Pollination dynamics in a *Platycladus orientalis* seed orchard as revealed by partial pedigree reconstruction

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Pollination dynamics in a *Platycladus orientalis* seed orchard as revealed by partial pedigree reconstruction

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Abstract

Pollination dynamics was studied in a first-generation *Platycladus orientalis* seed orchard with pedigree reconstruction using 8 nuclear and 4 chloroplast SSRs. The pedigree reconstruction assigned 371 out of studied 448 seeds to one of the orchards’ 192 candidate male parents and showed high level of outcrossing and pollen contamination in the orchard’s seed crop. While the orchard’s seed population showed greater allelic richness compared to the parental population, few alleles present in the parental population were missing in the seed crop. Additionally, we detected no significant correlation between male reproductive energy (pollen yield) and male reproductive success; however, uneven parental contribution was also observed. Pollen management practices were recommended to ensure the maintenance of genetic diversity in the seed crops and increase in genetic gain.

**Keyword:** seed orchard, pedigree reconstruction, gametic contribution, pollen contamination, selfing rate, genetic diversity.
Introduction

*Platycladus orientalis*, the only member of the family Cupressaceae, is native to northwestern China, Korea, and Russian Far East (Cheng and Fu 1978). As a pioneer species, *P. orientalis* is known for its wide adaptability, resistance to salt and insects (Hashemi and Safavi 2012) as well as drought tolerance (Li et al. 2011), thus it is commonly used in ecological restoration projects. Moreover, the species has an outstanding ability to absorb atmospheric and heavy metal pollutants (Chu et al. 2012). The species is known for its wood’s high density and decay-resistance, desirable properties for used in construction, furniture, and various industrial uses, creating an increasing demand for planting high quality trees.

In China, tree improvement and selective breeding activities of *P. orientalis* started in the 1980s with the establishment of provenance trials and phenotypic selection based on growth attributes. Following phenotypic selection, the selected parents were grafted and planted in breeding archives for maintaining and saving their genetic legacy and seed orchards were established for the production of genetically improved seeds for reforestation. The genetic diversity of the base breeding population determines the expected magnitude of breeding programs’ genetic potential (i.e., gain) and long-term viability of the breeding endeavor (i.e., genetic diversity). Genetic diversity in seed orchard populations is an important factor affecting their seed and seedling production’s genetic quality and resilience (Chaisurisri and El-Kassaby 1994; El-Kassaby and Ritland 1996). However, seed orchard populations are vulnerable to extraneous gene flow (i.e., pollen contamination) which impacts the genetic diversity as well as the adaptive potential of their seed crops (Funda and El-Kassaby 2012). Therefore, assessment of the pollination dynamics of seed orchard populations, including *P.*
orientalis, is of great importance as it provides references for better genetic management.

First-generation seed orchards are often composed of selected unrelated parents with unknown genetic worth, while advanced generation orchard consists of proven parents following progeny testing (El-Kassaby et al. 2011; El-Kassaby and Lstiburek 2009). Seed orchards mating system is always influenced by various factors such as reproductive phenology synchrony among parents, the degree and extent of pollen contamination, the trade-off between reproductive and vegetative investment (El-Kassaby and Barclay 1992), parental reproductive output, and the implemented cone crop management practices (El-Kassaby 1995). Thus, it is expected that seed orchard populations to deviate from Hardy-Weinberg equilibrium expectations (Eriksson 1973).

Pollen dynamics is an important factor affecting the mating system, its assessment can yield some important information, such as the rate of pollen contamination and parental gametic contribution. With the advance in molecular markers, DNA fingerprinting technologies and pedigree reconstruction (i.e., parental assignment), pollen dynamics analysis can be carried out (Vendramin and Hansen 2005), leading to more accurate assessment of the genetic gain and diversity of seed crops.

Here, we used nuclear and the paternally inherited chloroplast simple sequence repeats (SSRs) (Sakaguchi et al. 2014) to analyse the mating system in a first-generation Platycladus orientalis seed orchard. We determined the level of genetic diversity, selfing and pollen contamination rates, parental reproductive success (gametic contribution), and the correlation between male reproductive energy and success. Finally, we offered some recommendations for the development of effective population management practices.

