Clonal variation in phenological synchronization and cone production in a *Pinus patula* seed orchard

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Abstract

Synchronization between female receptiveness and pollen dispersal, and fecundity of clones influence effective population size and genetic diversity of germplasm produced in seed orchards. Our objective was to determine clonal variation in phenological synchronization and in cone production in a *Pinus patula* seed orchard. Two-year phenology data of female and male strobili from a sample of 31 clones, and of male strobili in trees from neighboring natural stands of the same species were used. Synchronization indices between female receptiveness and pollen release of the same clone ($\overline{O}_i\overline{P}_i$), of other clones ($\overline{O}_i\overline{P}_j$), and of natural stands ($\overline{O}_i\overline{P}_{NS}$) were calculated for each clone and averaged per precocity group (early, intermediate, and late). Genetic parameters for cone production of clones during three consecutive cone harvests were estimated, as was their relationship to precocity and synchronization index $\overline{O}_i\overline{P}_{NS}$. Cone production showed a broad variation among clones and between years, with strong genetic control ($H^2_c \geq 0.80$) and stable ($r_B \geq 0.79$) between yeas. There was wide clonal variation in synchronization indices, but no significant variation was found in most cases among precocity groups, neither were they consistent between years. Negative correlations [r_p = -0.37 and -0.40 ($P < 0.05$)] in cone production per clone in two years with the $\overline{O}_i\overline{P}_{NS}$ index in the first year, indicated that the most prolific clones had lower synchronization with pollen release in natural stands in the orchard's vicinity, and thus less risk of genetic contamination.

Keywords: *Broad-sense heritability, clone fecundity, clonal stability, genetic contamination, genetic variation, Pinus patula, reproductive phenology*

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Introduction

The main objective of a seed orchard is to produce large amounts of seed with high genetic quality (El-Kassaby et al., 1984). 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 achieved in the seed crops. Genetic gain is the ultimate goal of tree breeding efforts, but genetic diversity is important for the populations' resilience and adaptability in the future (Koski, 2000; Funda et al., 2016). For a seed orchard progeny to reach the highest quality, gain and genetic diversity, a complete flowering synchronization and equality in reproductive capacity among parents is required (Reynolds & El-Kassaby, 1990; Kang, 2001), allowing for a panmictic equilibrium and parental balance in the seed crop (Kang et al., 2004). These conditions, associated with a sufficient number of clones, contribute, in addition, to reduce self-fertilization and minimize inbreeding in the progeny (Kang, 2001).

A weak synchronization between female receptiveness and pollen dispersal has negative effects on the orchard's panmictic equilibrium (Matziris, 1993), because it promotes imbalance in clone contribution, non-random crossings among them, or an increase in the proportion of empty seeds. Phenology affects the frequency of gene exchange among clones and the genetic composition of seed derived from seed orchard (Kang & Lindgren, 1998). In addition, as synchronization between the orchard's clones is reduced, the risk of contamination by pollen from off-orchard sources increases, thus reducing genetic gain (Slavov et al., 2005). Pollen

contamination is affected by various factors; among them, proximity and size of the contamination source, reproductive synchrony between the orchard and the off-orchard source, and phenological variation among clones within the orchard are most important. For instance, El-Kassaby & Ritland (1986) found that the level of genetic contamination by external pollen in orchards of *Pseudotsuga menziesii* (Mirb.) Franco, varied from 18 % in late-flowering clones, to 24 % in early-flowering clones. Similarly, in a seed orchard of the same species, contamination percentage by external pollen varied from 23.3 % in late-flowering clones, to 55.5 % in the early-flowering (Slavov et al., 2005).

However, the impact of pollen contamination on the genetic quality of the seed crop in the orchard will also depend on the variation in clone fecundity and its relationship with clone phenology. If the most prolific clones are more likely to be pollen-contaminated, the negative impact on genetic gain would be higher. Thus, the number of female and male strobili (reproductive energy) and the conversion rate of female strobili to seed cones (reproductive success) in a seed orchard are also important for determining the genetic quality of seed crops (Matziris, 1997; Kang & Lindgren, 1998; Gomory et al., 2000; Kang, 2000; Ertekin, 2010). It is commonly observed that seed orchard parents vary in both reproductive energy and success with consistently high and low producers resulting in a disproportionate contribution from a reduced subset of parents (El-Kassaby et al., 1989; Koski, 2000). This increases the genetic relatedness in the progeny, and reduces the genetic diversity of the seed crop (Kang, 2001). Commonly, 25 % of the most prolific clones in the orchard, produce more than half of the male and female strobili, and 50 % of the most productive clones contribute with 80-90 % of female or male cones (Chalupka, 1991).

