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ORIGINAL ARTICLE

Mating dynamics of Scots pine in isolation tents

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Abstract Seed orchards are forest tree production populations for supplying the forest industry with consistent and abundant seed crops of superior genetic quality. However, genetic quality can be severely affected by non-random mating among parents and the occurrence of background pollination. This study analyzed mating structure and background pollination in six large isolation tents established in a clonal Scots pine seed orchard in northern Sweden. The isolation tents were intended to form a physical barrier against background pollen and induce earlier flowering relative to the surrounding trees. We scored flowering phenology inside and outside the tents and tracked airborne pollen density inside and outside the seed orchard in three consecutive pollination seasons. We genotyped 5683 offspring collected from the tents and open controls using nine microsatellite loci, and assigned paternity using simple exclusion method. We found that tent trees shed pollen and exhibited maximum female receptivity approximately 1 week earlier than trees in open control. The majority of matings in tents (78.3 %) occurred at distances within two trees apart (about 5 m). Self-fertilization was relatively high (average 21.8 %) in tents without supplemental pollination (SP), but it was substantially reduced in tents with SP (average 7.7 %). Pollen contamination was low in open controls (4.8–7.1 %), and all tents remained entirely free of foreign pollen. Our study demonstrates that tent isolation is effective in blocking pollen immigration and in manipulating flowering phenology. When complimented with supplemental pollination, it could become a useful seed orchard management practice to optimize the gain and diversity of seed orchard crops.

Keywords Genetic diversity · Isolation tent · Mating structure · Paternity assignment · Seed orchard

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Introduction

Seed orchards are production populations of forest trees, established with the primary objective to provide consistent and abundant yields of high genetic quality seed for reforestation purposes. The majority of conifer seed orchards worldwide is clonal, i.e., established using vegetatively propagated material collected from genetically superior trees. Depending on species, their population structure, and the pace of genetic improvement programs, they commonly consist of 20 to 50 different genotypes (parents), which are replicated across the plantation in several copies (ramets). Under an ideal scenario, mating among all ramets is random, self-fertilization is low, and gene flow from background natural stands into the seed orchard population does not occur. Meeting these underlying assumptions secures the genetic quality of the seed crop and delivers the actual progress of tree breeding programs into production forests. However, these assumptions are rarely

met in reality, as female and male gametic contributions are often greatly unbalanced, self-fertilization is common and the gene flow from unselected pollen sources, known as pollen contamination, may also be substantial. Consequently, the genetic quality of seed crops is often lower than theoretical expectation.

The genetic quality of seed orchard crops is evaluated by two major components: genetic gain (i.e., the shift between the mean phenotypic value in the offspring of the selected parents and that of the parental generation due to selection) and the genetic diversity encompassed in the crops. The two elements require actions in the opposite directions, and while the former is achieved through the progress of intensive tree breeding programs, the latter is regarded as a prerequisite for the populations' resilience and adaptability in the future.

Pollen immigration from unimproved background stands poses a major problem in seed orchard management. Background pollen may enrich the genetic diversity in the offspring population, but it introduces genes from unselected, likely low-breeding-value parents, which slows and limits the success of ongoing tree improvement programs. Moreover, when seed orchards are established in geographic regions other than those from where their selections originate (e.g., to enhance seed quality and production), background pollen may cause maladaptation of the offspring to target locations.

Great efforts have been invested into developing measures to avoid or minimize pollen contamination in seed orchards, such as the creation of pollen dilution zones (Sarvas 1970) or buffer stands (Squillace 1967), establishing larger orchards (Wright 1953), applying supplemental pollen (Wakeley et al. 1966), or delaying orchard trees' reproductive phenology by manipulating environmental conditions (Silen and Keane 1969). In Finland and Sweden, several pine and spruce orchards were transferred up to 6–8 latitudinal degrees southwards (650–900 km) in the 1970s in order to accelerate flowering and increase seed-cone production and seed quality. However, transferring seed orchards does not completely eliminate pollen contamination (El-Kassaby et al. 1989; Pulkkinen et al. 1994). Supplemental pollination can reduce pollen contamination to a detectable extent, but is primarily used to introduce new parents into a seed orchard population at a reasonable cost (Eriksson and Wilhelmsson 1991) and thus to increase the genetic diversity in the orchard's crops (El-Kassaby and Ritland 1986; Eriksson et al. 1994; Lai et al. 2010). Other attempts to reduce pollen contamination used physical isolation of ramets, such as: storing large containerized grafts in a plant cooler and bringing them back when no conspecific pollen was present in the open environment (Eriksson and Wilhelmsson 1991); moving grafts into a greenhouse (Hörnsten et al. 1997); or using umbrella cover as a mechanical hinder to pollen contamination (Lindgren 1994). While the former two were relatively expensive and facility-dependent, the latter did not prove effective due to deficient physical protection of whole ramets.

To explore the possibilities of improving the genetic quality of seed orchard crops, we established large isolation tents within a Scots pine (*Pinus sylvestris*) seed orchard. Similar tents, also known as high tunnels, are commonly utilized for the production of horticultural crops such as cucumbers, strawberries, or tomatoes with the objective to extend the production season and/or to protect the crops against severe weather (e.g., Lamont 2009; O'Connell et al. 2012). The primary function of tents in our study was to reduce pollen contamination by creating a physical barrier between the protected trees and the outside environment (Wennström et al. 2012) and simultaneously shifting the trees' reproductive phenology from that of unprotected trees. Our preliminary investigation from one pollination season showed that pollen contamination within the tents was completely eliminated (Torimaru et al. 2013) but whether this was a representative result required validation from multiple seasons.

In this study, we conducted a detailed investigation of the mating structure of ramets protected by these isolation tents over three consecutive pollination seasons. In addition to tent isolation, we also introduced forced air circulation and supplementary pollination in the tents as different treatments. We were specifically interested in determining (1) differences in the rate of pollen contamination and self-fertilization between the tents and unprotected controls, (2) the fine-scale mating structure in tents, and (3) the effect of different tent treatments on the genetic diversity of seed crops compared with those outside the tents. This study represents the first large-scale controlled pollination experiment in Scots pine and provides adequate data for understanding the mating dynamics of pine trees in isolation tents and in open environment. Knowledge of pollen dispersal and variance in reproductive success is essential for evaluating seed orchard functioning and the implementation of management practices to optimize the gain and diversity of seed orchard crops.

Materials and methods

Isolation tents and experimental population

Six tents measuring 30.8 × 7.0 × 5.5 m (Fig. 1) were constructed in spring 2010 in a first-generation clonal Scots pine seed orchard "Västerhus," located in northern Sweden (63° 18′ N, 18° 32′ E). The orchard was established in 1991 on an area of 13.7 ha using 28 replicated parents, following the algorithm by Lindgren and Matheson (1986) to increase relative representation of high-breeding-value parents. Ramets were

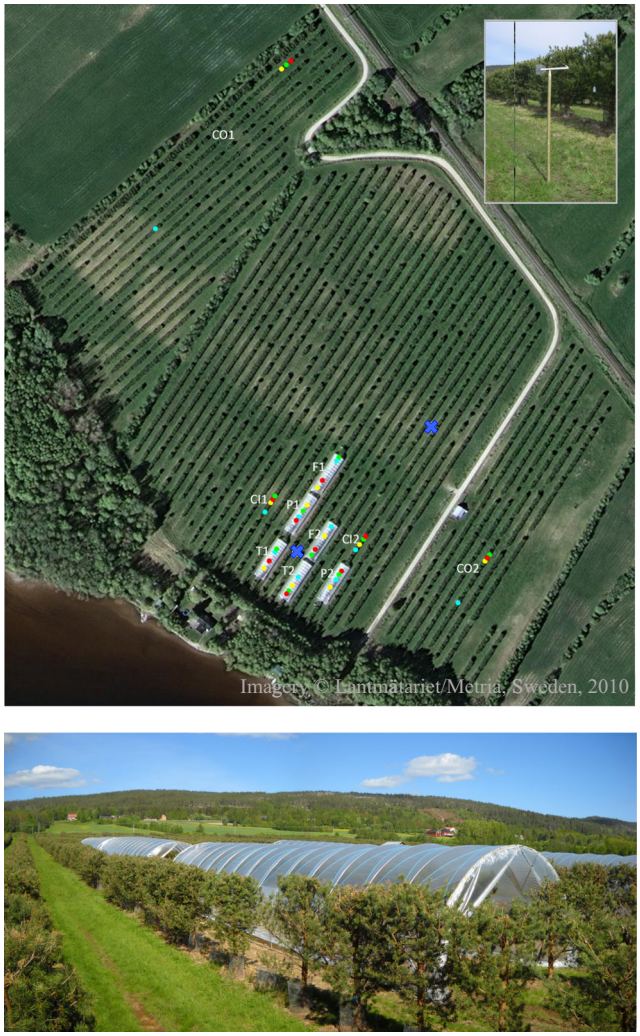

Fig. 1 The layout of seed orchard Västerhus with six isolation tents. Sampled mother trees are shown as *green* (Y3014), *red* (Z2081), *yellow* (AC3056), and *blue* (Y3012) *dots* and the two internal pollen traps (*top-right corner* for illustration) as *blue crosses*

planted in rows 7 m apart, with inter-tree distance within rows of 2.3 m. To avoid the presence of the same parent in adjacent rows, parents were split into three groups, each of which was planted in every third row in the whole orchard. For this experiment, one of the groups with a total of 10 parents was selected. At the last inventory in 2011, the orchard consisted of 3816 live ramets (136.3 ± 88.8 SD ramets per parent) and additional 67 trees that were visually identified as overgrowing rootstocks.

The tents were designed to protect a subset of the seed orchard's ramets (a total of 71 trees) from ambient pollen. The protection was assumed to take place in two ways: (1) through creating a physical barrier that would prevent ambient pollen produced by unselected, background trees from penetrating the protected environment within tents and (2) by inducing phenological separation between tent ramets and background pollen sources through altering microclimatic conditions within the tents and thus promoting mating among the protected ramets.