Materials and methods
**Seed orchard and seed crop**

We sampled 192 *P. orientalis* parent trees (with significant seed and pollen output) growing in a first-generation seed orchard located in the National Tree Breeding Station (Jiaxian, Henan Province, China), established in the mid-80s. The clonal seed orchard covers an area of 35 hectares. Originally, the orchard’s population consisted of 268 phenotypically selected parents from 24 provenances from Henan Province and were planted in 20 blocks following the permutated neighborhood design (Bell and Fletcher 1978). Due to mortality and management turnover, the parent population is reduced to 206 parents and the number of blocks is reduced to six. The number of the ramets per parent ranged from 1 to 65, among which the sampled 192 parents were of relatively good fecundity (male pollen and female seed outputs).

Seed and fresh needles were collected from 26 open-pollinated families (OP) from the block with highest seed-cone output (spatial distribution of the sampled families was shown in suppla), each OP family is represented by a single ramet. Additionally, the 192 seed producing families were sampled for DNA fingerprinting. DNA was extracted from 448 embryos of germinated seeds representing the 26 OP families. The time of our sampling coincided with seed shedding (September 2017). We used the cetyltrimethylammonium bromide (CTAB) method of Doyle (Doyle 1987) for DNA extraction. The number of seeds representing an OP family ranged between 28 (family #122) and 6 (family #65) with an average of 15 seed representing the majority of families.

**Pollen production observation and SSR genotyping**

We estimated the paternal reproductive energy by counting the abundance of male buds for all ramets across the seed orchard following the method of Woods (2005). Counting was conducted in March 2017 during the pollen shedding period. In this method, ramets
within each clone were classified into 10 male cone production classes ranging from 0 (no male buds present) to 10 (buds highly abundant) (Woods 2005; male gametic contribution, Method M3: visual assessment of pollen-cone production on every ramet of all parents).

Based on *P. orientalis* previous SSR marker screening and application, we chose 8 nuSSRs (Jin et al. 2016) and 4 cpSSRs (Huang et al. 2018) for genotyping. PCR was carried out in a total volume of 20 µl including: 10 µL 2×Taq PCR Mix, 4 µL (4 pmol) fluorescent-dye-labeled M13 primer, 2 µL (10 ng) genomic DNA, and 4 µL (4 pmol) mixed complementary forward and reverse primers. PCR amplifications were performed using Bio-Rad T100™ Thermal Cycler and Bio-Rad S100™ Thermal Cycler with the following profile: 4-min denaturing at 94°C; 20 cycles of 30-sec denaturing at 94°C, 30-sec annealing at 60°C (-0.5°C per cycle) and 45-sec extension at 72°C; 20 cycles of 30-sec denaturing at 94°C, 30-sec annealing at 50°C and 45-sec extension at 72°C, with a final extension step of 72°C for 5 min. The amplification products’ polymorphism levels were determined by fluorescent-based capillary electrophoresis with an ABI 3730 sequencer.

**Data analysis**

The number of observed alleles (*N*<sub>a</sub>), effective number of alleles (*N*<sub>ea</sub>), observed (*H*<sub>o</sub>) and expected heterozygosity (*H*<sub>e</sub>), inbreeding coefficient (*F*<sub>is</sub>), Shannon (*I*) and diversity (*h*) indices, and unbiased diversity (*uh*) were calculated using GenALEX version 6.5 (Peakall and Smouse 2012). The paternity analysis was conducted using CERVUS program with paternity assignment probability of 95% and 0.01 error rate allowance using the eight nuclear markers (Kalinowski et al. 2007). The proportion of candidate fathers sampled was set to 0.90 and simulation work indicated that 0.91 of the 10,000
simulated offspring could be assigned to parents under these parameters. GeneCap version 1.4 was used to match the haplotypes by contrasting chloroplast alleles of paternal parent and offspring (Wilberg and Dreher 2004). The outputs of GenCap and CERVUS jointly determined the paternal identity. If the only parent matched in CERVUS didn’t match the ID of the possible matched parent in GenCap, the offspring was considered to be a mismatch; if the only parent matched in CERVUS matched the possible paternal ID in the GenCap, the offspring was considered to have matched father; if more than one father were assigned in CERVUS, we accepted the parent assigned at the higher LOD score and also with support from GenCap; for other cases, it was considered to be a mismatch and no assignment was made. Estimate of correlation between reproductive energy (male cone scores) and reproductive success (gametic contribution based on genotyping results) was estimated. The multi-locus mixed-mating model of Ritland (2000) was used to estimate mating system parameters, including single- \( t_s \) and multi-locus \( t_m \) outcrossing rates and multi-locus correlated mating \( r_{p(m)} \) using the expectation-maximization (EM) procedure of the computer program MLTR 3.2 (Ritland 2002). Standard errors for mating system parameters were obtained from the construction of 1,000 bootstrap replicates.

