval Nos. F.526 and F.527, both from Tversted Dune Forest. The remaining 23 families in EX1415 are progeny from the clonal seed orchard (CSO) FP.620 Vallø, which only encompasses this relatively modest number of clones. Only these latter 23 families are analysed in the present study.

The clones in FP.620 were selected from older approved Danish stands at the beginning of the 1960s. Two clones were selected from a Danish stand of putative Borshomi origin, and the other 21 from Danish stands that presumably originated from northern Caucasus, shipped from the city of Pjatigorsk. The selection criterion was bough quality.

FP.620 was established in the years 1966–68 and covers an area of 3.0 ha. A total of 854 ramets were planted at approximately 6 × 6 m – each clone was represented at various frequencies ranging from 9 to 77 ramets. An examination of the seed orchard in 1986 and annual strobili scoring in a substantial part of it starting from 1992 (see paragraph below), made it possible to estimate the total number and clonal distribution of ramets in 1992, which was the year seed was harvested for EX1415.

Seed was harvested from three ramets of each clone and sown clone-wise in the nursery in spring 1993. In spring 1996 the three-year-old transplants were planted at the Arboretum, Hørsholm, in a balanced complete randomized block design with seven blocks and single-tree plots. Each block contained four non-contiguous plants of each OP family. With 23 OP families it implies that there were originally 644 (23×28) offspring from FP.620 in EX1415. By summer 2006 this number had decreased to 631 due to natural mortality.

### 2.2. Paternity analysis of half-sib offspring from FP.620

A needle sample was collected from each of the 631 remaining individuals in EX1415, and DNA was extracted with the DNeasy® Plant Mini Kit from QIAGEN (Germany). The same was done for three ramets of each of the 23 clones in FP.620. However, four of the original 23 clones had been rogued in 2001, and for those clones only two ramets, which had been left in FP.620 for gene conservation purposes, were available for DNA extraction.

All 696 individuals were subsequently genotyped with five microsatellites developed for Nordmann fir: NFF2, NFF3, NFF7, NFH3 and NFH15 (Hansen et al., 2005) and one microsatellite, SF78, developed for European silver fir (Cremer et al., 2006). Genotyping

through PCR was carried out with the QIAGEN® Multiplex kit (catalogue No. 206143). PCR conditions followed the given standard multiplex PCR protocol: 1X Multiplex master mix (providing a final concentration of 3 mM MgCl<sub>2</sub>), 0.2 μM of each primer, approximately 20 ng of DNA sample and water added to a final reaction volume of 15 μL. Thermal profile was: initially 15 min of denaturation at 95 °C, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s and extension at 72 °C for 60 s, with a final extension step at 60 °C for 30 min. Fragment sizes of the amplified labelled microsatellites were determined on the CEQ 2000XL Fragment analysis system from Beckman Coulter Inc. (Fullerton, California). Fathers of the trees were found via paternity analysis performed with the software CERVUS 3.0 (Kalinowski et al., 2007; Marshall et al., 1998). Trees with no paternal fit from the seed orchard clones were assumed to represent pollination from outside trees (pollen contamination).

### 2.3. Proportions of the various relatives and average genetic correlations

Offspring of an OP tree consist of selfs and outcrosses, and following Squillace (1974) there are four possible kinds of relatives (genetic correlation when parents are non-inbred and non-correlated in parentheses): self full-sibs (0.667), full-sibs (0.500), self half-sibs (0.408) and half-sibs (0.250). Self full-sibs (sfs) are two offspring produced by selfing, full-sibs (fs) are two outcrossed offspring that have both parents in common, self half-sibs (shs) are a self and an outcross, which share a common parent, and finally half-sibs (hs) are two outcrossed offspring that have a single parent in common (Squillace, 1974). By knowing the proportions of the different types of relatives, the average genetic correlation ( $r$ ) in a family produced by open pollinating can be calculated: average genetic correlation ( $OP\_FAM$ ) =  $r_{sfs} \times p_{sfs} + r_{fs} \times p_{fs} + r_{shs} \times p_{shs} + r_{hs} \times p_{hs}$ , where  $p$  is the proportion of the respective type of relatives (e.g.  $p_{fs}$  being the proportion of full-sib relatives). Inserting the genetic correlations between specific relatives therefore gives: average genetic correlation ( $OP\_FAM$ ) =  $0.667 \times p_{sfs} + 0.5 \times p_{fs} + 0.408 \times p_{shs} + 0.25 \times p_{hs}$ . Based on the results from the paternity analysis and formulas from Squillace (1974) the proportions of the various relatives as well as the average genetic correlations in the 23 half-sib families were calculated.