Each tent consisted of a supporting frame made of 40-mm-thick steel pipes and a polyethylene plastic foil that was spread all over the frame. The plastic foil could be unfolded at each end of the tent and lifted along the walls to enable ventilation during warm and sunny days. To protect strobili from possible pollen contamination, the plastic foil was kept on the frame until about 2–3 weeks after the cessation of female strobili receptivity.

Experimental design

The six tents were established close to one another within a small area near the southwestern edge of the seed orchard plantation (Fig. 1) to minimize spatial heterogeneity. Each tent covered 11–13 ramets and included at least one ramet of parents AC3056, Y3012, Y3014, and Z2081 (Fig. 1), which had been selected as mothers for seed sampling in this experiment. The initial plan was to establish all tents on patches with the same ramet composition and with a single ramet of each of the four parents; however, such arrangement was impossible to adhere to due to the original design of the seed orchard. As a result, the number of pollen parents present in the tents (ranging from 5 to 8) as well as the number of ramets of the sampled mother trees (ranging from 1 to 4) varied among tents.

Three different treatments were applied in the tents, each with two replicates. In T tents, ramets were protected by the tent, but no other action was conducted. In F tents, air circulation was promoted by a portable fan (Tanaka THB-2510N, 0.14 m^3/s), which blew pollen from the bottom of one ramet's crown onto the top of the ramet's two immediate neighbors' crowns at an angle of approximately 45°. This treatment was applied four to six times during the whole period of pollen shedding and each application lasted for approximately 10–15 min per tent, i.e., 1 min per tree. In P tents, we applied supplemental pollen from five pollen donors (AC1006, AC4221, Z3029, Z4003, and Z4022) that occurred in the seed orchard but not in the tents; the application was conducted three to five times during the female receptive period each season using a portable pollinator, constructed from a 2.5-m-long bamboo stem, a plastic tube, and a metal sprayer, to which a glass bottle with pollen was attached. Total weight of the applied pollen was 70.7, 106.1, and 75.7 g in 2010, 2011, and 2012, respectively, with equal proportions of the five donors in 2011 (21.2 g per pollen donor) and unequal in 2010 (14.1 g ± 4.33 SD) and 2012 (15.1 g ± 3.76 SD). In 2010 and 2011, each donor's pollen was applied separately in the order reflecting parental breeding values (AC1006 first, then Z4003, AC4221, Z3029, and Z4022) while in 2012, all pollen was applied as a mix. Each female strobili-bearing twig was pollinated at least two to three times per visit.

In addition to the tents, four open blocks in the orchard were used as controls, two in the tents' close vicinity (inner controls, CI) and two at the northern and southern margins of the seed orchard (outer controls, CO). One year after pollination (in autumn 2011, 2012, and 2013) the total cone production was harvested from the 40 sampled ramets and seeds were extracted separately by ramet and stored at −4 °C until germination. A sample of 36 seeds (2011 collection) and 60 seeds (2012 and 2013 collections) were randomly taken from each ramet for genotyping. The two outer controls were not sampled in 2012.

Seed germination

Seeds were soaked in 1 % H_2O_2 for 24 h and germinated in Petri dishes on moist filter paper at room temperate, with approximately 8 h of light and 16 h of dark. When seedlings reached ca. 3 cm in length, seed coats and megagametophytes were removed and the seedlings were stored in −80 °C until DNA isolation. To avoid bias in genetic composition of the analyzed seedlings, both fast and slowly growing seedlings were included.

DNA isolation and PCR amplification

Seedling tissue was disrupted with an oscillating mill (Retsch MM301) at 30 Hz. Total genomic DNA was isolated using E-Z 96 Plant DNA kit (OMEGA Bio-Tek, Norcross, GA) following the manufacturer's protocol. Genotypes were determined at nine nuclear SSR loci, eight developed for loblolly pine (PtTX2146, PtTX3025, PtTX3107, PtTX3116, PtTX4001 (Auckland et al. 2002), SsrPt_ctg1376, SsrPt_ctg4363 (Chagne et al. 2004), and LOP1 (Liewlaksaneeyanawin et al. 2004)) and one, SPAC12.5, developed for Scots pine (Soranzo et al. 1998). PCR amplification was performed in simplex reactions as described in Torimaru et al. (2009). DNA representing each of the 28 parents was isolated from frozen needles collected in the seed orchard in October 2007.

Genotyping

PCR products were mixed in two genotyping groups according to loci size and fluorescent color label (Group 1: PtTX2146, SsrPt_ctg1376, LOP1, SsrPt_ctg4363, PtTX4001; Group 2: PtTX3107, PtTx3025, SPAC12.5, PtTX3116) and were electrophoretically separated on a CEQ 8000 capillary sequencer (Beckman-Coulter, Brea, CA) along with 400-bp size standard. Allele identification and genotyping were performed using the CEQ 8000 Fragment Analysis software (Beckman-Coulter, Brea, CA). Offspring, whose genotypes did not match their respective mothers and/or any of the 28 candidate fathers on one or two loci were rescored or re-amplified on these loci to minimize the potential source of false exclusion, e.g., due to PCR amplification failure or the sizing standard being misread during electrophoresis.

Paternity analysis

Offspring were sampled from known mothers and assigned to candidate fathers using a modified, null-assuming simple paternity exclusion method of Moriguchi et al. (2004), described in Torimaru et al. (2009) using strict exclusion criteria. The combined exclusion probability of the paternal parent over all loci was calculated following Jamieson (1965) and a correction factor was applied to account for the probability of false exclusion of the true father due to presence of a null allele, as proposed by Dakin and Avise (2004)

$$Q_l = \sum_{i=1}^{n} p_i(1-p_i)^2 - \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\left(p_i p_j\right)^2\left[4-3\left(p_i+p_j\right)\right] - \sum_{i=1}^{k} p_i p_k (1-p_i),$$

where n is the number of visible alleles and p_i, p_j, and p_k are frequencies of visible alleles i and j and the null allele k, respectively, at locus l. A null allele is a mutation in binding site of a primer that causes poor or no amplification of the target microsatellite region (Chakraborty et al. 1992) and consequently results in an apparent excess of homozygotes (in the heterozygous state) or a missing product (in the homozygous state). Although null alleles at usual frequencies only slightly bias average exclusion probabilities (Dakin and Avise 2004), they were documented to introduce substantial errors into empirical assessments of particular mating events due to falsely excluded paternities.

Paternity was assigned when at least one father could not be excluded from the pool of candidates. When multiple fathers were determined, the father with a higher multilocus paternity index (Pena and Chakraborty 1994) was selected. When all candidates could be excluded, i.e., at least one mismatch was detected between an offspring and each of the 28 candidate fathers, the offspring was labeled as pollen contamination. In subsequent analyses, only offspring whose genotypes were recovered at all of the nine loci were included.

We defined two levels of pollen contamination in the tents: (1) pollen originating from seed orchard parents that were not present in a given tent (hereafter referred to as pollen leak) and (2) pollen from background sources occurring outside the seed orchard population that were not included in the pool of candidates (true pollen contamination). The distribution of paternal reproductive success within progenies of a single tree, within each tent and control plot, and across the three

studied years was determined using the sampling bias-corrected estimator of the effective number of fathers (Nielsen et al. 2003)

$$N_{e_f} = \frac{(n-1)^2}{\sum_{i=1}^{N_f} p_{f_i}^2 (n+1)(n-2) + 3 - n}$$

where n is the number of seeds sampled per category unit, N_f is the number of contributing fathers, and p_f is the relative reproductive success of ith father. This estimator has been commonly employed as an indirect quantification of the reduction in genetic diversity due to unbalanced mating success among contributing parents (e.g., Garcia et al. 2005; Jolivet et al. 2013).

Mating structure analysis

We used one-way ANOVA to test whether treatment, maternal parent and year had an effect on paternal reproductive success, mean number of alleles per locus (k), observed heterozygosity (H_o), effective number of fathers (N_{ef}), rate of pollen contamination (c), and self-fertilization (s) in the offspring. In P tents, we further evaluated the success rates of the pollen augmentation of the five pollen donors and the variation in receptivity of the four sampled maternal parents to the supplemental pollen. In all tents, we employed linear regression to determine the relationship between parental representation and pollen fecundity, pooled over all ramets of a given parent and male reproductive success. To test whether the presence of tents had an effect on the distribution of male reproductive success in nearby mother trees, we compared the reproductive success of fathers occurring in tents between inner and outer controls.

F tests at alpha of 0.05 (and, where applicable, pairwise t tests at alpha level corrected following Šidák's correction as $1-(1-\alpha)^{1/m}$ where m is the number of independent hypotheses tested) were conducted using PROC GLM and PROG REG in SAS 9.1.4 (SAS Institute Inc., Cary, NC). Interactions between factors were only presented if significant. Variables were either arcsine square root or log transformed to meet the underlying assumptions of the statistical analyses.

Next, we quantified the effect of inter-tree distance on pollination success. In this analysis, we only considered offspring produced by fathers occurring in a single copy in a given tent, as offspring of fathers with multiple copies could not be broken down into individual ramets of origin due to confounding effects. Furthermore, since each tent provided a different number of crosses representing a particular distance, we weighed each distance's sample size by the number of available crosses for that distance. For instance, tents F1 and F2 provided one and six usable crosses at the distance of two trees apart, respectively; therefore, the number of offspring produced in F2 was reduced sixfold to obtain the same reference level. The distance of zero, corresponding to self-fertilization of the sampled mother trees that existed in a given tent in just a single copy, was also included in the analysis.