Effective number of male parents \( N_e^{♂} \) was estimated as follows:

\[
N_e^{♂} = \frac{1}{\sum_{i=1}^{N} m_i^2}
\]

Where \( N \) is the orchard’s census number of males (192) and \( m \) denotes the proportional male gametic contributions of parent \( i \) to a seed crop \((0 \leq m_i \leq 1, \text{ and } \sum_{i=1}^{N} m_i = 1)\). Pollen contamination was not considered into \( N_e^{♂} \) (male) estimation based on the pedigree information.

Results
Assessment of genetic diversity

Comparing the genetic diversity parameters between the orchard’s parental population and its resultant seed crop indicated the presence of alleles gain and absence at some of the studied loci (Tables 1 and 2). Gain of alleles was observed at two loci (H4 and H7), which is indicative of gene flow and absence of alleles was observed at six loci (H8, H9, H11, N11, N20 and N27). This is confirming that rare alleles present in only few parents contributed minimally to gene pool, thus they were not adequately represented in the analyzed seed sample and more importantly, the sampled seed did not capture the parental population’s allelic profile.

A marked increase in the observed heterozygosity was detected between parents and their offspring (0.327 vs. 0.391) (Table 1). Additionally, the mean inbreeding coefficient within offspring is negative ($F_{is} = -0.002$) (Table 1), indicating that the observed heterozygosity is higher than expected heterozygosity and there is high heterozygosity among descendants. However, the genetic diversity of parents was significantly higher than offspring based on the polymorphism information of cpSSR (0.341 vs. 0.229) (Table 2). The low value of effective population size of male ($N_{e♂}$) was 19.67, accounting for only 10.24% of the census number of 192 parents.

Mating system and Paternity analyses

High single- ($t_s = 0.948 ± 0.025$) and multi-locus ($t_m = 0.986 ± 0.025$) outcrossing rates were obtained from the 26-OP family array, indicating that outcrossing is predominant in this population. The $t_m$ statistics were less than 1, suggesting the presence of a small amount of selfing. Additionally, the difference between the two outcrossing rate estimation methods ($t_m - t_s = 0.038 ± 0.023$) departed significantly from 0, suggesting that inbreeding probability is very low corresponding to the value of $F_{is}$. Multi-locus
correlated paternity $r_{pm}$ which presents the degree of correlation of outcross paternity within progeny arrays was low ($0.055 \pm 0.019$), indicating the presence of multiple parents participating in pollination.

NuSSR and cpSSR separately identified 183 and 50 unique genotypes, collectively indicated that all 192 parents had unique genotypes. Similarly, nuSSR and cpSSR, identified 381 and 33 unique genotypes, yielding a total of 428 unique genotypes in the offspring population (Table S1). According to CERVUS and GenCap results, a total of 371 out of 448 embryos (82.81%) were assigned to one of the 192 candidate males with 78 fathers matched with a given offspring, thus the remaining seed are representative of the gene flow/pollen contamination (17.19%). Additionally, we observed two individuals resulted from selfing, producing a selfing rate of 0.45%. The distribution of full-sib families and their size within and among male parents ranged from 0 to 9, with a large proportion with a small size (from 0 to 3) (Figure 1). Male half-sib family sizes ranged between 0 and 56 and male cumulative gametic contribution indicated that 80% of male reproductive success was contributed by 15.63% of parents (Figure 2), indicating the presence of few dominant males for the sampled 2017 reproductive season.

**Correlation between pollen production and male reproductive success**

Pearson’s product-moment correlation coefficients between male DNA-based reproductive success (gametic contributions) and pollen production (field surveys) was poor ($R^2 = 0.018$), indicating that the survey-based pollen yield is not a reliable indicator for pollination success in this study. This may be caused by the great variation in clone size (range: 1 to 65). The male-cone reproductive output coefficient of variations (cv) among individuals within parents (i.e., ramets within clones) and among parents were 0.327 and 0.587, respectively, suggesting that the difference in
Discussion