Studies on variation in cone production are the basis for first-generation seed-orchard management and for clone selection in advanced-generation orchards (Huwei et al., 2014). Production of female strobili largely determines seed production in clones, and, therefore, affects the parental balance and the effective population size in the seed crop (Burczyk & Chalupka, 1997). A cumulative contribution curve, known as a parental balance curve, is often used to quantify fecundity variation in seed orchards (Griffin, 1982; El-Kassaby & Reynolds, 1990; Adams & Kunze, 1996). In previous studies, a significant variation in cone production has been found among clones and years, in different coniferous species, such as *Pinus tabuliformis* Carriere (Huwei et al., 2014), *P. thunbergii* Parl. (Kang et al., 2004), and *Pseudotsuga menziesii* (El-Kassaby et al., 1989). However, such information for subtropical pines is scarce. Furthermore, the effect of an unequal fecundity of clones may be modified by phenological variation within the orchard. The most prolific clones can be partially or totally excluded from crossings with other clones if the male strobili release pollen before or after the female strobili in other clones are receptive; or vice versa, if the female strobili are receptive before or after pollen dispersal occurs in other clones (El-Kassaby & Ritland, 1986). Despite this, the relationship between cone production and floral synchronization of clones has been rarely addressed (Burczyk & Chalupka, 1997), so information on the genetic relationship between clone fecundity, reproductive phenology and risk of pollen contamination is limited.

The study was aimed at: 1) determining clonal variation in phenological synchronization between receptiveness of female ovules and pollen release from the same clone, from other clones, and from trees in nearby natural stands; 2) determining whether phenological synchronization of clones in the seed orchard is stable between years; and 3) determining clonal variation in production of female cones and its relationship with the risk of genetic contamination by external pollen.

Materials and Methods

Study area

The seed orchard of *Pinus patula* Schiede ex Schltdl. et. Cham. used in the study was established in 2003 by grafting of scions obtained from phenotypically selected, sexually mature, trees. The seed orchard is located in central Mexico, at the Reserva Forestal Multifuncional "El Manantial", in Aquixtla municipality, State of Puebla, México (19°43´13" N, 97°59´20" W, 2,860 m), within the species' natural distribution range. The orchard has an area of 1.2 ha, with a completely random design, a spacing of 3 x 3 m, with a different number of ramets per clone. At the beginning of the study, the orchard had 82 clones with a total of 657 ramets (1-22 ramets per clone). At that time, ramets were 12 years old, from grafting, and had already reached sexual maturity (Vargas-Hernández & Vargas-Abonce, 2016).

Evaluation of reproductive phenology and cone production

In 2014 and 2015, the phenology of male and female strobili was recorded for a sample of 31 clones from the orchard, with three ramets per clone, totaling 93 trees, selected on the basis of their cone production in the 2012 seed crop. The course of phenology of male strobili was evaluated in five branches marked in the lower portion of the tree crown during January to March each year, with a 3-4 d interval, until all the pollen was released; the same was done for female strobili in five branches marked in the upper portion of the crown, until they were not receptive anymore. Observations were done on the same ramets in both years, although not necessarily on the same branches, due to natural variation between years in the production of male and female strobili.

The phenology of male strobili and pollen dispersal from adult trees (e.g. in reproductive age) of *Pinus patula* was also evaluated during the same periods in four even-aged natural stands, along an altitudinal transect (2,807-3,011 m), at a distance of 150 to 1,000 m from the orchard's center. Ten trees from each stand were selected, with male-strobili production, easily accessible and visible in the tree crown, to allow sampling them. Matziris (1994) and Codesido & Merlo (2001) methodologies, modified by Hernández-Zaragoza et al., (2016) for *Pinus patula*, were used to determine the phenological stages for female and male strobili.

To determine the cone productive capacity of the sampled clones, all female mature strobili at the crown of the 31 clones, with their respective ramets, were collected and counted by the end of April in 2014, 2015, and 2016. These cone crops correspond to the flowering cycles in the spring of 2012, 2013, and 2014, respectively.

Data analysis

The start and end date (number of days from January 1st), and the length of the female receptiveness and pollen dispersal periods in each year were determined for each ramet and clone, with the phenological data obtained from each sampled branch. The same was done for pollen dispersal in trees sampled in the natural stands. The software program SYNCHRO (Zas et al., 2003) was used for this purpose. The index of average phenological synchronization ($\overline{OP}$) between the period of female receptiveness in each sampled clone in the orchard and: a) the period of pollen dispersal of the same clone ($\overline{O}_i\overline{P}_i$); b) the period of pollen dispersal of other clones in the orchard ($\overline{O}_i\overline{P}_j$); and c) the period of pollen dispersal of the trees in the neighboring natural stands ($\overline{O}_i\overline{P}_{NS}$) was calculated with each year's data. These indices were calculated following the procedure proposed by Askew & Blush (1990), which is a quantitative measure of the proportional symmetry of the female and male phenograms generated by the SYNCHRO program. The index ($\overline{OP}$) for the two mate trees is the ratio of the common area to the total (maximum) area between the female and male phenograms summed across all days. It varies from 0 (no overlap) to 1.0 (complete overlap); in this scoring scale, an index value above 0.5 is considered an appropriate phenological synchronization between mates (Askew & Blush, 1990). For instance, in *Pinus radiata* the synchronization index between pairs of mating clones varied from 0 to 0.82 across years (Codesido et al., 2005); in *Pinus sylvestris*, however, the index varied from 0 to 0.40 in two years (Burczyk & Chalupka, 1997).