Pollen production

Male fecundity was inferred from the count and mean length (cm) of pollen strobili (SC and SL, respectively) on all ramets included in the experiment, except for those in the two outer controls (in total 94 assessed ramets). The assessment was conducted on one half of each ramet's crown; in some cases, both sides were scored and the two records were averaged. Pollen production (g) per ramet was estimated as $2 \times SC \times SL \times 0.028$, where the former and latter coefficients represent an adjustment for strobili production on a whole crown of a ramet and the average yield of pollen produced by 1 cm of a pine strobilus, respectively (Koski 1975). We installed four pollen traps, two within and two outside the seed orchard, to track the abundance of ambient pollen cloud. Of the two traps inside the orchard, one was situated in between the tents and the other ca. 50 m from the tents (Fig. 1). The two outside traps were placed 400 m from the orchard's NW and SE edges in the respective directions. Pollen was collected on slides with a double-sided tape and assessed on daily basis. Test surface corresponded to 31.25 mm^2 in 2010 and 2011 and 50 mm^2 in 2012 (five and eight squares of 2.5 × 2.5 mm each, respectively). Male and female reproductive phenology was monitored on two ramets of the four sampled parents (AC3056, Y3012, Y3014, and Z2081), one of which was inside and one outside the tents.

Results

Paternity assignment

The analyzed sample size consisted of 28 candidate parents and 5683 offspring that were genotyped over all nine simple sequence repeat (SSR) loci (1413, 1895 and 2375 in pollination seasons 2010, 2011, and 2012, respectively). Paternity was assigned to 5590 offspring, of which 5571 (99.7 %) were assigned to a single father in the orchard while the remaining 19 were assigned to two fathers. Among the 5590 assigned paternities, 5147 exceeded the paternity index (W) of 0.95 (mean 0.994 ± 0.010 SD). The remaining 443 paternities had an average W of 0.861 (median = 0.904). For the 19 offspring with two unexcluded fathers, the difference between W for the first and second most likely father was on average 0.291 ± 0.169 SD and was statistically significant ($F_{1,35} = 21.0$; $p < 0.0001$). Multilocus exclusion probabilities for father were between 0.9981 and 0.9995 in the six tents and 0.9994 in open controls.

Mating distribution in tents

We analyzed 3692 offspring in the six tents (870, 1415, and 1407 from pollination seasons 2010, 2011, and 2012, respectively; Tables 1, 2, and 3). All but one offspring perfectly matched one of the seed orchard's 28 candidate fathers. The only mismatching offspring occurred in tent P2 in 2012 at locus SPAC12.5, which differed from the genetically closest father Z2081 by an additional repeat of 2 bp. Thus, this mismatch could be either due to true pollen contamination or a mutation. Five offspring were sired by fathers that were present in the seed orchard but did not occur within a given tent (pollen leak), of which four were detected in 2010 and one in 2011. Two of the leaks in 2010 originated from father Y4507, which ranked #1 in both pollen fecundity and reproductive success that year.

Paternal reproductive success was highly variable within tents (Fig. 2a). This variation was mainly attributed to fathers ($F_{9, 119} = 13.40, p < 0.0001$); their relative representation in an environment ($F_{1, 127} = 107.68$, $p < 0.0001$); pollen fecundity ($F_{1, 127} = 70.98$, $p < 0.0001$); and distance to the sampled mother trees ($F_{2, 114} = 163.67, p < 0.0001$), which individually explained 50.3, 45.9, 35.9, and 74.2 % of the total variation, respectively. Treatment and year were not significant ($F_{2, 126} = 0.17$, $p = 0.843$ and $F_{2, 126} = 0.39$, $p = 0.675$, respectively). After excluding the pollen leak, the mismatching offspring and mating success of the supplemental pollen, Pearson's product-moment correlations between the reproductive success and parental representation in T and F tents ranged from 0.28 to 0.96, with 75 % of the correlations being significant ($\alpha = 0.05$), while in the P tents, the correlations ranged from 0.27 to 0.90, with all but one being non-significant (Table 4). Correlations between the reproductive success and parental pollen fecundity (range 0.17–0.82 in T and F tents and 0.58–0.91 in P tents) showed no consistent pattern across years and treatments (Table 4). In contrast, these correlations ranging from 0.62 to 0.82 were significant in the open controls in all 3 years.

In all treatments, the vast majority of detectable mating events occurred at short distances; for example, the cumulative mating success reached 51.5 to 95.4 % in T, 78.0 to 91.1 % in F, and 64.5 to 96.7 % in P tents (Fig. 3) for the distances between zero (i.e., self-fertilization of the sampled mother trees) and two trees apart. Mating at larger distances was generally rare and only ca 5 % of all events occurred between ramets more than six trees apart (5.4, 3.4, and 6.6 % in the three treatments, respectively, pooled over the 3 years) (Fig. 3).

Supplemental pollen from the five donor fathers fertilized 61.6 % of all analyzed seeds (771 of 1251) in P1 and P2 tents, with 59.8, 48.2, and 76.3 % in pollination seasons 2010, 2011, and 2012, respectively (Tables 1, 2, and 3). All five pollen donors participated in mating and their reproductive success reached on average 11.2 ± 3.9 (SD), 10.2 ± 3.5, and 15.4 ± 4.8 % in tent P1 and 12.6 ± 4.0 (SD), 9.2 ± 4.0, and 15.1 ± 8.0 % in tent P2 in the 3 years; the minimum and maximum values were 3.3 % (AC1006; P2 in 2012) and 22.2 % (AC4221; P2 in 2012), respectively (Fig. 2a). The success of the pollination treatment was independent of year of application ($F_{2, 27} = 2.83$, $p = 0.077$) and the amount of pollen applied ($F_{1, 28} = 1.16, p = 0.291$), but the effect of pollen donor was significant ($F_{4, 25} = 3.04$, $p = 0.036$; Fig. 4a). At individual donor level, only father AC1006 was reproductively less successful than Z4022 ($|t| = 3.17$, $p = 0.004 < \alpha \approx 0.005$); other pairwise comparisons were non-significant. Sampled mother tree was also a significant factor affecting pollen donors' reproductive success ($F_{3, 20} = 5.76$, $p = 0.005$; Fig. 4b), indicating that there was variation among the four mothers in receptivity of the supplemental pollen. For instance, mother Z2081 was less receptive of supplemental pollen than Y3012 and Y3014 ($|t| = 3.24$ and 3.81, $p = 0.004$ and $0.001 < \alpha \approx 0.009$) and its offspring had the lowest proportion of supplemental pollen as a source of fertilization in all years in tent P1 and, with the exception of year 2010, also in tent P2 (average 40.2 %, range 22.2–60.0 %) whereas mother Y3014 had on average 76.0 % of offspring sired by supplemental pollen with a range from 50.0 to 94.4 %.

Mating distribution in open controls

We analyzed 543, 480, and 968 offspring in pollination seasons 2010, 2011, and 2012 in the four control blocks (Tables 1, 2, and 3). The assigned portion to orchard fathers reached 95.6, 93.8, and 96.1 %, respectively, resulting in average pollen immigration from unselected background trees of 4.7 %. Following correction for cryptic gene flow, the probability of which was estimated to be 0.0089, the annual pollen contamination reached 5.3, 7.1, and 4.8 % in the 3 years.

Father was a significant factor in determining paternal reproductive success, explaining 69.7 % of variation in the data ($F_{27, 252} = 21.48$, $p < 0.0001$; Fig. 2b). Pairwise comparisons of individual fathers' success showed that 146 of 378 were significant ($|t| > 3.86, p < \alpha \approx 0.0001$). In all 3 years, the most reproductively successful fathers were Y4507, AC3056, Z3007, Y3012, and AC2064, which collectively fertilized 48.4, 46.5, and 52.4 % of the analyzed seeds in each year. The Spearman rank correlations showed that this pattern was consistent for the remaining fathers too, as the correlations were high and significant for all three combinations of years ($r_{2010–2011} = 0.78$, $r_{2010–2012} = 0.84$, and $r_{2011–2012} = 0.85$; $n = 28$); ANOVA confirmed that year was not a significant factor for determining reproductive success among fathers ($F_{2, 277} = 0.01$, $p = 0.989$). The location of the sampled mother trees in CI or CO blocks was non-significant for mating composition ($F_{1, 98} = 0.45$, $p = 0.502$), which indicates that tents did not upward-bias the mating success in nearby trees.

Table 1 Results summary for Västerhus seed orchard's seed collections from pollination season 2010