### 2.4. Male strobili data for the seed collection year

In the spring 1992, same year as seed were collected for the half sib trial in the autumn, the amount of male strobili in FP.620 was assessed by ocular scoring, using a logarithmic scale from 1 to 8 (score 1 = 0 strobili, 2 = 1–3, 3 = 4–15, 4 = 16–60, 5 = 61–250, 6 = 251–1 000, 7 = 1 001–4 000, 8 = 4 001–16 000) (Sirikul et al., 1991). Scoring was carried out on a subset of the ramets in the seed orchard: 340 trees out of the total number of 701 trees remaining in the CSO (49%). Counts were made from the ground.

To find the clonal proportions of the overall amount of male strobili, each strobili score was multiplied by the mid-range value of that particular strobili score; e.g. individuals with the strobili score 5 were assigned  $(61 + 250)/2 = 155.5$  male strobili. The clonal mean number of strobili was then estimated by Least Square means in PROC GLM (SAS v. 9.1), and this value was multiplied by the number of ramets in the whole CSO for the respective clones. Finally the estimated number of male strobili per clone was divided by the sum of

estimates from all clones. With these data it was possible to compare the differences in proportions of male strobili with the actual outcome of paternity to offspring.

### 2.5. Analysed quantitative data – progeny trial

For the study of potential bias in breeding values and heritabilities due to unknown paternity, a set of previously collected data (growth traits, phenology and resistance to adelgids (Nielsen et al., 2002) as well as new data (post-harvest quality) was used.

*Growth traits:* tree height after four growing seasons in the field trial and the number of branches in the uppermost whorl. *Phenology:* flushing measured according to a modified Langlet scale going from 0 (buds in winter condition) to 6 (shoots elongated, needles in final position). *Resistance to adelgids:* EX1415 was originally established with the specific purpose of testing for genetic differences between provenances and families with regards to their suitability as host plants for the silver fir woolly adelgid (*Dreyfusia nordmanniana* Eckst.). The investigation was based on artificial infestations, and later counting of two different morphs of the adelgid: sistentes and progredientes. For results and a detailed description of the methodology see Nielsen et al. (2002). *Post-harvest quality:* the 631 progeny from FP.620 in EX1415 were assessed for needle loss in autumn 2006. This trait is assessed by cutting of branches, placing them indoor and subsequently, after 10 d, rate the needle loss on a 8-step scale. For a detailed description of the importance of this trait and further details concerning the methodology the reader is referred to Nielsen and Chastagner (2005).

### 2.6. Statistical analysis

A general linear mixed model was fitted to the quantitative data for the 596 trees in EX1415 for which paternity could be assigned. Two different models were used:

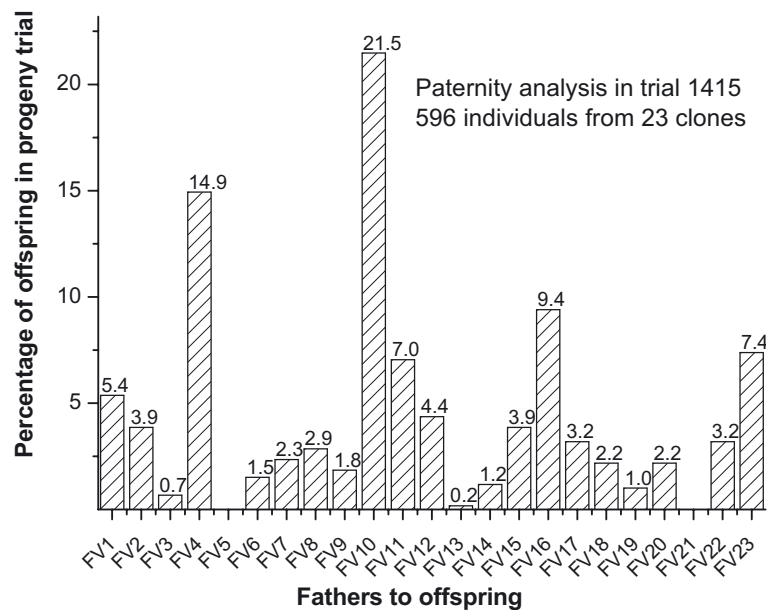
$$\mathbf{Y} = \mathbf{Xb} + \mathbf{Z}_1\mathbf{a} + \mathbf{e} \quad (1)$$