Synchronization indices were calculated for each single clone. Then, clones were grouped according to their precocity (defined according to the start date of the female receptiveness period), in early, intermediate, and late-flowering clones. To determine differences in synchronization indices ($\overline{O}_i\overline{P}_i$, $\overline{O}_i\overline{P}_j$ and $\overline{O}_i\overline{P}_{NS}$) between early and late-flowering clones, the Student´s 'T' test for mean comparison was applied on the average values of these indices for each clone group, considering the individual clone indices as sample values. The stability between years in the phenological synchronization indices of the orchard clones ($\overline{O}_i\overline{P}_i$, $\overline{O}_i\overline{P}_j$ and $\overline{O}_i\overline{P}_{NS}$) was evaluated by estimating the Pearson correlation coefficient (r_p) between the clone index values obtained each year.

Genetic and between-year variation in cone production in the orchard clones were estimated by variance analysis of the number of cones collected each year, using the Mixed procedure from SAS (SAS Institute, 2002), and the following lineal model:

$$Y_{ijk} = \mu + A_i + C_j + AC_{ij} + \mathcal{E}_{ijk}$$

Where: Y_{ijk} is the observation of the k^{th} ramet in the j^{th} clone in the i^{th} year, μ is the mean, A_i is the random effect in the i^{th} evaluation year [($i = 1, 2, 3$) ~ NID ($0, \sigma^2_a$)], C_j is the random effect in the j^{th} clone [($j = 1, 2,\ldots 31$) ~ NID ($0, \sigma^2_c$)], AC_{ij} is the random effect of the year x clone interaction ~ NID ($0, \sigma^2_{ac}$), and $\mathcal{E}_{ijk}$ is the error ~ NID ($0, \sigma^2_e$). Before the analysis, the original data of number of cones (x) were transformed with the function $\sqrt{x}$ to homogenize the variance between years and among clones (Steel & Torrie, 1980).

Broad sense heritability of the clone means (H^2_c), also called "clonal repeatability" was calculated with the following equation:

$$H_c^2 = \frac{\sigma_c^2}{\sigma_c^2 + \left(\frac{\sigma_{ac}^2}{a}\right) + \left(\frac{\sigma_e^2}{ra}\right)}$$

Where: σ^2_c is the variance among clones; σ^2_{ac} is the variance due to year x clone interaction; σ^2_e is the error variance; a is the number of years, and r is the number of ramets per clone. The genetic stability between years (reproductive cycles) in the clone contribution to the total cone crop was determined by estimating the type-B genetic correlation (r_B) using the procedure described by Falconer and Mckay (1996), applying the following equation:

$$r_B = (\sigma^2_c) / (\sigma^2_c + \sigma^2_{ac})$$

The Spearman correlation coefficient for average cone production per clone was also estimated between years. To evaluate the relationship between cone production and risk of genetic contamination by external pollen in the orchard clones, the phenotypic correlations (r_p) of the average number of cones produced per year by each clone with the synchronization index ($\overline{O}_i\overline{P}_{NS}$), and with the start date and length of the female receptiveness period of clones were estimated separately for each year's phenology data.

Results

Clonal variation in cone production

Cone production in the seed orchard varied significantly ($P \leq 0.001$) both between years and among clones; moreover, the year x clone interaction was also significant. Average cone production per tree varied from 6.3 cones in 2015, to 43.5 in 2016. In absolute terms, differences among clones were greater in 2014 and 2016, but the variation coefficient was greater in 2015 (191.14 %) due to a lower production that year.

Three clones (32, 33, and 86) were consistently less productive in all three years; however, in 2015, six other clones did not produce cones. The 12 most productive clones together produced 70 %, 74 %, and 80 % of the total cone crop in 2014, 2015, and 2016 respectively (Figure 1). Despite fluctuations in the ordering of clones according to their cone production across years, as indicated by the significant effect of the year x clone interaction, the Spearman correlation coefficient between years 2014 and 2016 was 0.74 ($P \leq 0.0001$), and between 2014-2015 and 2015-2016 was 0.60 ($P \leq 0.001$).

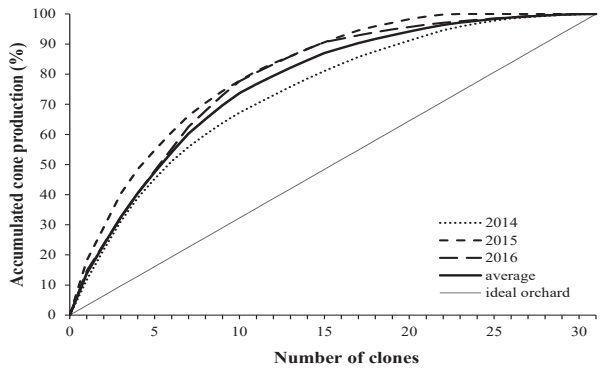


Figure 1
Accumulated contribution of *Pinus patula* clones to the cone crop in the seed orchard in three consecutive years.