Unit	Mother N_{fa} (N_t)	Offspring analyzed	Offspring assigned	N_{fc}	N_{ef}	k	k_e	H_o	H_e	PC count (rate)	Selfing count (rate)	SP count (rate)
T1	AC3056	36	36	6	5.05	4.78	2.65	0.620	0.595	0	9 (0.250)	
	Y3012	34	34	6	4.57	5.33	3.43	0.771	0.688	0	0 (0.000)	
	Y3014	34	34	4	2.74	5.00	3.11	0.712	0.651	0	0 (0.000)	
	Z2081	36	36	7	4.78	5.33	2.79	0.648	0.591	0	6 (0.167)	
	8 (11)	*140*	*140*	*8*	*6.45*	*5.67*	*3.80*	*0.687*	*0.697*	*0*	*15 (0.107)*	
T2	AC3056	47	47	5	2.93	4.67	2.35	0.574	0.552	0	25 (0.532)	
	Y3012	2	2	2	1.00	2.22	3.55	0.722	0.685	0	0 (0.000)	
	Y3014	36	36	3	1.82	3.67	2.76	0.657	0.594	0	5 (0.139)	
	Z2081	46	46	6	3.57	5.33	2.42	0.546	0.532	0	17 (0.370)	
	6 (13)	*131*	*131*	*6*	*4.09*	*5.44*	*3.26*	*0.590*	*0.635*	*0*	*47 (0.359)*	
F1	AC3056	36	36	6	5.13	4.89	2.81	0.660	0.628	0	7 (0.194)	
	Y3012	34	34	5	3.31	4.11	3.00	0.712	0.631	0	3 (0.088)	
	Y3014	36	36	8	5.39	5.22	3.05	0.673	0.653	0	9 (0.250)	
	Z2081	37	37	5	3.44	4.11	2.38	0.631	0.551	0	12 (0.324)	
	8 (12)	*143*	*143*	*9*	*5.55*	*5.33*	*3.60*	*0.668*	*0.693*	*0*	*31 (0.217)*	
F2	AC3056	40	40	5	2.95	4.56	2.32	0.608	0.550	0	22 (0.550)	
	Y3012	34	34	5	2.38	4.56	2.96	0.690	0.627	0	2 (0.059)	
	Y3014	38	38	5	3.50	4.11	2.89	0.649	0.614	0	14 (0.368)	
	Z2081	38	38	5	3.50	4.11	2.45	0.623	0.542	0	13 (0.342)	
	8 (12)	*150*	*150*	*8*	*3.65*	*5.56*	*3.54*	*0.641*	*0.678*	*0*	*51 (0.340)*	
T + F	*10 (48)*	*564*	*564*	*11*	*5.66*	*6.67*	*3.62*	*0.647*	*0.683*	*0*	*144 (0.255)*	
P1	AC3056	33	33	9	7.56	5.89	2.89	0.650	0.618	0	5 (0.152)	21 (0.636)
	Y3012	36	36	8	7.01	6.33	3.32	0.731	0.658	0	1 (0.028)	25 (0.694)
	Y3014	41	41	9	7.89	6.44	3.30	0.710	0.673	0	3 (0.073)	28 (0.683)
	Z2081	36	36	11	5.58	6.11	2.91	0.670	0.605	0	5 (0.139)	8 (0.222)
	5 + 5 (12)	*146*	*146*	*12*	*9.02*	*7.33*	*4.17*	*0.692*	*0.707*	*0*	*14 (0.096)*	*82 (0.562)*
P2	AC3056	35	35	13	11.92	6.00	2.87	0.651	0.622	0	5 (0.143)	12 (0.343)
	Y3012	41	41	11	9.33	6.78	3.46	0.743	0.687	0	1 (0.024)	30 (0.732)
	Y3014	36	36	7	4.71	5.67	3.18	0.698	0.643	0	1 (0.028)	34 (0.944)
	Z2081	48	48	9	7.15	6.22	2.98	0.648	0.591	0	5 (0.104)	25 (0.521)
	8 + 5 (11)	*160*	*160*	*14*	*9.65*	*7.78*	*4.36*	*0.684*	*0.707*	*0*	*12 (0.075)*	*101 (0.631)*
P	*10 + 5 (23)*	*306*	*306*	*16*	*9.75*	*8.11*	*4.27*	*0.687*	*0.708*	*0*	*26 (0.085)*	*183 (0.598)*
T + P + F	*10 + 5 (71)*	*870*	*870*	*17*	*8.44*	*8.44*	*3.89*	*0.661*	*0.695*	*0*	*170 (0.195)*	
CI1	AC3056	36	34	13	11.96	6.78	3.26	0.775	0.671	2 (0.056)	0 (0.000)	
	Y3012	35	34	19	19.38	6.89	3.32	0.740	0.668	1 (0.029)	1 (0.029)	
	Y3014	36	35	17	11.92	7.89	3.63	0.704	0.689	1 (0.028)	0 (0.000)	
	Z2081	35	33	16	11.25	7.11	3.21	0.733	0.634	2 (0.057)	1 (0.029)	
	28 (3745)*	*142*	*136*	*25*	*16.14*	*8.89*	*4.48*	*0.738*	*0.728*	*6 (0.042)*	*2 (0.014)*	
CI2	AC3056	38	36	11	7.51	5.67	2.84	0.687	0.635	2 (0.053)	1 (0.026)	
	Y3012	37	36	11	6.44	5.44	3.36	0.778	0.673	1 (0.027)	0 (0.000)	
	Y3014	36	35	14	4.03	6.67	3.37	0.732	0.675	1 (0.028)	0 (0.000)	
	Z2081	35	33	17	16.03	7.00	3.25	0.746	0.641	2 (0.057)	1 (0.029)	
	28 (3745)*	*146*	*140*	*26*	*10.41*	*8.56*	*4.24*	*0.735*	*0.718*	*6 (0.041)*	*2 (0.014)*	
CO1	AC3056	36	33	12	9.80	6.22	2.95	0.694	0.633	3 (0.083)	3 (0.083)	
	Y3012	9	9	7	12.23	5.11	3.61	0.728	0.687	0 (0.000)	0 (0.000)	
	Y3014	36	34	13	7.30	6.33	3.42	0.694	0.676	2 (0.056)	0 (0.000)	
	Z2081	48	46	16	11.02	7.00	2.71	0.632	0.592	2 (0.042)	9 (0.188)	
	28 (3745)*	*129*	*122*	*23*	*12.97*	*8.67*	*3.91*	*0.674*	*0.690*	*7 (0.054)*	*12 (0.093)*	
CO2	AC3056	36	35	16	15.28	6.56	2.90	0.676	0.631	1 (0.028)	2 (0.056)	
	Y3012	38	36	15	14.34	6.56	3.51	0.722	0.690	2 (0.053)	0 (0.000)	
	Y3014	15	14	10	15.31	6.11	3.91	0.763	0.707	1 (0.067)	0 (0.000)	
	Z2081	37	36	15	18.03	6.89	2.85	0.694	0.606	1 (0.027)	4 (0.108)	
	28 (3745)*	*126*	*121*	*21*	*15.92*	*8.89*	*4.23*	*0.705*	*0.709*	*5 (0.040)*	*6 (0.048)*	
CI + CO	*28 (3745*)*	*543*	*519*	*28*	*14.90*	*9.78*	*4.25*	*0.714*	*0.714*	*24 (0.044)*	*22 (0.041)*	

Unit(s) summaries are presented in Italics

N_{fa} number of available fathers, N_t number of trees, N_{fc} number of fathers contributing to the offspring, N_{ef} effective number of fathers, k and k_e number and effective number of alleles, H_o and H_e observed and expected heterozygosity, *PC* pollen contamination, *SP* supplemental pollination

*All seed orchard ramets outside tents

Table 2 Results summary for Västerhus seed orchard's seed collections from pollination season 2011

Unit	Mother N_{fa} (N_t)	Offspring analyzed	Offspring assigned	N_{fc}	N_{ef}	k	k_e	H_o	H_e	PC count (rate)	Selfing count (rate)	SP count (rate)
T1	AC3056	60	60	6	4.16	4.56	2.62	0.594	0.599	0	11 (0.183)	
	Y3012	70	70	8	5.17	5.33	3.18	0.705	0.657	0	4 (0.057)	
	Y3014	43	43	7	4.11	5.44	3.43	0.703	0.673	0	1 (0.023)	
	Z2081	60	60	7	5.10	5.33	3.03	0.717	0.627	0	2 (0.033)	
	8 (11)	*233*	*233*	*8*	*6.39*	*5.78*	*3.80*	*0.679*	*0.695*	*0*	*18 (0.077)*	
T2	AC3056	61	61	6	3.74	5.44	2.66	0.617	0.602	0	23 (0.377)	
	Y3012	59	59	5	2.80	4.89	3.07	0.676	0.647	0	3 (0.051)	
	Y3014	60	60	6	3.35	5.33	2.61	0.570	0.589	0	28 (0.467)	
	Z2081	60	60	5	2.08	4.44	2.51	0.644	0.553	0	15 (0.250)	
	6 (13)	*240*	*240*	*6*	*3.56*	*5.67*	*3.60*	*0.627*	*0.681*	*0*	*69 (0.288)*	
F1	AC3056	60	60	5	2.75	4.56	2.66	0.635	0.606	0	11 (0.183)	
	Y3012	60	60	5	1.72	4.33	2.82	0.672	0.620	0	7 (0.117)	
	Y3014	60	60	4	2.87	4.11	2.81	0.620	0.611	0	19 (0.317)	
	Z2081	55	55	6	2.91	4.67	2.61	0.640	0.567	0	7 (0.127)	
	8 (12)	*235*	*235*	*8*	*3.44*	*5.56*	*3.46*	*0.642*	*0.675*	*0*	*44 (0.187)*	
F2	AC3056	62	62	6	3.19	5.00	2.81	0.695	0.623	0	3 (0.048)	
	Y3012	54	54	4	2.86	3.89	2.85	0.617	0.617	0	9 (0.167)	
	Y3014	56	56	5	1.82	4.22	2.98	0.710	0.621	0	6 (0.107)	
	Z2081	62	62	6	4.32	5.00	2.60	0.643	0.573	0	11 (0.177)	
	8 (12)	*234*	*234*	*9*	*4.81*	*5.78*	*3.61*	*0.667*	*0.683*	*0*	*29 (0.124)*	
T + F	*10 (48)*	*942*	*942*	*10*	*5.41*	*6.78*	*3.67*	*0.654*	*0.687*	*0*	*160 (0.170)*	
P1	AC3056	52	52	9	7.81	6.56	3.10	0.703	0.655	0	5 (0.096)	19 (0.365)
	Y3012	60	60	9	6.84	6.67	3.21	0.691	0.661	0	5 (0.083)	42 (0.700)
	Y3014	60	60	8	6.79	6.33	3.36	0.694	0.674	0	3 (0.050)	43 (0.717)
	Z2081	60	60	9	4.77	6.44	2.99	0.706	0.618	0	8 (0.133)	14 (0.233)
	5 + 5 (12)	*232*	*232*	*10*	*7.79*	*7.11*	*4.21*	*0.698*	*0.715*	*0*	*21 (0.091)*	*118 (0.509)*
P2	AC3056	60	60	12	7.91	6.89	3.08	0.698	0.651	0	5 (0.083)	32 (0.533)
	Y3012	60	60	9	5.60	6.56	3.30	0.698	0.679	0	3 (0.050)	32 (0.533)
	Y3014	60	60	9	5.21	6.33	3.12	0.657	0.660	0	5 (0.083)	30 (0.500)
	Z2081	61	61	9	5.85	6.33	2.72	0.619	0.576	0	13 (0.213)	16 (0.262)
	8 + 5 (11)	*241*	*241*	*13*	*8.67*	*7.67*	*4.08*	*0.668*	*0.708*	*0*	*26 (0.108)*	*110 (0.456)*
P	*10 + 5 (23)*	*473*	*473*	*14*	*8.52*	*7.78*	*4.15*	*0.683*	*0.712*	*0*	*47 (0.099)*	*228 (0.482)*
T + F + P	*10 + 5 (71)*	*1415*	*1415*	*15*	*7.73*	*8.11*	*3.91*	*0.663*	*0.699*	*0*	*207 (0.146)*	
CI1	AC3056	60	53	20	15.15	8.22	2.93	0.661	0.639	7 (0.117)	8 (0.133)	
	Y3012	59	53	18	11.99	7.67	3.50	0.721	0.695	6 (0.102)	3 (0.051)	
	Y3014	60	55	16	6.85	7.89	3.32	0.695	0.662	5 (0.083)	4 (0.067)	
	Z2081	60	59	17	8.35	7.33	2.96	0.702	0.613	1 (0.017)	4 (0.067)	
	28 (3745-)*	*239*	*220*	*25*	*10.86*	*9.89*	*4.22*	*0.695*	*0.717*	*19 (0.079)*	*19 (0.079)*	
CI2	AC3056	61	58	18	13.02	7.22	2.92	0.656	0.635	3 (0.049)	11 (0.180)	
	Y3012	60	57	21	15.21	8.22	3.42	0.698	0.686	3 (0.050)	3 (0.050)	
	Y3014	60	58	18	9.73	7.22	3.25	0.696	0.657	2 (0.033)	2 (0.033)	
	Z2081	60	57	18	14.93	8.00	3.14	0.717	0.626	3 (0.050)	1 (0.017)	
	28 (3745-)*	*241*	*230*	*24*	*15.62*	*9.67*	*4.23*	*0.691*	*0.709*	*11 (0.046)*	*17 (0.071)*	
CI	*28 (3745-*)*	*480*	*450*	*28*	*13.75*	*10.22*	*4.22*	*0.693*	*0.714*	*30 (0.063)*	*36 (0.075)*	