$$\mathbf{Y} = \mathbf{Xb} + \mathbf{Z}_1\mathbf{a} + \mathbf{Z}_2\mathbf{f} + \mathbf{e} \quad (2)$$

where  $\mathbf{y}$  is a column vector containing the phenotypic values for the traits measured in the 596 individuals,  $\mathbf{b}$  is a vector of fixed effects (overall mean and block effect),  $\mathbf{a}$  is a vector of random genetic effects of individual trees (offspring as well as parents),  $\mathbf{f}$  is a vector of random full-sib family effects and  $\mathbf{e}$  is a vector of random residual deviations, the latter being assumed to be distributed independently of the random genetic effects.  $\mathbf{X}$ ,  $\mathbf{Z}_1$  and  $\mathbf{Z}_2$  are incidence matrices which relate the observations in  $\mathbf{y}$  to the effects in  $\mathbf{b}$ ,  $\mathbf{a}$  and  $\mathbf{f}$ .

Variance components and the variances of variance components were estimated by restricted maximum likelihood (REML, Patterson and Thompson, 1971). From these the heritabilities and their corresponding standard errors were calculated. Best linear unbiased predictions, here designated breeding values (BVs), were estimated for the 23 parent clones. All procedures were performed in the software ASReml version 2 (Gilmour et al., 2006).

Three different situations were analysed: (1) traditional half-sib analysis (only mother known) – only additive effects estimated (Model 1); (2) full-sib analysis (mother and father known) – only additive effects estimated (Model 1); (3) full-sib analysis (mother and father known) – both additive and dominance effects estimated



**Figure 1.** Overview of the distribution of fathers to the FP.620 offspring in EX1415 – based on paternity analysis with six microsatellites. Numbers on top of bars are the actual values – given as percentage of the total number of offspring.

(Model 2). In situation 1 the pedigree file ( $Z_1$ ) in ASReml only contained the identity of the mothers, while in situation 2 and 3 both the identity of the mother (known from collection of seed for the trial) and the father (known from paternity analysis) were included in the pedigree file. The statistical significance of the additive as well as dominant genetic effects was tested with the REML likelihood ratio test (Gilmour et al., 2006, p. 17).

### 3. RESULTS

#### 3.1. Paternity analysis and establishment of full pedigree in EX1415

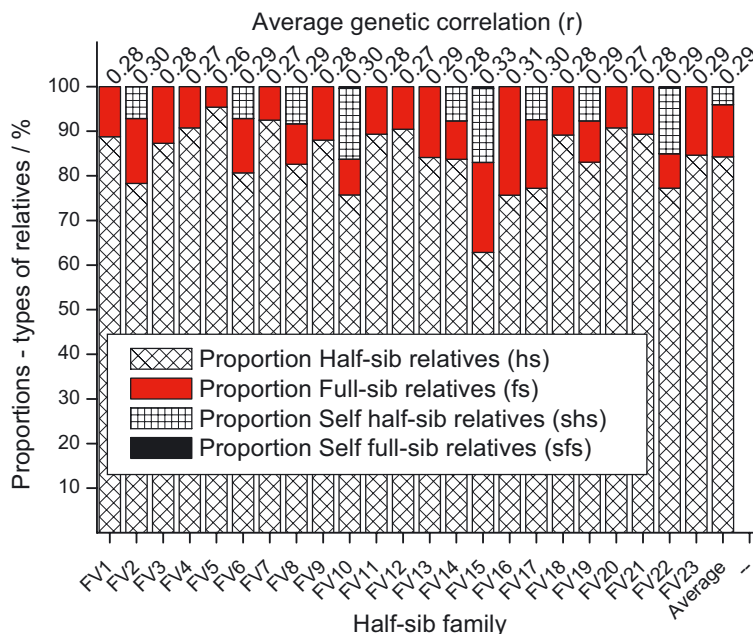
Paternity analysis in CERVUS assigned paternity to 596 trees with 95% confidence, which equates to 94.5% of the trees of FP.620 origin in EX1415. Some minor manual adjustments were made to the output from CERVUS according to the following criteria: (1) all individuals with three or more trio-mismatches were excluded from the analysis. Trio-mismatch is mismatch among the progeny tree, assumed mother tree and assigned father clone (Sixteen trees were excluded due to this criterion); (2) all individuals with two trio-mismatches were excluded, unless: (a) 2 pair-mismatches could be explained by null-alleles or (b) 1 pair-mismatch could be explained by null-alleles and the trio-LOD score given by CERVUS was positive (eleven trees excluded); (3) individuals with 1 trio-mismatch were excluded if the mismatch could not be explained by null-alleles, and both the pair-LOD score and the trio-LOD score were negative (eight trees excluded). In a few cases, the manual adjustments went the other way. For example, for some trees with paternity assignment below 95% confidence, a father was assigned anyway, because some mismatches could be explained by null-alleles.