A wide genetic variation in cone production was found in the sampled clones in the orchard, both in the overall analysis as in the paired-year analysis (Table 1). The variance component of the year *x* clone interaction was relatively low (< 27 %) in relation to genetic variation, except in the 2015-2016 cone crop analysis, where its contribution to total variance was almost equal to genetic variance. Broad-sense heritability of clonal cone production (clonal repeatability) was 0.80, although estimated values fluctuated from 0.57 to 0.85 in the analysis carried out for different paired-years. Phenotypic correlations of clone means (r_p) between years were positive and relatively high (r_p ≥ 0.57), while the type-B genetic correlation (r_B) was 0.79 in the overall analysis, with values from 0.52 to 0.97 between the different paired-years (Table 1).

Table 1
Variance components, σ^2_{ac}/σ^2_c relation, broad-sense heritability of clone means (H^2_c), type-B genetic correlations (r_B) of cone production, and phenotypic correlations of clone means (r_p) in a *Pinus patula* seed orchard during three consecutive years.

Genetic parameter	Cone production			
	Overall analysis	2014-2015	2014-2016	2015-2016
σ^2_c	4.29	2.25	7.53	3.11
σ^2_{ac}	1.17	0.47	0.20	2.85
σ^2_e	5.94	4.94	7.31	5.56
σ^2_{ac}/σ^2_c	0.27	0.21	0.03	0.92
H^2_c	0.80	0.68	0.85	0.57
r_B	0.79	0.83	0.97	0.52
r_p		0.61**	0.73**	0.57**

**Statistically significant (P <0.01)

Clonal variation in phenological synchronization indices

The synchronization index $\overline{O}_i\overline{P}_i$ varied from 0.39 to 0.78 in 2014, and from 0.21 to 0.75 in 2015 (Table 2), but the average values were significantly different (P ≤ 0.05) between years (0.60 vs. 0.52). Of the 31 clones evaluated, 58 % in 2014, and 23 % in 2015 had $\overline{O}_i\overline{P}_i$ values higher than 0.6. However, no significant differences were found in $\overline{O}_i\overline{P}_i$ among the early, intermediate, and late-flowering groups in both years. Thus, the $\overline{O}_i\overline{P}_i$ index value was not related to phenological precocity of clones.

The value of $\overline{O}_i\overline{P}_j$ index varied from 0.42 to 0.71 in 2014, and from 0.26 to 0.59 in 2015, with no significant differences between years in the average values (0.54 vs. 0.50, Table 2), nor between precocity groups, except for the intermediate and late-flowering clones (P ≤ 0.05) in 2015. On the other hand, the value of the $\overline{O}_i\overline{P}_{NS}$ index varied from 0.35 to 0.64 in 2014, and from 0.25 to 0.66 in 2015, with no significant differences between years in the average values (Table 2) nor between early and late-flowering clones. Late-flowering clones showed a greater overlap with external pollen release than the early-flowering clones in 2014, but in 2015 the overlap was similar for both clone groups (Figure 2).

Table 2
Average and extreme values of the phenological synchronization index ($\overline{OP}$) between female receptiveness in the orchard clone and pollen of the same clone ($\overline{O}_i\overline{P}_i$), other clones ($\overline{O}_i\overline{P}_j$), and natural stands ($\overline{O}_i\overline{P}_{NS}$) for clones with different precocity in two consecutive years.

	2014			2015		
Clone$_i$ group	$\overline{O}_i\overline{P}_i$	$\overline{O}_i\overline{P}_j$[a]	$\overline{O}_i\overline{P}_{NS}$[a]	$\overline{O}_i\overline{P}_i$	$\overline{O}_i\overline{P}_j$[a]	$\overline{O}_i\overline{P}_{NS}$[a]
a) Early-flowering						
Average	0.57	0.49±0.25	0.42±0.22	0.48	0.49±0.23	0.55±0.08
Minimum	0.48	0.42±0.23	0.35±0.20	0.28	0.43±0.17	0.40±0.05
Maximum	0.64	0.58±0.27	0.54±0.25	0.65	0.58±0.29	0.66±0.14
b) Intermediate-flowering						
Average	0.61	0.55±0.21	0.50±0.18	0.53	0.49±0.20	0.49±0.11
Minimum	0.39	0.42±0.13	0.41±0.06	0.21	0.26±0.13	0.25±0.04
Maximum	0.74	0.71±0.27	0.59±0.23	0.73	0.59±0.25	0.59±0.16
c) Late-flowering						
Average	0.62	0.57±0.20	0.55±0.14	0.53	0.50±0.22	0.45±0.10
Minimum	0.49	0.44±0.14	0.42±0.03	0.29	0.42±0.16	0.34±0.05
Maximum	0.78	0.68±0.31	0.64±0.22	0.75	0.57±0.29	0.64±0.15
Global $\overline{OP}$	**0.60**	**0.54**	**0.49**	**0.52**	**0.50**	**0.48**

[a]Average values $\overline{OP}$ ± standard deviation.