Unit(s) summaries are presented in Italics

N_{fa} number of available fathers, N_t number of trees, N_{fc} number of fathers contributing to the offspring, N_{ef} effective number of fathers, k and k_e number and effective number of alleles, H_o and H_e observed and expected heterozygosity, *PC* pollen contamination, *SP* supplemental pollination

*All seed orchard ramets outside tents

Table 3 Results summary for Västerhus seed orchard's seed collections from pollination season 2012

Unit	Mother N_{fa} (N_t)	Offspring analyzed	Offspring assigned	N_{fc}	N_{ef}	k	k_e	H_o	H_e	PC count (rate)	Selfing count (rate)	SP count (rate)
T1	AC3056	60	60	4	2.69	4.11	2.34	0.589	0.557	0	32 (0.533)	
	Y3012	60	60	3	2.96	3.67	2.68	0.607	0.609	0	22 (0.367)	
	Y3014	60	60	3	2.60	3.78	2.86	0.682	0.629	0	9 (0.150)	
	Z2081	60	60	5	2.55	4.33	2.63	0.646	0.582	0	11 (0.183)	
	8 (11)	*240*	*240*	*6*	*4.02*	*5.00*	*3.58*	*0.631*	*0.680*	*0*	74 (0.308)	
T2	AC3056	60	60	5	2.49	4.78	2.87	0.667	0.613	0	10 (0.167)	
	Y3012	41	41	5	3.26	4.67	2.88	0.610	0.626	0	9 (0.220)	
	Y3014	56	56	4	2.80	3.89	2.84	0.661	0.601	0	10 (0.179)	
	Z2081	59	59	2	2.00	2.78	2.33	0.574	0.521	0	26 (0.441)	
	6 (13)	*216*	*216*	*6*	*3.32*	*5.44*	*3.55*	*0.629*	*0.669*	*0*	55 (0.255)	
F1	AC3056	60	60	6	3.27	4.78	2.68	0.622	0.609	0	9 (0.150)	
	Y3012	60	60	5	2.68	4.11	3.02	0.670	0.643	0	4 (0.067)	
	Y3014	60	60	5	3.31	4.33	2.77	0.620	0.620	0	24 (0.400)	
	Z2081	60	60	6	3.39	5.00	2.65	0.680	0.578	0	8 (0.133)	
	8 (12)	*240*	*240*	*8*	*4.68*	*6.11*	*3.60*	*0.648*	*0.687*	*0*	45 (0.188)	
F2	AC3056	60	60	8	5.71	5.67	2.80	0.643	0.620	0	7 (0.117)	
	Y3012	59	59	3	2.82	3.56	2.71	0.616	0.605	0	20 (0.339)	
	Y3014	60	60	4	1.32	4.22	2.92	0.691	0.604	0	5 (0.083)	
	Z2081	60	60	5	3.97	4.56	2.34	0.578	0.522	0	22 (0.367)	
	8 (12)	*239*	*239*	*8*	*4.06*	*5.78*	*3.52*	*0.632*	*0.678*	*0*	54 (0.226)	
T + F	*10 (48)*	*935*	*935*	*10*	*5.28*	*6.78*	*3.68*	*0.635*	*0.685*	*0*	228 (0.244)	
P1	AC3056	60	60	9	8.16	6.67	3.14	0.717	0.659	0	2 (0.033)	42 (0.700)
	Y3012	57	57	8	5.89	6.11	3.27	0.694	0.669	0	3 (0.053)	51 (0.895)
	Y3014	60	60	8	5.50	6.11	3.39	0.700	0.667	0	2 (0.033)	56 (0.933)
	Z2081	56	56	8	6.39	6.56	3.28	0.710	0.634	0	3 (0.054)	30 (0.536)
	5 + 5 (12)	*233*	*233*	*9*	*6.93*	*6.89*	*4.42*	*0.705*	*0.718*	*0*	*10 (0.043)*	*179 (0.768)*
P2	AC3056	58	58	9	5.76	6.67	3.31	0.762	0.662	0	0 (0.000)	51 (0.879)
	Y3012	61	61	7	5.76	6.00	3.37	0.725	0.687	0	0 (0.000)	44 (0.721)
	Y3014	60	60	8	5.77	6.22	3.22	0.659	0.643	0	7 (0.117)	50 (0.833)
	Z2081	60	59	8	7.13	6.33	3.01	0.681	0.602	1 (0.017)	6 (0.100)	36 (0.600)
	8 + 5 (11)	*239*	*238*	*12*	*6.59*	*7.22*	*4.40*	*0.707*	*0.717*	*1 (0.004)*	*13 (0.054)*	*181 (0.757)*
P	*10 + 5 (23)*	*472*	*471*	*12*	*6.95*	*7.22*	*4.40*	*0.706*	*0.718*	*1 (0.002)*	*23 (0.049)*	*360 (0.763)*
T + P + F	*10 + 5 (71)*	*1407*	*1406*	*15*	*8.28*	*8.11*	*3.97*	*0.659*	*0.701*	*1 (0.001)*	*251 (0.178)*	
CI1	AC3056	60	57	18	10.24	7.11	2.95	0.720	0.643	3 (0.050)	2 (0.033)	
	Y3012	57	54	14	6.99	7.67	3.33	0.704	0.661	3 (0.053)	0 (0.000)	
	Y3014	64	59	18	11.41	8.67	3.40	0.705	0.666	5 (0.078)	0 (0.000)	
	Z2081	60	57	21	11.01	8.11	3.18	0.721	0.627	3 (0.050)	0 (0.000)	
	28 (3745)*	*241*	*227*	*26*	*11.12*	*10.44*	*4.27*	*0.712*	*0.711*	*14 (0.058)*	*2 (0.008)*	
CI2	AC3056	60	58	19	10.67	7.78	2.95	0.707	0.639	2 (0.033)	5 (0.083)	
	Y3012	60	58	19	12.07	7.33	3.42	0.693	0.688	2 (0.033)	2 (0.033)	
	Y3014	63	62	20	13.61	8.11	3.40	0.713	0.672	1 (0.016)	1 (0.016)	
	Z2081	60	58	18	11.49	8.00	3.09	0.678	0.626	2 (0.033)	1 (0.017)	
	28 (3745)*	*243*	*236*	*25*	*12.63*	*9.56*	*4.15*	*0.698*	*0.713*	*7 (0.029)*	*9 (0.037)*	
CO1	AC3056	61	60	15	9.73	7.00	2.83	0.641	0.629	1 (0.016)	11 (0.180)	
	Y3012	60	59	16	7.71	7.44	3.26	0.719	0.671	1 (0.017)	6 (0.100)	
	Y3014	60	58	14	8.48	7.00	3.39	0.717	0.671	2 (0.033)	1 (0.017)	
	Z2081	60	55	15	8.40	7.78	3.11	0.704	0.629	5 (0.083)	0 (0.000)	
	28 (3745)*	*241*	*232*	*24*	*9.31*	*9.67*	*4.09*	*0.695*	*0.712*	*9 (0.037)*	*18 (0.075)*	
CO2	AC3056	63	62	18	11.20	7.33	2.80	0.637	0.621	1 (0.016)	14 (0.222)	
	Y3012	60	60	18	16.10	7.11	3.24	0.685	0.666	0 (0.000)	4 (0.067)	
	Y3014	60	54	16	14.46	8.22	3.30	0.691	0.670	6 (0.100)	5 (0.083)	
	Z2081	60	59	15	10.38	7.11	2.78	0.650	0.594	1 (0.017)	13 (0.217)	
	28 (3745)*	*243*	*235*	*23*	*14.90*	*9.00*	*4.18*	*0.665*	*0.708*	*8 (0.033)*	*36 (0.148)*	
CI + CO	*28 (3745*)*	*968*	*930*	*28*	*12.81*	*11.33*	*4.18*	*0.692*	*0.711*	*38 (0.039)*	*65 (0.067)*	

Unit(s) summaries are presented in Italics

N_{fa} number of available fathers, N_t number of trees, N_{fc} number of fathers contributing to the offspring, N_{ef} effective number of fathers, k and k_e number and effective number of alleles, H_o and H_e observed and expected heterozygosity, *PC* pollen contamination, *SP* supplemental pollination