Results from the mating system and pedigree reconstruction provided tangible evidence that outcrossing is the predominant mating mode in this seed orchard. The observed high outcrossing may have been the results of the permutated neighborhood design (Bell and Fletcher 1978) which restricted the proximity of individual ramet of a particular clone, thus effectively promoting mating among unrelated individuals. Several factors could have also contributed to the observed high outcrossing, the species reproductive biology (Dong et al. 1992) and reproductive synchrony among the orchard’s parents. Although most conifers practice outcrossing as their predominant mating system (Adams and Birkes 1991), some closely related species to our study object in the Cupressaceae family reported to have lower outcrossing rate. Xie et al. (1991) studied the mating system of the same species, and found a mean multi-locus outcrossing rate of 0.75 (Xie et al. 1991), a much lower estimate than the observation here (0.986). It is worth noting that Xie et al. (1991) study was conducted in a natural population, where significant family structure could have been present. They also concluded that sibling mating is one of the main causes of the low outcrossing rate, which is not applicable in a first-generation seed orchard as the present study. Another possible explanation for the differences in outcrossing rate between these two studies is the choice of marker. The SSRs in our study have much higher resolution ($P_{ID} = 0.004$; calculation referred to Waits et al. 2001) than the allozyme markers used in Xie et al. (1991), which could reveal in more depth the mating dynamics. An outcrossing rate of 0.63 was also observed in natural populations of eastern white cedar (Thuja occidentalis) (Perry and Knowles 1990), where significant isolation among individuals and small clusters were present in the studied area. The isolated nature among trees in this study...
may have prevented gene exchange among different individuals, leading to a relatively low level of outcrossing. Lamy et al. (1999) reported even lower outcrossing rates of 0.240 and 0.335 in two eastern white cedar populations in Quebec, Canada (Lamy et al. 1999). The authors attributed this phenomenon to deep substructure within the populations originating from a genetic bottleneck that occurred in North America during the Holocene (Hebda and Mathewes 1984). Another related species, *Thuja plicata*, was found to have outcrossing rate of 0.715 and 0.32 in a natural population (O’Connell et al. 2001) and a seed orchard population (El-Kassaby et al. 1994), respectively. All these findings suggest the existence of unique mating pattern and tolerance to selfing in the family Cupressaceae compared to other conifers. The selfing rate estimated in our study is relatively low. The phenomenon of inbreeding depression may exist in *P. orientalis*, such as seeds abortion, empty seeds, low germination rate, and reduced seed set (O’Connell et al. 2005; Stoehr and Newton 2002). If it is true, then the estimated selfing is underestimated. However, inbreeding depression corresponds to majority of conifer species, mainly in family Pinaceae rather than Cupressaceae (Klekowski 1988; Sorensen 1969), as all studies mentioned above indicated high survival rate for inbred seeds. Thus, further work is needed to address the specific reasons for low selfing rate revealed in the present study.

The remaining unassigned 77 offspring (17.19%) could be the product of gene flow from outside the seed orchard or from the remaining few un-sampled parents. Pollen contamination cannot be ruled out since the seed orchard is in proximity to provenance trial of the same species. The genetic gain may be diminished as contamination happens in seed orchard (Lindgren and Mullin 1998), but genetic diversity of the seed crop usually increases with a relatively low rate pollen contamination (Adams and Kunze 1996). However, when the rate of pollen contamination is high, the influence on genetic
gain and genetic diversity depends on the genetic quality of the contamination source. Since the orchard is wind pollinated and the pollination situation is extensively affected by climate, the genetic quality of the contamination source is difficult to assess. In order to ensure the genetic quality of seed orchards, it is necessary to avoid the occurrence of pollen contamination. The pollen contamination rate of the seed samples was high and reductions in pollen contamination may require effective implementation of pollen management methods such as substantial spatial isolation from other orchard blocks, supplemental-mass-pollination and/or bloom delay (Adams and Burczyk 2000; El-Kassaby and Ritland 1986; Wheeler and Jech 1986).