In general, mismatches can be caused by mistakes either during seed harvest, handling of plants during field trial establishment or simply because of errors in labelling/grafting in the seed orchard. The latter category also includes cases in which rootstocks have overtaken their grafts and become the stem in the tree. In fact, of the 65 ramets genotyped in FP.620, disagreement was found among the genotypes of ramets in three clones: e.g. one of the three ramets had a different genotype from the two other genotyped ramets of the same clone.

At the end, 596 trees were retained for further analysis, which was the same number as originally found in CERVUS, although a very small fraction of the included individuals differed. The 35 excluded trees (5.5%) would either be a result of pollination from incoming pollen from the forest that surrounds the seed orchard, or simply individuals for which human error (e.g. mislabelling) had made the identity of the true mother tree unknown. An overview of the distribution of fathers among the clones is illustrated in Figure 1. It can be observed that the paternal contribution to the FP.620 offspring in EX1415 varies a lot among clones. For example, clone FV10 sired 21.5% of the offspring while clones FV5 and FV21 did not sire any of the offspring in the progeny trial.

#### 3.2. Proportions of various relatives – average genetic correlations

The distribution of the four possible kinds of relatives, calculated from the output of the paternity analysis, can be seen in Figure 2. It is here important to realize that all offspring are relatives to each other, and that is what decides the average genetic correlation. None of the 23 OP families consisted solely



**Figure 2.** Proportions of the four possible kinds of relatives in the 23 OP families harvested in FP.620. Self full-sibs (Sfs) were only seen in families FV10, FV15 and FV22 (range 0.31 to 0.40%). Based on these proportions the average genetic correlation in each OP family was calculated and is shown on top of each bar. The bar on the right side of the diagram shows the average distribution and genetic correlation (0.29) for all 23 families in the CSO. Average size of OP-family: 25.9 offspring. Range: 22 to 28 offspring.

of half-sibs. In the OP-family harvested from FV15 only 63% of the relationships were of the half-sib type, while the highest proportion of half-sib relationships was found in the OP-family of FV5 (95%) (Fig. 2). The latter also had the lowest proportion of full-sib relationships (5%), while the OP-family of FV16 had the highest percentage of full-sib relationships (24%). Overall, the average proportions of the four different relationships were: 84% half-sib relatives, 12% full-sibs, 4% self half-sibs and 0.05% self full-sibs – see right-hand bar in Figure 2. Self full-sibs (Sfs) only occurred in three families (FV10, FV15 and FV22), and in very small proportions (range 0.31 to 0.40%). Based on the relationship proportions, the calculated average genetic correlations were in the range from 0.26 (FV5) to 0.33 (FV15) – the clonal values can be seen on top of the bars in Figure 2. The overall average genetic correlation for all offspring was 0.29.

### 3.3. Male strobili data – imbalances in the CSO

The analysis of variance revealed a highly significant difference among the 23 clones in amount of male strobili ( $P < 0.0001$ ). The least square mean estimates ranged from 295 male strobili per tree (clone FV5) to 10 000 male strobili (clone FV7) (Fig. 3A). The number of ramets at the time of flowering in 1992 also showed large variation. Clone FV6 was only represented by nine ramets in 1992 while there were 68 ramets of clone FV4 (Fig. 3B). Finally, combining these two types of imbalances by simple multiplication and division with the total sum revealed substantial imbalance among clones in the estimated proportion of total male strobili. Clones FV4 and

FV10 had the highest estimated proportion of male strobili – both around 13%. Clones FV5 and FV13 had the lowest – both below 0.2% (Fig. 3C).

### 3.4. Correlation between male strobili score in CSO and distribution of paternity

The connection between the estimated clonal proportion of male strobili in the CSO and the distribution of paternity found in EX1415 by using microsatellites is illustrated in Figure 4. There appears to be a strong correlation between the two proportions, with a highly significant correlation coefficient ( $r$ ) of 0.87 ( $p < 0.0001$ ). The clones that deviated the most from the expectation, based on male strobili, were clones FV10, FV23 and FV2 (Fig. 4).