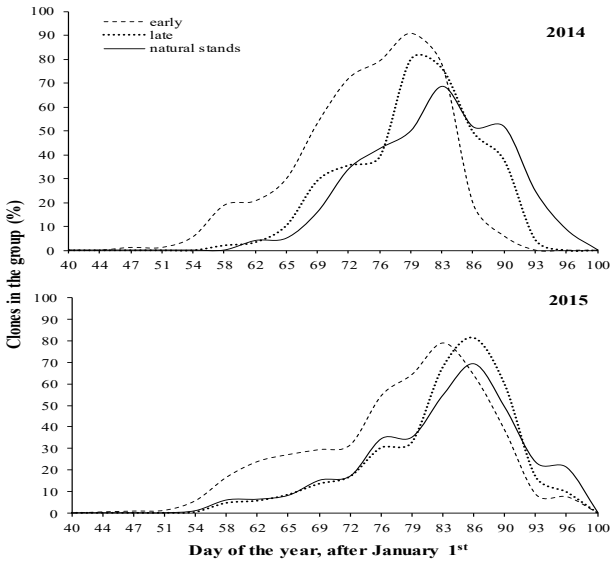


Figure 2
Seasonal variation in female receptiveness periods for early- and late-flowering clone groups as related to the pollen release in natural stands.

$\bar{O}_i\bar{P}_j$ values were slightly higher than those of $\bar{O}_i\bar{P}_{NS}$ for most clones in both years (Table 3). However, except for the late-flowering clone group in 2015, no significant differences ($P \leq 0.05$) between the average values of $\bar{O}_i\bar{P}_j$ and $\bar{O}_i\bar{P}_{NS}$ were found in any group. The between-year correlation coefficient for $\bar{O}_i\bar{P}_i$ and $\bar{O}_i\bar{P}_j$ indices was nearly zero ($|r| \leq 0.05$, $P \geq 0.79$), indicating that the possibility of self-pollination and cross-pollination for each clone is not stable in successive years. In contrast, the between-year correlation for $\bar{O}_i\bar{P}_{NS}$ index was -0.48 ($P \leq 0.01$), which implies that clones with greatest synchronization in 2014 showed a lower synchronization in 2015 and vice versa, due to changes in both ovule receptiveness in orchard clones and pollen dispersal periods in neighboring stands across years.

Table 3
Comparison of average synchronization index of female receptiveness period with pollen from other clones in the orchard ($\bar{O}_i\bar{P}_j$) and from natural stands ($\bar{O}_i\bar{P}_{NS}$) for clones with different precocity in two consecutive years.

Year	Clone group	N	Phenological synchronization index ($\overline{OP}$)		Pr > F
			$\bar{O}_i\bar{P}_j$	$\bar{O}_i\bar{P}_{NS}$	
2014	Early-flowering	8	0.49	0.42	0.50
	Intermediate	14	0.55	0.50	0.07
	Late-flowering	9	0.57	0.55	0.92
2015	Early-flowering	7	0.49	0.55	0.18
	Intermediate	10	0.49	0.49	0.93
	Late-flowering	13	0.50	0.45	0.02

Relationship of cone production with precocity and risk of genetic contamination

The phenotypic correlations between cone production in the orchard clones and the starting date, and duration of the female receptiveness period were low and non-significant in general (Table 4). The exception was with the starting date of the receptiveness period in 2014, and when using the three-year average of cone production, which showed a moderate negative relationship ($r_p \leq -0.39$). The negative sign in this correlation indicates that the most productive clones tend to be the most precocious in female receptiveness, and, therefore, they would be expected to have lower risk of genetic contamination due to their lower overlap with pollen dispersal in natural stands. The negative correlations ($r_p = -0.37$ and -0.40, $P < 0.05$) between cone production in the 2014 and 2016 cone crop and the $\bar{O}_i\bar{P}_{NS}$ value in 2014 shows that the most productive clones had a lower synchronization index with external pollen dispersal in nearby natural stands. However, cone production in the 2016 cone crop also had a positive correlation ($r_p = 0.44$, $P < 0.05$) with the $\bar{O}_i\bar{P}_{NS}$ value in 2015. Using the three-year average of cone production (a better estimate of clone fecundity), however, no significant correlation was found between this trait and the average $\bar{O}_i\bar{P}_{NS}$ index value (Table 4).

Table 4
Phenotypic correlation (r_p) of cone production in clones with the $\bar{O}_i\bar{P}_{NS}$ value and with the starting date, and duration of female receptiveness in consecutive years in the seed orchard.

Trait and year		Phenotypic correlation (r_p)		
Cone harvest	Pollen in natural stands	$\bar{O}_i\bar{P}_{NS}$	Receptiveness	
			Starting date	Duration
2014	2014	-0.37*	-0.40*	0.09
2015	2014	-0.28	-0.17	0.02
	2015	0.28	0.02	-0.03
2016[1]	**2014**[1]	-0.40*	-0.29	-0.11
	2015	0.44*	-0.27	0.25
r_p (average values across years)		0.13	-0.39*	0.21

[1]Cones harvested in April 2016 come from the flowering cycle in 2014 (See Materials and Methods). *Statistically significant ($P < 0.05$).