The calculation of genetic diversity revealed by nuSSR suggested that the offspring lost several low-frequency alleles that existed in the parental population; however, the offspring possess even greater observed heterozygosity that is on average found in the parental population. The value of observed heterozygosity in offspring population is greater than that of expected heterozygosity and causes its negative value of inbreeding coefficient within individual. This phenomenon is probably likely to occur in first-generation seed orchards because the breadth and depth of parent tree selection that is carried out in a wider geographic area, including individual representing great number of populations (Funda 2012), which means that individuals that can not normally exchange gene due to physical barriers achieve genetic interaction (Chaisurisri et al. 1994). This situation reflects the positive role of the seed orchards. However, the genetic diversity calculated by the polymorphic information of cpSSR indicating that offspring owned low level of allelic richness. The lack of recombination in the chloroplast genome coupled with male imbalance contribution of gametes led to a reduction in chloroplast genetic polymorphisms in the offspring population. The effective population size ($N_e$) can be used to measure the genetic diversity in a breeding
population (Wright 1931). The effective population size ($N_e$) of 10 was determined to be adequate for capturing 95% of the quantitative genetic diversity of the original population (Yanchuk 2001), so $N_e$ of 10 is considered as a minimum standard (Snetsinger 2004). Above all, $N_e^♂$ met this minimal standard requirement (19.67 >10); however, it is significantly lower than the census number, suggesting the reduction of genetic diversity caused by reproductive success variation should not be underestimated. Small effective population size would result in the build-up of co-ancestry and high selfing in the resultant seed crop and thus it is expected to influence the genetic gain (Lai et al. 2010).

We estimated 80% of the gametic contribution was attributable to 15.63% of the parental population, which is consistent with the “80/20” rule commonly observed in many conifers seed orchards (Anonymous 1976). The “80/20” rule asserting that 20% of an orchard’s parents produce as much as 80% of the entire seed-cone crop. The results of male gametic contribution should be considered in crops genetic worth estimation as parents with low breeding value might have large gametic contribution, which can significantly reduce the genetic quality of seed crops. Thus, first-generation seed orchard management needs to reduce its adverse effects by roguing lower breeding value parents and strict monitoring of parental male reproductive output as it is often overlooked.

The male reproductive output coefficient of variations among individuals within parents and among parents were remarkably high, indicating the serious polarization of paternal reproductive energy. There is almost no correlation between reproductive energy (cone count) and reproductive success (pollination success rate), and this can be caused by a variety of reasons. The considerable variation in the amount of pollen cones among individuals within clones and the varied number of ramets of clone coupled with
the limited spatial location of the mother tree has some influence on the imbalance of gamete contribution. Additionally, the climatic conditions (i.e., wind direction) at the time of pollination also have an effect on the pollination success rate of specific parents or the observed results could be the product of non-random mating within the seed orchard, a situation commonly reported for many seed orchards (Burczyk et al. 1997; Cheliak 1985; Mitton 1992).

**Conclusion**

Evaluation of genetic diversity and mating system of open-pollinated seedlots sampled from 26 selected clones using 8 nuSSRs and 4 cpSSRs indicated low level of selfing, relatively small effective number of male parents, high level of pollen contamination, and differential male reproductive success. Low correlation between male reproductive energy and reproductive success was also observed suggesting the need for implementing proactive cone crop management practices.

**Acknowledgement**

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**References**


Tables captions

Table 1 Genetic diversity of seed orchard parents and offspring based on the polymorphism information of 8 nuclear SSRs.

Table 2 Genetic diversity of seed orchard parents and offspring based on the polymorphism information of 4 chloroplast SSRs.

Figures captions

Figure 1 The distribution of 448 full-sib families from 26 open-pollinated *P. orientalis* seeds donors.

Figure 2 Cumulative reproductive success of 192 *P. orientalis* male parents determined from partial pedigree reconstruction. Paternal cumulative reproductive success represented by the dotted solid line and 80% contribution is represented by the solid line. The diagonal line represents hypothetical scenario with equal success among parents.
Table 1 Genetic diversity of seed orchard parents and offspring based on the polymorphism information of 8 nuclear SSRs.

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$N_a$, observed number of alleles; $H_o$, the observed heterozygosity; $H_e$, the expected heterozygosity; $F_{is}$, the inbreeding coefficient within individual.
Table 2 Genetic diversity of seed orchard parents and offspring based on the polymorphism information of 4 chloroplast SSRs.

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$N_a$, observed number of alleles; $N_{ea}$, effective number of alleles; $I$, Shannon's information index; $h$, diversity; $uh$, unbiased diversity.
Figure 1 The distribution of 448 full-sib families from 26 open-pollinated P. orientalis seeds donors.
Figure 2: Cumulative reproductive success of 192 *P. orientalis* male parents determined from partial pedigree reconstruction. Paternal cumulative reproductive success represented by the dotted solid line and 80% contribution is represented by the solid line. The diagonal line represents hypothetical scenario with equal success among parents.