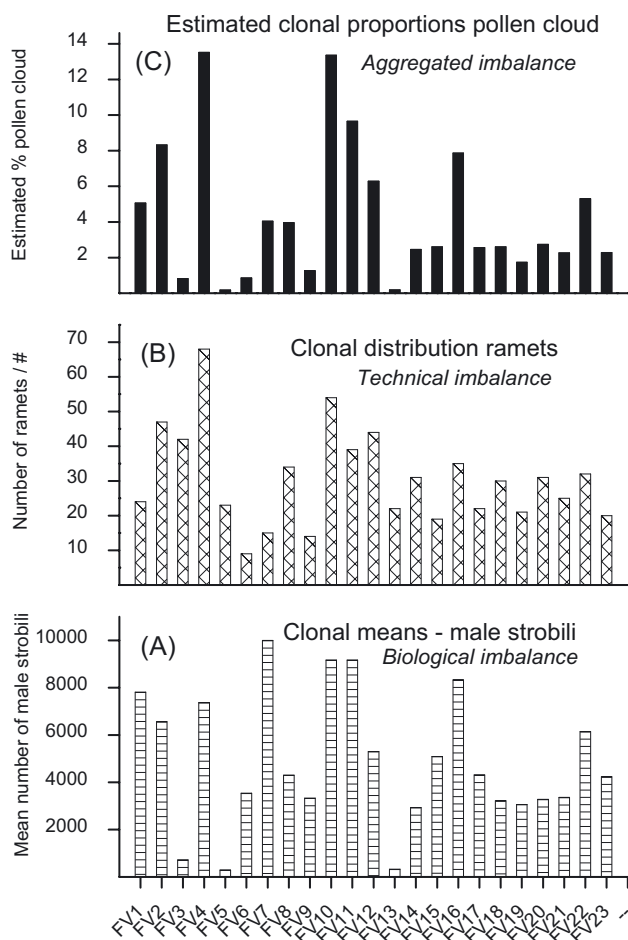
### 3.5. Heritabilities, breeding values and dominance effects

The ASReml half-sib analysis (model 1 without paternity analysis) revealed significant or highly significant additive genetic effects for counting of sistentes, needle loss, height and flushing, while number of branches and counting of progredientes were non-significant (Tab. I, Col. 2). Using the same data in a full-sib analysis (model 1 with paternity analysis) resulted in substantially higher significance levels, showing significant additive effects for all characters (Tab. I, Col. 4). The corresponding heritabilities estimated by half- and full-sib analysis are also given in Table I (Cols. 3 and 5). The heritability for

**Table I.** Results from ASReml analyses. Three types of analyses were run: (a) half-sib analysis (model 1); (b) full-sib analysis – only additive effects estimated (model 1) and (c) full-sib analysis – both additive and dominance effects estimated (model 2). Columns 2 and 4:  $D_A$  is the test statistic of the REML likelihood ratio test, used to test the significance of the additive genetic effects estimated in model 1. Columns 3 and 5: single tree heritabilities ( $h^2$ ) and corresponding standard errors for six characters, using variance components from either half- or full-sib analysis (model 1). Column 6: correlation between breeding values (BVs) estimated for the 23 parent clones in FP.620 using either half- or full-sib analysis (both model 1). Column 7:  $D_D$  is the test statistic of the REML likelihood ratio test, used to test the significance of the dominance effects estimated in model 2.

Character	Half-sib model 1		Full-sib model 1 = only additive effects		Correlation HS-FS	Full-sib model 2 = additive and dominance effects
	$D_A = 2 [\log(l_{M2}) - \log(l_{M1})]$	$h^2$ (SE)	$D_A = 2 [\log(l_{M2}) - \log(l_{M1})]$	$h^2$ (SE)	$r_{(BVs)}$	$D_D = 2 [\log(l_{M2}) - \log(l_{M1})]$
Height 1999	50.40 ***	0.57 (0.19)	46.88 ***	0.35 (0.12)	0.91	6.94 **
No. branches	1.16 ns	0.06 (0.06)	5.91 *	0.08 (0.05)	0.88	0.21 ns
Needle loss	10.75 **	0.20 (0.10)	49.76 ***	0.27 (0.09)	0.95	0.00 ns
Flushing	108.98 ***	0.96 (0.25)	226.81 ***	0.89 (0.16)	0.97	2.18 ns
Progreidentes	0.10 ns	0.02 (0.05)	7.64 **	0.08 (0.05)	0.76	1.79 ns
Sistentes	4.64 *	0.12 (0.08)	21.96 ***	0.16 (0.07)	0.91	1.62 ns

\*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , ns non-significant. The critical  $\chi^2$ - value = 3.84 for significance on a 5% level.



