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Clonal fertility and heritability in strobilus production, and gene diversity of seed crops in a second-generation seed orchard of *Pinus thunbergii*

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Abstract

Variation of female and male strobilus production among clones over 4 consecutive years was estimated, and genetic variance, heritability, and genetic correlation in strobilus production were investigated in the second-generation seed orchard of *Pinus thunbergii*. This seed orchard was created with grafts selected according to the volume growth of their offspring. The production of female strobili continued to increase, whereas the production of male strobili fluctuated throughout the investigated period. The number of female strobili was highest in rich cone years, but the production of male strobili was not the same in rich or poor years. The maleness index showed a balance between female and male parents that occurred when male strobilus production was in rich or poor years. The parental balance curve for female strobilus production was closer to the expectation (i.e., more equal contribution) than that for male strobilus production in all years. The analysis of variance revealed significant differences among clones in female and male strobilus production over the four-year period. The narrow-sense heritability was higher in male strobilus production than female strobilus, implying that male strobilus production was under genetic control. The Pearson's correlation was positive and mostly significant, indicating that female strobilus production was reflected in male strobilus production. The effective parent number was lowest in moderate and good years for cone production. The loss of gene diversity in seed crops was lower than expected based on clonal fertility variation.

Keywords: *Pinus thunbergii · advanced-generation seed orchard · strobilus assessment · genetic parameter · genetic diversity · effective population size*

Introduction

Pinus thunbergii, Korean black pine, is a conifer species that is economically and ornamentally valuable because of its strong resistance to pollution and salinity. It is widely distributed in coastal areas of Japan and Korea, but is extensively killed by pine nematodes, which are transmitted by beetle vectors. Subsequently, drastic declines and deaths are leading to the species becoming endangered in its natural range (KNA, 2015; KFS, 2021).

As a first-generation orchard, a clonal seed orchard of 23ha has been established, fully supplying enough high-quality seeds for national reforestation in Korea. Second-generation seed orchards are being created by hybridization among selected families based on volume growth through progeny testing. Accordingly, the second-generation seed orchard was established in 2 ha (KFS, 2021). For tree breeding of *P. thunbergii*, it is important to investigate the degree of variation in the production of female and male strobili and to predict the genetic composition of seed crops derived from seed orchards.

Investigation of strobili production in seed orchards is very important because the flowering process affects gene exchange among clones and the genetic makeup of seed crops (Eriksson et al. 1973; Matziris 1994; Burczyk and Chalupka 1997). Male and female fecundity can be influenced by genetic factors (Brünjes and Link, 2021), including the genetic diversity of the seed orchard population (Bilir et al., 2003; Nikkanen and

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Ruotsalainen, 2000). Also, high genetic diversity positively impacts fecundity by increasing the likelihood of successful pollination and fertilization (Torimaru et al., 2012). It is presumed that individual trees produce an equal amount of female and male gametes in a seed orchard. In addition, the contribution of each clone in seed production is evaluated as an important factor in determining the genetic value of seeds produced from the seed orchard.

The quantitative number of strobili is of paramount importance, as the genetic composition of orchard seeds depends primarily on the number of female and male strobili produced by each clone. Effects of other contributing variables, such as pollen divergence and female receptivity, are often masked by the number of strobili (Jonsson et al. 1976; O'Reilly et al. 1982; Griffin 1984).

The main objectives of this study are: 1) to survey changes in female and male strobilus production among clones over four consecutive years, 2) to investigate the abundance of strobilus production for each ramet in terms of maleness index and parental balance curve, 3) to estimate genetic parameters (e.g., variance, heritability, and genetic correlation) in strobilus production, and 4) to estimate gene diversity of seed crops from the second-generation seed orchard of *Pinus thunbergii*.

Materials and methods

Seed orchard and strobilus assessment

The second-generation seed orchard of *Pinus thunbergii* Parl. is located in the west coast of Chungnam-do province (at N36°46', E126°39', 10m asl) in South Korea. The seed orchard was established by grafts with 26 full-sib families in 2006. The parental families were selected backwardly for the volume growth of offspring by open-pollinated progeny tests. The design of orchard deployment was essentially random with respect to the allocation of ramets, and all grafts were planted at 5m×5m spacing in the seed orchard.

The number of female and male strobili was assessed for all grafts over a four-year period (2015-2018) in the clonal seed orchard. At the time of data collection, the total number of ramets was 312, ranging from 3 to 16 per clone, and the average number of ramets was 12. The female strobili were counted individually throughout the crown. the number of male strobili was estimated by multiplying the number of strobili on a branch representing the overall male flower density in a single tree by the total number of branches bearing male strobili.

Pearson's product-moment and Kendall's rank correlation coefficients among annual female and male strobilus production were performed in the surveyed four years.

Maleness index, parental balance, and heritability estimation

The maleness index (M_i, sexual unbalancing) was calculated based on the proportion of female and male strobili production as follows (Lloyd, 1979),

$$M_i = \frac{pm_i}{pf_i + pm_i} \qquad (1)$$

where pm_i and pf_i are the relative proportion (contribution) of the female and male strobilus production of the *i*-th family on the total strobilus production.

The cumulative gamete contribution curve was used to assess parental balance (Griffin, 1982). The contribution of each clone to the degree of equal contribution was analyzed based on the parental balance curve within the seed orchard. The numbers of female and male strobili were ordered by clones from high to low production in descending order, and then the percentages of cumulative contribution were plotted against the proportion of clones.

One-way analysis of variance (ANOVA) was performed to estimate the variance of female and male strobilus production as formula 2, which analyzes a significant difference in the strobilus production among clones in each year using R version 3.5.3 (R Core Team, 2019) and RStudio version 1.2 (RStudio Team, 2019).