*All seed orchard ramets outside tents

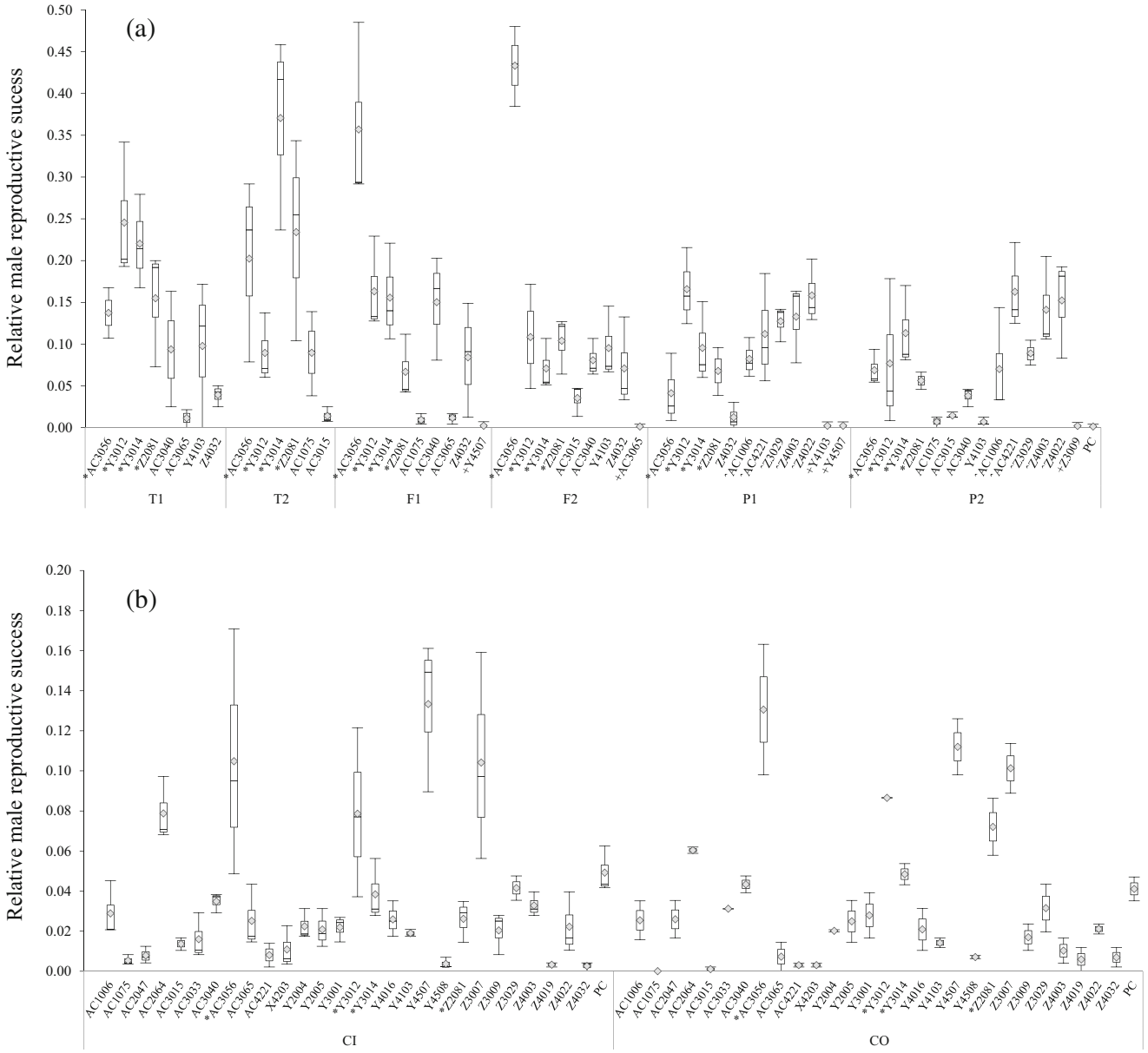

Fig. 2 Relative male reproductive success in seed orchard Västerhus in pollination seasons 2010, 2011, and 2012 in isolation tents (**a**) and open controls (**b**). *Asterisks*, *carets*, and *plus signs* denote the four sampled mother trees, five pollen donors, and parents not occurring in the tents, respectively. *PC* pollen contamination. Outer controls (CO) were not sampled in 2011

Reproductive phenology and pollen traps

Both male and female reproductive phenology was accelerated within tents. Tent trees started to shed pollen approximately 1 week earlier compared with open seed orchard trees in all 3 years, with the shift being most pronounced in 2012 (11 days). There was only a little overlap in pollen shedding between the tents and seed orchard in 2010 and 2011 and no overlap in 2012, when the seed orchard started shedding 5 days after shedding's cessation in the tents (Fig. 5). Female cone receptivity followed a similar pattern with the peak receptivity inside tents clearly distinct from the open orchard in all 3 years. The most marked shift of 9 days occurred in 2011, which completely separated the inside female receptivity from outside. In all three pollination seasons, there was very little overlap between female cone receptivity in tents and pollen shedding of the seed orchard (Fig. 5).

Pollen traps captured substantially larger amounts of pollen within the seed orchard than at 400 m outside. During peak periods in 2010 (June 3–9), 2011 (June 2–11), and 2012 (June 9–18), internal traps collectively caught 126.0, 263.0, and 178.7 pollen grains per square millimeter per day whereas the external two only 11.5, 42.5, and 32.7, respectively (Fig. 5). Two small pollen peaks occurred on external traps

Table 4 Pearson's product-moment correlations between paternal reproductive success and (1) relative parental representation and (2) pollen fecundity

		Relative parental representation			Parental pollen fecundity/pollen applied		
Tent	N_{fa}	2010	2011	2012	2010	2011	2012
T1	8	0.83*	0.28 ns	0.86*	0.78*	0.50 ns	0.62 ns
T2	6	0.96*	0.74 ns	0.62 ns	0.82*	0.31 ns	0.17 ns
F1	8	0.76*	0.80*	0.76*	0.75*	0.63 ns	0.79*
F2	8	0.83*	0.88*	0.96*	0.73*	0.79*	0.71*
P1[a]	5	0.90*	0.28 ns	0.57 ns	0.87 ns	0.78 ns	0.91*
P2[a]	8	0.55ns	0.47 ns	0.27 ns	0.58 ns	0.60 ns	0.79*
P1[b]	5				-0.73 ns	n/a	0.10 ns
P2[b]	5				-0.62 ns	n/a	0.24 ns
CI + CO[c]	28	0.72*	0.62*	0.64*	0.82*, d	0.70*, d	0.76*, d

N_{fa} number of available fathers

[a] Tent trees only

[b] Pollen from supplemental pollination donors only

[c] Pooled over the two replicates

[d] Pollen fecundity estimated for the whole seed orchard population

ns Non-significant at $\alpha = 0.05$

* Significant at $\alpha = 0.05$ (N_{fa} minus 2 degrees of freedom)

(but not on the internal ones) in 2011 prior to the beginning of pollen shedding of seed orchard parents, suggesting background pollination, but these early pollen occurred before seed orchard females were receptive, thus were unlikely to have a direct impact on the genetic quality of the seed crop.

Genetic diversity parameters

To determine the effect of isolation treatments on genetic diversity in the offspring, we compared the mean number of alleles per locus (k), observed heterozygosity (H_o), effective number of fathers (N_{ef}) and selfing rate (s) in each group (Tables 1, 2, and 3). For all four measures, treatment was a significant factor, explaining 78.3, 31.1, 72.6, and 33.4 % of variation, respectively ($F_{4,\ 107} > 12.1$; $p < 0.0001$). Based on all four parameters, pairwise t tests divided the treatments into three groups with significant differences between them: one consisted of T and F tents, another of P tents, and the last one of the open controls.

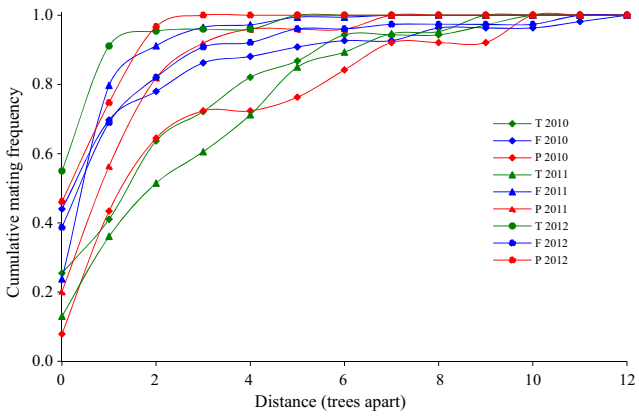

Fig. 3 Cumulative mating frequency as a function of distance between pollen source (father) and receptor (mother) in tents with different treatments across three studied years

The highest genetic diversity was reached in open controls while the lowest in TF tents. K was, on average, more than a third lower in TF tents compared with that in the controls (Tables 1, 2, and 3). Even a larger distortion was detected for N_{ef} in TF tents that reached only 38.0, 39.3, and 41.2 % of the values attained in the controls (N_{ef} = 5.66, 5.41, and 5.28 versus 14.90, 13.75, and 12.81 in the 3 years, respectively; Tables 1, 2, and 3). This variation in N_{ef} was mainly due to the unbalanced number of fathers available for mating (denoted hereafter as N_{fa}), which was 28 in open controls and only between 6 and 13 in tents. N_{ef} and N_{fa} were highly correlated ($r = 0.906$; $F_{1,\ 110} = 248.95$; $p < 0.0001$). High variability in N_{ef} was also observed among individual ramets, ranging from 1.32 to 5.71 in TF tents, from 4.71 to 11.92 in P tents and from 4.03 to 19.38 in open controls.

Supplemental pollination substantially improved N_{ef}: when pooled over the two P tents, the offspring reached N_{ef} of 9.75, 8.52, and 6.95 in the 3 years, respectively (Tables 1, 2, and 3), while it would have dropped to 5.47, 3.79, and 4.44 without the treatment. N_{ef} of the five pollen donors alone was 4.88, 4.97, and 4.44 in the 3 years, approaching the census number of all fathers involved the supplemental pollination. In 2012, the improvement in N_{ef} between TF and P tents was relatively small and reached only 13.1 %. This was due to the enormous success of the supplemental pollination treatment, which fertilized more than three fourths of all of the analyzed seed.