**Figure 3.** Clonal differences in number of male strobili, number of ramets and estimated proportion of total amount of male strobili. (A) Clonal least square means for number of male strobili, estimated via a scoring scale. (B) Clonal differences in the number of ramets in the CSO – estimated for the year 1992. (C) Clonal proportions of the total amount of male strobili in 1992 – based on data from (A) and (B) clonal mean number of strobili multiplied by number of ramets.

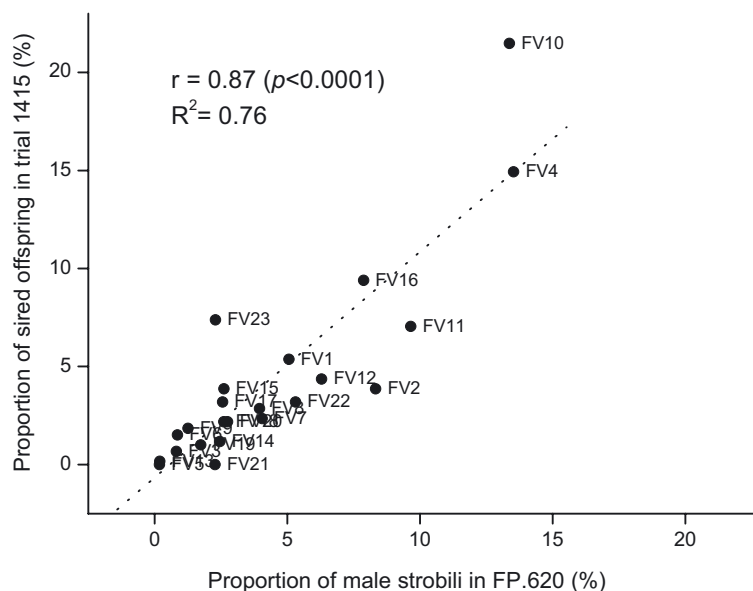
height decreased substantially from 0.57 in the half-sib analysis to 0.35 in the full-sib analysis, but only minor differences were seen for the other characters, except counting of progredientes, which was non-significant in the half-sib analysis. In all instances the standard error of the heritability estimates is equal or, more often, smaller when the full-sib analysis was used.

Where the differences in heritability reflects how much genetic gain in breeding is over- or underestimated when we only use half-sib testing, the resulting breeding values from each type of analysis may indicate whether the missing pedigree information may lead to direct errors in the breeding process, where trees are selected. In other words, how strong is the correlation between breeding values estimated in the two situations? We therefore calculated the correlation between clonal BVs for the six characters, estimated both by half- and full-sib analysis, and these are seen in column 6 in Table I. It appears that the correlation among the breeding values from the two types of analysis is rather strong for most of the characters – typically around 0.9 or higher. The lowest correlation was seen for the number of progredientes adelgids ( $r = 0.76$ ) while the strongest correlation was observed for flushing ( $r = 0.97$ ). The strong correlation between breeding values obtained from half- and full-sib analyses respectively, taken together with the very skewed paternal contribution to the analysed offspring, gives an indication that there is little specific combining ability and thereby dominance.

This point was formally tested in the comparison between the analyses from model 1 (full-sib analysis – only additive effects estimated) and model 2 (full-sib analysis – both additive and dominance effects estimated). It turned out that the estimated dominance effect only was statistically significant for height 1999 (Col. 7 in Tab. I).

#### 4. DISCUSSION

The fundamental issues treated in this study were highlighted more than 40 y ago (Namkoong, 1966) – but it is



**Figure 4.** Correlation between clonal proportions of the total amount of male strobili in 1992 (CSO FP.620) and the distribution of paternity in EX1415.

only within the last 10–15 y that we have obtained the tools to investigate it experimentally. One prerequisite has been the emergence of highly polymorphic DNA-markers such as microsatellites, which can unravel the exact pollination patterns in seed orchards (e.g. Moriguchi et al., 2004). Another has been the development of maximum likelihood methods (e.g. Searle, 1995) and matching software, which can estimate variance components in the highly un-balanced crossing schemes that are often the result of OP offspring.