Discussion

Clonal variation in cone production and its relation to phenology

Cone production in the orchard varied annually, as it naturally occurs in seed orchards of other coniferous species such as *Pseudotsuga menziesii* (El-Kassaby et al., 1989), *Pinus nigra* Arnold (Matziris, 1993), and *P. halepensis* Mill. (Matziris, 1997). This annual variation is more common in young seed orchards, since at the initial reproductive stages, some clones have not reached sexual maturity and they fail to produce female or male strobili, or produce them irregularly (Li et al., 2011). *Pinus*

patula is a reproductively precocious species, where flowering commonly begins at 3 years of age in trees derived from seed, and heavy annual cone production is common by ages 8-10 years (Wormald, 1975). Flowering at the *Pinus patula* seed orchard began the second year after planting in some clones and it was well established across clones when they were 4 years old, but a wide variation in fecundity among clones has already been observed (Vargas-Hernández & Vargas-Abonce, 2016).

Despite the annual and clonal effects on cone production, variance of the year x clone interaction was lower than the genetic variance attributable to clones (Table 1), which indicates that this fecundity trait is under high genetic control and is relatively genetically stable across cone crops. Genetic variation in cone production could be accounted for by various factors. First, clone variation in sexual maturity, as mentioned by Li et al. (2011) in *P. tabuliformis*; second, variation in relative growth rate among clones, which produces differences in crown size; third, clone variation in flowering synchronization with environmental conditions in the site, as mentioned for *Pinus tabuliformis* (Huwei et al., 2014); and fourth, intrinsic differences in genotype fecundity. Studies on cone production in *Pinus contorta* Dougl. (Ying et al., 1985) and *P. thunbergii* Parl. (Kang et al., 2004) indicated that some clones are highly prolific and have a greater participation in seed production. Apparently, differences in strobili production are less pronounced in seeding years (O´Reilly et al., 1982).

A wide variation in cone production among clones (El-Kassaby et al., 1989), under high genetic control (El-Kassaby & Cook, 1994), has also been found in seed orchards of *Pseudotsuga menziesii*. Estimated values of broad-sense heritability for cone production were similar to those found in 11-year-old clones of *Pinus nigra*, which fluctuated from 0.61 to 0.71 (Matziris, 1993), but are higher than those found in clones of *Pseudotsuga menziesii* (El-Kassaby & Cook, 1994) and *Pinus halepensis* (Matziris, 1997), where H^2_c values were below 0.48. However, a higher genetic control has been found in clones of *Pinus radiata*, with $H^2_c = 0.91$ in two years of evaluation (Codesido & Fernández-López, 2014).

The slight changes in estimated heritability across years reflect the effect of annual changes in cone production associated to within-clone variation in response to variation in environmental conditions (Matziris, 1993). However, such variation is expected to diminish and stabilize with age of trees, as was the case for a 6-year-old *Pinus pinaster* Ait. seed orchard, where heritability increased with age from 0.76 to 0.82 (Merlo & Fernández-López, 2004). The high heritability estimates suggest that observations carried out in the seed orchard are a useful tool for a better selection of clones to be established in second-generation orchards, by combining information about fecundity with productivity and wood quality.

Clonal participation in the cone crop was relatively low for this *Pinus patula* seed orchard. About 20 % of the clones sampled produced 50 % of the cone crop, and 39 % of the clones produced between 70 and 80 % of the cones harvested across the three years of evaluation (Figure 1). However, these data are similar to clonal contribution found in seed orchards of other species with ages below 20 years, as is the case of *Pinus sylvestris* (Burczyk & Chalupka, 1997) and *P. pinaster* (Merlo & Fernández-López, 2004), where 25 and 35 % of the clones contributed 50 to 65 % of the cone production in the orchard. In a *Pinus tabuliformis* seed orchard, the estimated genetic contribution of each clones varied substantially across years, with up to 21-fold differences between highest and lowest cone producers (Li et al., 2011). It demonstrates clonal effects on gametic contribution and on expected genetic gains. This information could be useful for genetic roguing of the seed orchard, by removing the low producer clones (Li et al., 2011).

Negative phenotypic correlations of clonal cone harvest in 2016 (from the 2014 flowering cycle) with $\overline{O}_i\overline{P}_{NS}$ index and start and duration of female receptiveness in 2014 (Table 4) indicate that more productive clones are less synchronized with pollen dispersal from natural stands and tend to start receptiveness earlier. This is a favorable situation, because it means that clones with higher potential for seed contribution to the cone crop had lower genetic-contamination risk, due to a lower overlap with pollen release in natural stands. However, when considering average clone values across sampled years, the correlation is weak and it might change from year to year (Table 4), mainly because of annual variation in flowering phenology. Thus, the relationship doesn´t seem to be stable and there may be years in which the most productive clones may be exposed to high genetic-contamination risks, affecting germplasm quality and expected gains the orchard seed lots.