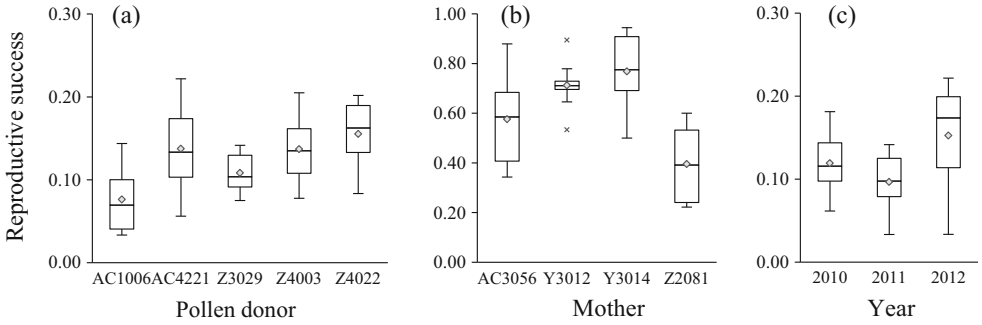

Fig. 4 Relative reproductive successes of five external pollen donors (**a**), their distribution among the progeny of four sampled mothers in tents with supplemental pollination treatment (**b**), and across pollination seasons 2010–2012 (**c**)

Self-fertilization varied significantly among treatments ($F_{4,\ 107} = 13.43$, $p < 0.0001$; Fig. 6); however, the pairwise t tests divided treatments into two groups only: one consisting of T and F tents ($|t| = 0.56$, $p = 0.575$) and the other of P tents along with the open controls ($|t| < 2.13$, $p < \alpha \approx 0.005$). Selfing was higher in the TF tents with average rate of 25.5, 17.0, and 24.4 % in the 3 years while the P tents had markedly lower values of 8.5, 9.9, and 4.9 %, approaching those found in the open control (4.1, 7.5, and 6.7 %) each year (Tables 1, 2, and 3). The distortion in selfing was even higher at individual tree level, ranging from 0.0 to 55.0 % (mean 12.2 ± 14.8 % SD) (Tables 1, 2, and 3). Zero selfing was detected in 11 of the 40 sampled trees in 2010 and in 6 trees in 2012, of which 12 were in controls and the remaining five in tents. The highest value of 55.0 % was found in mother AC3056 in 2010 in tent F2, which existed in the tent in four copies (33.3 % of all ramets in the tent). The second and third highest selfing values (53.2 and 53.3 %) were also detected in mother AC3056 in T2 in 2010 and in T1 in 2012, respectively. Self-fertilization was significantly related to the estimated parental pollen production in an environment ($F_{1,\ 110} = 27.42$; $p < 0.0001$). Thus, the difference in s among mothers was likely due to unequal pollen fecundity of the parent in T and F tents rather than their genetic or phenological predisposition to self-fertilization. We detected no significant differences between years ($F_{2,\ 109} = 0.76$; $p = 0.468$), indicating that self-fertilization was consistent across the studied pollination seasons.

Discussion

Isolation tent as an effective barrier to pollen flow

We analyzed nearly 3700 offspring collected in isolation tents over three consecutive pollination seasons. All but six were sired by fathers present in the tents or by supplementary pollen donors, which demonstrates an enormous success of the isolation experiment. Five of these cases were pollen leakage into the tents by orchard fathers. Pollen leakage is expected during operations in tent treatments such as air circulation by fan and supplemental pollination, in which tents have to be visited regularly during pollen shedding and female strobili receptivity period, and thus, ambient pollen can easily leak in through the openings. We consider these results to be a reasonable approximation of the immigration rate because only 5 to 8 out of the 28 fathers were present in the tents, for which the origin (tent versus open seed orchard) could not be distinguished. Thus, the probability of overlapping fathers from outside the tents causing a serious bias in our estimation is not very likely. This reasoning is further supported by the reproductive phenology shift inside the tents, which separated tent females' receptivity from outside males' pollen shedding in all 3 years. Pollen trapping recorded only negligible amounts of pollen both inside and outside the orchard at maximum female receptivity of tent trees, illustrating that there

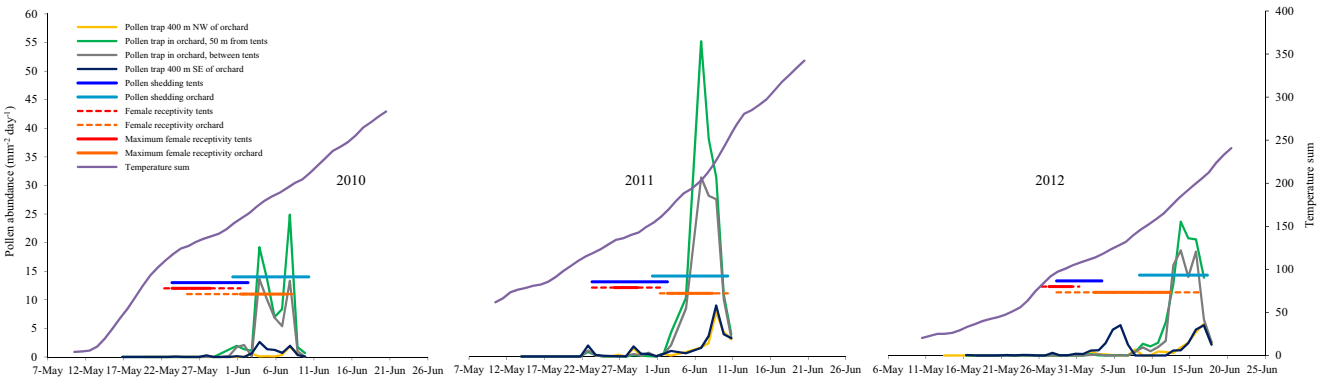

Fig. 5 Male and female reproductive phenology and pollen abundance measured using pollen traps in the Västerhus seed orchard in pollination seasons 2010–2012. Temperature sum is based on data from a nearby weather station Järved (distance about 10 km to the orchard)

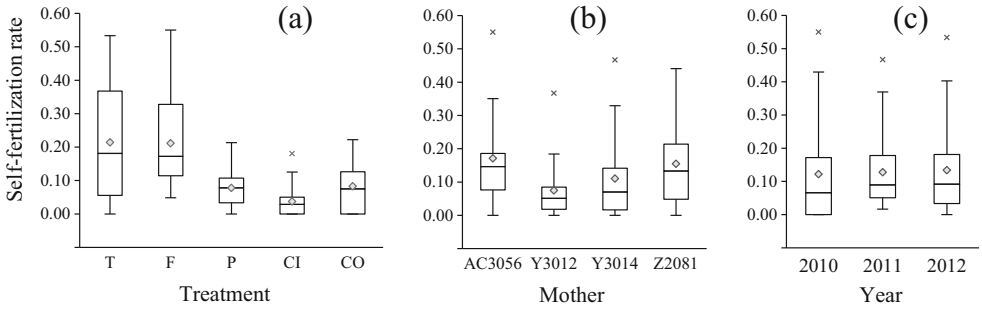

Fig. 6 Distribution of self-fertilization rates across treatments (**a**), sampled mothers (**b**), and pollination seasons 2010–2012 (**c**)

was very little pollen (whether background or produced by unprotected seed orchard parents) that could serve as a potential source of contamination into the tents. Additionally, the early pollen released from tents did not reach appreciable abundance that could be tracked by pollen traps or upbias tent fathers' reproductive success on mothers standing nearby the tents in the open controls.

Aside from the diversity measures, which are discussed later in this section, one immediate and greatly encouraging output from this study is that the mating success of foreign pollen can be substantially reduced. Since only 0.14 % of leak and only a single pollination from background sources were detected (provided it was not a result of mutation), the tested isolation tents can be considered to be capable of fully eliminating the penetration by outside pollen. As reviewed in the "Introduction" section, many different methods have been proposed to reduce pollen contamination in seed orchards; however, while their effectiveness was often detectable, they mostly failed to provide an appreciable improvement. The tent system evaluated here seems to be the most successful method of reducing pollen contamination developed to date. Although only small fraction of the control seed was found to be sired by background sources in each year (4.8–7.1 %), which is relatively much less than in majority of earlier studies (e.g., El-Kassaby et al. 1989; Harju and Nikkanen 1996; Nagasaka and Szmidt 1985; Torimaru et al. 2009, 2012; Wang et al. 1991; Yazdani and Lindgren 1991), our results suggest that under high pollen contamination rates typically reported in conifer seed orchards in Scandinavia, the significance of the tent isolation system on the economic revenue may be great.

This seed orchard was selected for the isolation tent experiment because pollen contamination of ca. 50 % had been detected in it in pollination season 2006 (Torimaru et al. 2009, 2012). This estimate may represent an extreme situation in the orchard or an up-biased estimation due to complicated banding patterns of some of the SSR loci that could have led to false paternity exclusion (see Funda et al. 2015 for details). The chance of false paternity assignment due to cryptic gene flow, where an offspring that perfectly matches with one of the candidate fathers had actually been sired by a random individual from the background population, was less than 1 %; thus, the pollen contamination rates reported here are unlikely to be seriously underestimated. Pollen trapping data also support our conclusion as the amount of pollen detected on the two internal traps within the seed orchard was substantially higher than that detected on the external traps, reaching 11.0-, 6.2-, and 5.5-fold difference during the peak periods of 2010–2012, which suggests that the majority of pollen available for fertilizing seed orchard mothers had been produced by seed orchard fathers. In addition, in 2010 and 2011, the internal traps' pollen peaks nearly fully overlapped with maximum female receptivity of seed orchard trees, which is in line with the low pollen contamination rates determined by the paternity analyses in the unprotected controls.

Mating structure in isolation tents and genetic consequences

Male and female reproductive successes are influenced by several factors including patterns of pollen dispersal, flowering phenology, and fecundity traits (Torimaru et al. 2012). Variance in individual reproductive success generated through those factors can impact the genetic value of seed orchard crops. The isolation tents created controlled pollination environment to quantify fine-scale mating dynamics in Scots pine.