#### 4.1. Violation of true half-sibs assumption in progeny trials

The mating probabilities of trees in a CSO can differ, and this may warrant for a correction of the average genetic correlation, if the variance components are to be estimated from the progeny coming from the CSO seed crop (Askew and El-Kassaby, 1994). The highly skewed distribution of paternal contributions to the offspring revealed by the paternity analysis is very much in line with previous studies in *Abies* CSOs. For example Hansen and Kjær (2006) found that three clones out of 13 sired around 75% of the seeds in a small *A. nordmanniana* CSO, although only comprising 24% of the number of ramets. Four clones did not sire any seeds at all. In a very similar study of an *A. alba* CSO with 12 clones, Hansen (2008) established that four clones had sired more than 80% of the seeds. The effective population size on the male side, represented by the status number ( $N_s$ ), was  $N_s = 4.2$  and  $N_s = 4.6$  for *A. nordmanniana* and *A. alba* respectively. In the present study, five clones out of 23 sired 60% of the trees in the half-sib trial (Fig. 1), two clones did not contribute anything as male parents, and in general a similarly skewed paternal contribution seems to be the rule in conifer CSOs (e.g. Moriguchi

et al., 2004). The rather skewed distribution of fathers, combined with the limited number (23) of potential fathers in the CSO, resulted in 84% of the four possible relationships being of the half-sib type, giving an average genetic correlation of 0.29 among half-sibs (range for OP families 0.26–0.33).

Some of the earliest studies dealing with the influence of mating patterns in forest trees in relation to the genetic relatedness among offspring were carried out by Surles et al. (1990) and Askew and El-Kassaby (1994). The former investigated two leguminous tree species, and the latter dealt with two conifer CSOs. None of the studies had the option of full paternity analysis – e.g. Askew and El-Kassaby (1994) combined a simulation approach with data on reproductive overlap. For a 15-clone CSO of Loblolly pine (*Pinus taeda*) without selfing and pollen contamination Askew and El-Kassaby (1994) estimated that 88.7 to 92.3% of the pair wise progeny relationships were of the half-sib type. We found little selfing, low pollen contamination and 84% half-sib relationships, so both conditions as well as our results are therefore in line with the predictions of Askew and El-Kassaby (1994).

A few other more recent studies have been conducted in conifers, investigating the issue of relatedness in OP families with the help of DNA markers. In a breeding population of radiata pine (*Pinus radiata*) Kumar and Richardson (2005) found that the estimated genetic correlation (they use the equivalent term coefficient of relationship) was in the range of 0.00 to 1.0 with an average of 0.29 – the exact same value as in our study. However, Kumar and Richardson (2005) did not link this higher than expected genetic correlation to possible pollination patterns, but instead suggested that some of the selected plus trees were not unrelated as initially assumed. Dorksen and Herbing (2008) also performed a paternity analysis in an OP test with 38 families of red spruce (*Picea rubens*) and found a highly skewed paternal reproductive success, but



no calculation of genetic correlation between the half-sibs which could be assigned a father was given. Very recently a study by Gaspar et al. (2009) in *Pinus pinaster* estimated the average genetic correlation among trees in a progeny trial from a CSO to be 0.26 – close to the 0.25 which holds for true half-sibs.

#### 4.2. Value of male strobili scoring to predict paternal contributions in CSOs

The study found a rather strong correlation between the amount of male strobili in the CSO and the paternal contribution to the field trial offspring, and the simple linear regression model explained 76% of the variation in siring success (Fig. 4). The magnitude and significance of this relation between strobili amount and siring success in conifers is very interesting. For example there have been a large number of studies of the variation in male strobili in conifer CSOs (e.g. Gömöry et al., 2003; Kjær, 1996), but how these often profound genotypic differences are actually reflected in the paternal distribution to the plants which end up in the plantings, is unknown. In *A. nordmanniana* it seems that male strobili scoring is a valuable practical tool for the seed orchard manager to estimate paternal contributions to the seed crop – much faster and cheaper than paternity analysis with markers. The effectiveness of the visual scoring in combination with the actual ramet numbers indicates a proportional system in which pollination success is related to the actual amount of pollen, but also indicates that there are probably no or only limited differences among clones in the orchard regarding floral synchrony (confirmed by observations available from 1992). This is in accordance with our experience of carrying out controlled crossings in *A. nordmanniana* (Hansen and Nielsen, 2008), and also for *Abies* species in general (Andrej Kormutak, personal communication 2000), which shows that there is only a short period of pollen shedding and female receptivity – often only a week.