Clonal variation in phenological synchronization

Although similar average values were found in $\overline{O}_i\overline{P}_i$ and $\overline{O}_i\overline{P}_j$, there was a wide variation among clones in these values, indicating that the possibility of self-fertilization and cross-pollination varies among them. However, this possibility was not related to precocity of clone receptiveness, nor were they consistent over the two consecutive years. Rather, they seem to depend on environmental circumstances and on year-to-year phenology variation of clones. Other coniferous species have also shown a wide variation in the phenological synchronization between female-receptiveness with pollen release from other clones, as is the case of *Pinus sylvestris* L. (Burczyk & Chalupka, 1997), *P. radiata* D. Don (Zas et al., 2003; Codesido & Fernández-López, 2008) and *P. tabuliformis* (Li et al., 2012).

A considerable proportion of the clones sampled (over 50 % in 2014) showed a relatively high value of $\overline{O}_i\overline{P}_i$ (above 0.6), but similar to what has been found in other pine species, since a higher synchronization is commonly expected between ramets of the same clone than between ramets of different clones (Li et al., 2012; Tan et al., 2019). However, the probability of self-fertilization in the orchard would be expected to be relatively low, even in those clones, for various reasons. First, due to the high number of clones in the orchard, the number of different mates that can bring pollen to the ovules of each clone is at least 30 times larger (considering only the number of clones sampled) than that coming from the clone itself. In addition, the ramets of each clone are distributed in the orchard in a spatial design which reduces the possibility of pollen exchange among them. Assuming a similar pollen production

for all the clones, and all other factors remaining constant, the probability of self-fertilization is below 5 % for all the clones. Thus, self-fertilization does not seem to be an important problem in the orchard, despite moderate and high $\overline{O}_i\overline{P}_i$ values. In *Pinus tabuliformis* orchards, 80 % of the clones had values from 0.60 to 0.90 in the $\overline{O}_i\overline{P}_i$ synchronization index (Li et al., 2012).

Average $\overline{O}_i\overline{P}_{NS}$ values were similar in both years, but the wide variation in this value among clones shows that genetic contamination risk varies widely among them, as has been found in *Pseudotsuga menziesii*, where early and late flowering clones had higher pollen contamination from natural stands than the intermediate class (El-Kassaby & Ritland, 1986). The greater phenological overlap in the late-flowering clones in 2014 (Figure 2) and the variation in $\overline{O}_i\overline{P}_j$ and $\overline{O}_i\overline{P}_{NS}$ within each group shows that risk is associated to phenological earliness, as well as to annual variation in pollen dispersal period in nearby stands, which depends mainly on environmental conditions (Muñoz-Gutiérrez et al., 2017). Even though no difference was found in phenological synchronization among clone groups, it has been shown in other species that early-flowering clones have longer receptiveness and dispersal periods (Codesido et al., 2005). In our study, the starting date of female receptiveness had a high negative correlation with the duration of the receptive period ($r_p \leq$ -0.85), indicating that clones initiating earlier have a longer female receptiveness period. On the contrary, intermediate and late-flowering clones, despite having a shorter receptiveness period, have a more homogeneous synchronization index with pollen dispersal in other clones, which promotes mating among all clone pairs in the orchard (El-Kassaby & Ritland, 1986).

Overall, contamination risk in the orchard is greater than self-fertilization due to the number of trees producing pollen in the orchard's neighborhood, although it depends, to a great extent, on the total amount of pollen produced and the overlap with the ovule receptiveness period. Muñoz-Gutiérrez et al. (2017) found that pollen production in the orchard is much higher than in natural stands, with a longer dispersal period. In addition, $\overline{O}_i\overline{P}_j$ and $\overline{O}_i\overline{P}_{NS}$ data show that, globally, synchronization with the orchard's pollen is slightly higher, which can, as a whole, significantly reduce the possibility of genetic contamination.

Even so, low reproductive synchronization among some clones in the orchard can create scenarios where some of them are more exposed to contamination by external pollen, or less represented in the orchard's progeny. This is the case of those clone mates with $\overline{O}_i\overline{P}_j$ values lower than 0.5. It is possible for orchard clones to be grouped in small breeding groups due to the wide variation in phenological synchronization among them, as has been found in seed orchards of *Pseudotsuga menziesii* (El-Kassaby & Ritland, 1986; Kess & El-Kassaby, 2015). This scenario may have negative effects on the orchard's genetic efficiency, since it may reduce effective population size and genetic diversity of progeny, as compared to the panmixia ideal scenario. However, this negative effect could be reduced by the orchard's design and management, given that ramets from a given clone are not surrounded by the same clones across the orchard, and management activities, such as supplemental mass pollination, water spray, flowering stimulation, among others, could increase phenological synchronization and clonal participation in the cone crop.