We expected that the genetic diversity would be lowest in T and F tents, unless a larger amount of external pollen would leak into the tents to boost it, intermediate in P tents, provided the application of supplemental pollen were successful, and highest in unprotected controls. As the proportion of external pollen was negligible in all tents across years, the genetic diversity attained in the tents could not exceed that encompassed in the limited number of available fathers in T and F tents and that of the available fathers plus external donors supplied in P tents. Compared with controls, T and F tents produced offspring with 28, 31, and 41 fewer alleles at the nine loci in 2010, 2011, and 2012, respectively. Some portion of these differences was due to pollen contamination, which brought 5, 7, and 17 foreign alleles (i.e., alleles that did not occur among the 28 seed orchard parents) into the open-pollinated offspring during the 3 years. On the other hand,

while the foreign alleles might have improved genetic diversity of the seed orchard crops, they could be genetically inferior and thus unsuitable from breeders' and seed users' perspective.

We employed the concept of effective number of fathers to determine the level of reduction from the census number due to unequal reproductive success of fathers (or, strictly speaking, due to non-random sampling of gametes causing genetic drift), which has been widely adopted in quantitative and population genetics studies and included in many animal and tree breeding programs. Owing to the recognition of high importance of the genetic diversity of forest stands, the concept of effective number of parents was implemented in forestry jurisdictions, e.g., in Alberta and British Columbia where N_e (paternal and maternal combined) of any seed lot to be used for reforestation of Crown land must not drop below 18 and 10, respectively (Anonymous 2009; Stoehr et al. 2004). The latter value is derived from the presumption that N_e of 10 secures capturing of 95 % of the genetic diversity existing in the base population (Nei 1973; Yanchuk 2001). According to Lindgren and Prescher (2005), the optimum N_e for Scots pine is 16.

In this study, the paternal N_{ef} in T and F tents were reduced to a little over one half of the fathers' censuses (57, 54, and 53 % in the 3 years) and a similar ratio was attained in the controls (53, 49, and 46 %), although the absolute values here were greater, owing to the higher number of fathers available for mating that comprised the whole seed orchard population. This reduction in N_{ef} is in accordance with those observed in seed orchards of many other conifer species, such as Douglas fir, lodgepole pine, and western larch (Funda et al. 2011). High variability in the number of contributing fathers and their mating success on the sampled mother trees caused great differences in N_{ef} estimates in the offspring of individual trees, ranging from 1.32 to 5.71 in TF tents, 4.71 to 11.92 in P tents, and 4.03 to 19.38 in the controls. The lowest estimate of 1.32 was found in mother Y3014 in F2 tent and was a result of only four out of eight available fathers participating in mating with that mother tree, of which the most successful father AC3056 sired 87 of 60 analyzed offspring. The remaining three fathers sired only 5, 2, and 1 offspring. This great variation in paternal mating success was likely the results of the tent's unbalanced pollen production (e.g., AC3056 produced 28.6 % of all pollen in tent F2 in 2012) as well as the spatial distribution of sampled mothers relative to the available fathers. For example, two copies of AC3056 were the immediate neighbors of the sampled mother Y3014 in tent F2; thus, the effect of overrepresentation of AC3056 was accentuated by the application of fan that was always directed to blow pollen at a ramet's neighbors. Therefore, while the fan application succeeded in maintaining self-fertilization at a reasonably low level (8.3 %), it failed to produce offspring with sufficient genetic diversity. The highest TF tent value of 5.71 was a result of all eight available fathers contributing to the offspring, although still with a highly imbalanced mating success (contribution range 3–21 offspring, average 7.5 ± 5.7 SD).

Conifer species, including pines, typically reach high outcrossing rates. According to a review by O'Connell (2003), the mean outcrossing rate of 52 different conifer species was 83.5 or 87.8 % for pine species. Although conifers are monoecious, wind-pollinated, and had not developed strong genetic incompatibility systems, a large portion of self-fertilized zygotes abort prior to seed maturation because of the segregation of lethals in the homozygous state. This substitute for genetic incompatibility is beneficial (Seavey and Bawa 1986), but it does not fully protect seed from further development in all instances. Self-fertilized seeds that progress to maturation and reach the seedling stage are often subject to inbreeding depression linked with adverse effects on the phenotypic performance (Kärkkäinen et al. 1996; Lynch and Walsh 1998). Knowledge of the selfing rate in a seed lot is desirable because inbreeding depression may not become apparent until later phases of forest stands development.

Assuming equal reproductive success among all ramets, self-fertilization is often predicted from relative parental representation. In this seed orchard, the expected selfing rate was ca. 5.1 %, which is fairly close to the estimates determined by the SSR-based parentage analyses that averaged 6.1 % over 3 years. This level of selfing is acceptably low and is in accordance with many studies conducted on conifer seed orchards to date (Dering et al. 2014; Funda et al. 2014; Goto et al. 2002; Slavov et al. 2005). In T and F tents, both the predicted (from 14.0 % in T1 to 19.5 % in T2) and observed (from 17.5 % in T1 to 29.1 % in T2) values were higher than in the controls. The higher than expected selfing observed in the T and F tents was likely due to the existence of multiple ramets of the sampled mothers in the tents.

Supplemental pollination and genetic improvement

Supplemental pollination (SP) is a technique developed for increasing genetic quality of seed crops. Owing to a relative ease of handling pollen, it has encountered a broad utilization in seed orchard management for improving filled seed yields (Bridgwater and Bramlett 1982; Webber 1987), in particular, in young seed orchards with limited production of own pollen, and for manipulating genetic composition of seed crops (Askew 1992; Bridgwater et al. 1998) through, e.g., facilitating mating among asynchronous parents (El-Kassaby et al. 1988), balancing male reproductive success (Lai et al. 2010), introducing specific male parents into the population (Woessner and Franklin 1973), and reducing self-fertilization (El-Kassaby et al. 1990) and pollen contamination (Stoehr et al. 1998; Webber 1995). A great variety of SP success rates have been estimated for pine species to date, ranging from 4 % (Yazdani et al. 1986) to 80 % (Bridgwater et al. 1987).

We determined high and consistent success rates across all three studied years, ranging from 48.2 to 76.8 %, with negligible differences between replicates within years. The high success can be attributed to a combination of several factors—the isolation tents created a greenhouse-like environment that compacted reproductive phenology among mother trees whereby female strobili receptivity peaked at a similar time (Figure 5); the application was repeated several times each season so that most female strobili were covered by one of the applications at peak receptivity; each female strobilus was pollinated individually and multiple times per visit; the applied pollen was highly viable and competitive; and the isolation tents protected the applied pollen from being blown away by wind or washed away by rain. While repeated application had been reported to provide inconsistent improvement of the SP success rate, timing (Owens et al. 1981) and pollination of individual strobili (Eriksson et al. 1994) were shown to play an important role. For instance, Eriksson et al. (1994) observed that average success rates following individual strobili pollination ranged from 66 to 84 %, whereas in an operational study where whole trees were pollinated, it declined to 7 to 26 %.

The high success rate of the SP application in the P tents substantially reduced self-fertilization rates compared to the T and F tents, and approached rates observed in the unprotected controls (Table 4). In 2012, owing to the tremendous success of SP application, which left little mating opportunities for intent fathers, the self-fertilization rate in P tents was even 2 % lower than in the controls. Compared to the low genetic diversity observed in offspring of T and F tents, the application of additional pollen in P tents saw a substantial improvement in k and N_{ef}. The N_{ef}/census ratio in 2010 and 2011 increased to 65 and 57 %, respectively. In 2012, the ratio declined slightly, but it was because most seeds had been fertilized by the five SP donors, while only a small portion (ca 24 %) by the tent fathers. Because of the high success of SP treatment, pollen mix from a larger number of donors could be applied to make the N_{ef} in seed crops comparable to that in unprotected controls. This would also secure the retainment of all alleles among the offspring generation, including rare ones. Along with pollen contamination from background sources being fully eliminated, this strategy would have secured the production of higher-quality seed crops through lowering the inbreeding depression of subsequent forest stands as well as through realizing higher genetic gains attained during previous phases of tree improvement programs.

Conclusion and recommendation

The tested isolation tents have proven to be a highly effective method of preventing seed orchard trees from undesired, background pollen, as no pollen contamination was detected in any of the six tents within three consecutive years. The tents created a physical barrier against background pollen, and, at the same time, accelerated the reproductive phenology of trees within them and induced temporal reproductive isolation between trees inside and those growing outside. Due to the limited number of available fathers in each tent, their seed lots exhibited low male effective population sizes and high rates of inbreeding; however, supplemental pollination that accounted for more than 60 % of all seeds produced under this treatment substantially improved the seed lots' status, indicating that the tents provide a suitable environment for pollen augmentation. Air circulation by portable fans did not bring notable improvement in creating a more mixed mating, at least not under the limited exposure times.

In order to keep pollen contamination at reasonably low levels and, at the same time, to meet seed lots' genetic diversity requirements, more fathers can be used as pollen donors during the supplemental pollination treatment and/or seed crops from several small tents with different parental compositions can be mixed. Furthermore, seed orchards can be designed to accommodate tents at maturity by covering a specific number or composition of trees (e.g., fewer ramets representing more parents) in order to maximize the merit. The tent system also offers the opportunity to cover longer stripes of trees, and by establishing tents in neighboring columns a larger area can be isolated, theoretically comprising of entire seed orchards. Such large-scale tents would be particularly useful for seed orchards that are not sufficiently separated from conspecific stands and thus may suffer from high rates of pollen contamination, or for orchards whose design would not enable small-scale tents to encompass sufficient parental representation, leading to a low genetic diversity in their seed crops. We also see a great potential of the tents in protection of south-transferred seed orchards (e.g., to promote their seed production or avoid late-frost damages), in which pollen contamination may have more severe genetic consequences than in orchards comprising of local parents.

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Data archiving statement The genotype data reported in this study are provided in the Supplementary data file.

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