Only a few other studies have investigated this link between male strobili amount and siring success in conifers. Moriguchi et al. (2007) investigated the influence of floral synchrony on paternity contribution in *Cryptomeria japonica*, but did not find any significant effect in a pollen shedding period of 1.5 months (Moriguchi et al., 2005), and thereby considerably longer than in *A. nordmanniana*. Like us, Moriguchi et al. (2007) found a significant relationship between male strobili production and male reproductive success, but their linear regression model only explained around 15% of the variation in siring success. In *Pinus thunbergii*, Goto et al. (2005) also found a significant association between male fecundity and male reproductive success – their regression model explained around 43% of the variance – again with a non-significant effect of floral synchrony.

In the case presented here, using only clonal male strobili averages, and not taking into account the differences in ramet numbers, the correlation between male strobili amount and siring success was much weaker – only explaining around 43% of the variation (data not shown). It seems rather clear, by the great improvement in paternity precision by multiplying num-

ber of ramets by significant genotypic values, that for example linear deployment of clones (Lindgren and Matheson, 1986) really means deployment of different numbers of ramets – “it works” – “it’s metric”.

#### 4.3. Bias in estimated genetic parameters due to unknown paternity

For the tree breeder the bias in the estimated genetic parameters coming from the use and underlying assumptions of OP families harvested in CSOs is of primary interest! We illustrated this bias by comparing  $h^2$  estimates calculated with and without information about paternity, based on real data, and by looking at the correlation among breeding values coming from these two models.

Borralho (1994) used simulated data to investigate the bias of  $h^2$  estimates, and focused on the impact of varying selfing rates among families and dominance effects. He found upwardly biased estimates due to both types of factors, although the impact of varying selfing rates was more influential than the dominance effects.

Kumar et al. (2007) also employed molecular markers to test whether parental GCA values would be biased due to unequal pollen contribution – their system was a polycross mating design where pollen from 15 *Pinus radiata* parents was used in equal proportions. The resulting GCA values obtained from the polycross were then compared with GCA values obtained from a subset of the same parents, but where the GCA values had been estimated by a complete-pedigree design (female-tester). Despite evidence for significant unequal pollen contribution in some of the polycross families, a good correspondence between GCA estimates obtained from the polycross and female-tester design was found, which again is consistent with our results.

In our study, inclusion of paternity information increased significance levels of the additive genetic effect for all characters and diminished standard error of the heritability estimates for several of them. For height, the estimated and significant dominance effect, combined with the highly skewed paternal contribution could cause an overestimated additive genetic variance in the half-sib situation. For the other characters, except counting of progredientes, which was non-significant in the half-sib analysis, the heritability estimates were roughly on the same level when pedigree information about fathers was included in the analysis. One may of course claim that none of the observed differences in heritability estimated in half-sib versus full-sib analysis are true, due to the relatively large standard errors of the estimates. This is, however, a problem that is relevant to many/most studies of heritability. As described above Gaspar et al. (2009) used molecular markers in a *Pinus pinaster* progeny trial. For the two traits height and wood density, the differences between the heritabilities unadjusted and adjusted for paternity contributions (= true coancestry coefficients) were very small, namely 0.609 vs. 0.586 (wood density) and 0.166 vs. 0.160 (diameter) – the latter being the adjusted values. In contrast to our study they only obtained paternity estimates for a subset of the families included in the

trial (5 out of 46 families). However, through simulation studies Gaspar et al. (2009) found that increasing the number of studied/genotyped families (e.g. from 5 to 10) would not affect heritability estimates, as long as the mean genetic correlation within families was correctly estimated (unbiased).

Our trial is actually too small to make clear cut statistically reliable estimates of the SCA (Greg Dutkowski, personal communication 2007), but the estimates we did obtain indicate that SCA does not play an important role in the investigated characters, although significant for height. The estimated low levels of SCA by itself helps to diminish the impact of the observed uneven siring, because this implies no or minor added value to breeding values of specific combinations.

## 5. CONCLUSIONS

Although there were a limited number of potential fathers in the CSO and a rather skewed paternal contribution among these, the violation of the assumption of true half-sibs in the progeny trial was only modest. On average 84% of the relationships among the offspring were true half-sib, resulting in an average genetic correlation of 0.29 (range 0.26 to 0.33) – a slight increase compared to the theoretical value of 0.25. Male strobili scorings from the seed harvest year showed promise for forecasting the paternal contribution to the offspring.

The large variation in siring success and existence of other types of relationships than true half-sibs among the offspring only gave minor bias in estimated genetic parameters. Overall, this gives confidence to the present approach of breeding of *A. nordmanniana* based on OP families.

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