Annual variation in phenological synchronization

Absence of between-years correlation of $\overline{O}_i\overline{P}_i$ and $\overline{O}_i\overline{P}_j$ values indicates that self-fertilization and cross-pollination likelihood in each clone change from year to year. These results differ from the stability in synchronization indices found among *Pinus tabuliformis* (Li et al., 2012) and *Pinus sylvestris* (Burczyk & Chalupka, 1997) clones; in *Pinus radiata*, synchronization indices improved with age (Codesido & Fernández-López, 2008).

Year-to-year changes in phenology of the receptive period, due to its greater dependence on environmental conditions and lower genetic control (Muñoz-Gutiérrez et al., 2019), were the main factor affecting the stability of $\overline{O}_i\overline{P}_i$ and $\overline{O}_i\overline{P}_j$ values across years. On the other hand, the negative between-years correlation in $\overline{O}_i\overline{P}_{NS}$ values seems to be due both to instability of the receptive period and to annual variation in pollen dispersal period in natural stands (Muñoz-Gutiérrez et al., 2017). The correlation between annual $\overline{O}_i\overline{P}_{NS}$ values is not high (r =-0.48), indicating, for practical purposes, that risk of genetic contamination of clones changes from year to year, so it is difficult to make predictions based on previous $\overline{O}_i\overline{P}_{NS}$ values.

Implications for seed production

The present study showed that cone production in *Pinus patula* was under strong genetic control and genetically stable, but it varied widely from year to year and among clones, which should affect the gene diversity of resulting seed crops, as has been shown for other coniferous species (Park et al., 2017). This information about the differential clone contribution to the gamete gene pool is important for the proper estimation of the expected genetic composition and genetic gain in planting seedlots (Burczyk & Chalupka, 1997; Stoehr & El-Kassaby, 1997; Koski, 2000). Genetic composition of the seed crop in a seed orchard is important. The main goals in seed-orchard management include maximization of genetic gain, avoidance of inbreeding depression, and increase in genetic diversity in the seed crop (Koski, 2000). These goals may be affected by factors such as self-fertilization, contamination with external pollen, synchronization in reproductive phenology among orchard clones, and differences in female and male fertility (Kang, 2001), among others. Avoiding or reducing self-fertilization and increasing synchronization among orchard clones will help increase cross-fertilization rates and reduce inbreeding and risk of contamination from external pollen.

In our case, wide clonal variation in $\overline{O}_i\overline{P}_i$ and $\overline{O}_i\overline{P}_j$ values associated with a broad genetic variation in clonal reproductive phenology warn about possible breeding groups inside the orchard, with some clones more prone to self-fertilization, as has been found in *Pseudotsuga menziesii* (Kess & El-Kassaby, 2015). In addition, lack of phenological isolation of the orchard, and broad clonal variation in $\overline{O}_i\overline{P}_{NS}$ index also warn about risk of genetic contamination, particularly for late-flowering clones. These two factors have negative effects on the genetic quality expected in the seeds produced in the orchard.

However, due to the large number of clones in the orchard, the overall proportion of self-fertilization in relation to cross-fertilization is expected to be low. In addition, clone fecundity (female-cone production) showed a negative moderate correlation with the $\bar{O}_i\bar{P}_{NS}$ index, which reduces the potential impact of genetic contamination on the genetic quality of the seed crop.

Various authors recommend several management practices in similar situations, including the use of techniques: a) to induce, increase, and homogenize flowering among clones; b) to increase and homogenize synchronization of reproductive phenology inside the orchard (Ross et al., 1985); c) to increase phenological isolation of the orchard in relation to external pollen dispersal; and d) to increase orchard-pollen competitiveness and success in fertilization, especially in those clones with low reproductive capacity and low $\bar{O}_i\bar{P}_j$ values.

Conclusions

The study showed that cone production in *Pinus patula* is highly variable, both among clones and years, but it has a strong genetic control and low genotype x environment interaction across cone crops, so it is genetically stable. The strong imbalance in clone contribution to annual cone crops negatively affects the effective population size and genetic efficiency of the orchard, and so the genetic diversity of seed lots produced. Even so, the most prolific clones tend to be receptive earlier in the flowering season and have lower synchronization with external pollen from natural stands, so they have lower risk of genetic contamination from outside the orchard.

Synchronization indices between female receptiveness and pollen of same clone $\bar{O}_i\bar{P}_i$ and with pollen of other clones in the orchard ($\bar{O}_i\bar{P}_j$) varied widely among clones, so the likelihood of self-fertilization and cross-fertilization would vary among them. The risk of genetic contamination also varied among clones and seed crops, given the broad variation found across clones and years in the synchronization index between female receptiveness of clones in the orchard and pollen from neighboring natural stands. Considered together, pollen contamination seems more important than self-fertilization in affecting the genetic efficiency of the orchard, given the amount of trees releasing pollen in the orchard's neighborhood. Because of large clonal variability across years in the genetic contamination risk, however, it is difficult to predict the impact on the seed crop based on previous flowering cycles. In order to minimize this risk, management practices to increase and homogenize flowering potential and phenology among clones, and to increase phenological isolation of the orchard, would be required.

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