The narrow-sense heritability (h^2) was estimated by the ANOVA results as,

$$h^2 = \frac{\sigma_c^2}{\sigma_P^2} = \frac{\sigma_c^2}{\sigma_c^2 + \sigma_e^2/k} \qquad (2)$$

where σ^2_P, σ^2_c, and σ^2_e are the variances of total (phenotypic), clone (additive), and error (environmental), and *k* is the coefficient of variance component, respectively.

Fertility variation, effective parent number, and gene diversity

Fertility as female and male gametic contribution was estimated based on the assessment of female and male strobilus production. Variation in fertility (Ψ) among clones was calculated as

$$\Psi = CV_c^{\,2} + 1 \qquad (3)$$

where CV_c is the coefficient of variation in clonal fertility (i.e., an average of female and male fertility). The clonal fertility variation could then be separated with female (ψ_f) and male (ψ_m) fertility variation based on the coefficient of variation in female and male strobilus production (CV_f and CV_m) as,

$$\psi_f = CV_f^{\,2} + 1 \text{ and } \psi_m = CV_m^{\,2} + 1 \qquad (4)$$

Effective parent number (N_p) is defined as the number of genotypes divided by the fertility variation (Ψ, sibling coefficient) (Kang and Lindgren, 1999; Kang and El-Kassaby, 2002; Bilir et al., 2003) as below,

$$N_p = \frac{N}{\Psi} = \frac{N}{CV_c^2 + 1} \qquad (5)$$

where *N* is the census number of clones.

The effective parent number is divided into female [$N_{p(f)}$] and male [$N_{p(m)}$] gametic parents as,

$$N_{p(f)} = \frac{N}{\Psi_f} = \frac{N}{CV_f^2+1} \text{ and } N_{p(m)} = \frac{N}{\Psi_m} = \frac{N}{CV_m^2+1} \qquad (6)$$

where CV_f and CV_m are the coefficients of variation in female and male strobilus production and *N* is the number of clones in the seed orchard (Roberds et al., 1991).

Relative effective parent number (N_r) is calculated relative to the census number of clones (*N*). In addition, this can be divided into relative effective female [$N_{r(f)}$] and male [$N_{r(m)}$] parents, which represent the ratio of the effective numbers of female and male parents to the census number.

Gene diversity (*GD*) of seed crops was estimated for parental clone level, based on the effective parent number (N_p) as,

$$GD = 1 - \frac{0.5}{N_p} \qquad (7)$$

Results

Variation of female and male strobilus production

The production of female strobili continued to increase as the ramet matured, whereas the production of male strobili fluctuated over a four-year period (Figure 1 and Table 1). The average number of female strobili was highest in 2017 and lowest in 2015. However, the production of male strobilus peaked in 206 with an average of 28,326 male strobili per ramet, followed by 2015 and 2018 (Table 1).

All ramets (312 grafts) produced female strobili over four years, but there were 24 (2015) and 8 (2018) ramets that were non-fertilized with zero male strobilus production. These variations reflected the coefficient of variation (*CV*), which resulted in greater *CV*s for male strobilus production in 2015 and 2018, respectively (Table 1). The *CV* of male strobilus production among clones was higher than that of female strobilus production except in 2016 (Table 1). The simple correlation coefficients between female and male strobilus production were statistically significant for 2015 and 2016 (Table 1).

The maleness index (M_i) shows how sexual symmetry exists between female and male strobilus production in each clone (Lloyd, 1979). The M_i was somewhat far from expectations for the gender balance of grades in 2015 and 2018 (Figure 2). The average of the maleness index was the highest at 0.51 in 2016 and the lowest at 0.45 in 2015, while the sums of M_i were close to half of the census number (1/2*N*=13); 13.2 in 2016 and 12.7 in 2017. This result implies that the contributions between female and male parents was more sexually balanced in good (2016) and poor (2017) male year (see also Figure 3).

The parental equilibrium curves for female strobilus production were closer to the expectation (equal contribution) than those for male strobilus production in all years (Figure 3). The accumulative contribution line for male strobilus production in good male year (2016) was closer to equal contribution than those in poor years (2015 and 2017). This indicates that clones contributed more equally to male gamete productions in good years than in other years.

For female strobilus production, the most productive 20 % of clones produced from 31.8 % in 2016 to 26.0 % in 2017 of the total female strobili over four years. On the other hand, the strobilus production of the most fertile 20 % clones ranged from 42.0 % in 2015 to 28.0 % in 2016 of the total male strobilus productions, indicating that only a few clones were dominating in the female and male strobili production (Figure 3).

Estimation of variance, heritability, and correlation

Table 2 presents the estimation of variance components and narrow-sense heritability for female and male strobilus production. The ANOVA showed significant differences among clones in female and male strobilus production over a four-year period. The narrow-sense heritability of female strobilus was lower than those of male strobilus for all years, ranging from 0.066 in 2016 to 0.219 in 2018 (Table 2), suggesting that environmental factors such as temperature, wind, and climate might have great effect on female strobili compared to male strobili production. The narrow-sense heritability of male strobilus ranged from 0.216 in 2015 to 0.293 in 2018, implying under genetic control (Table 2).

Pearson's product-moment and Kendall's rank correlation coefficients between female and male strobilus production over four years are shown in Table 3 (see also Figure 1). All of Pearson's correlations were positive and mostly significant, indicating that the female strobilus production mirrored the male strobilus production. Kendall's rank correlation coefficients were also positive only except between male strobilus production in 2015 and female strobilus production in 2017 and 2018 (Table 3).

Effective parent number and gene diversity of seed crops

The fluctuations for female fertility variation (ψ_f) were lower than for the male counterparts (ψ_m) with the exception of 2017 (Table 4). On the other hand, the effective number of female parents [$N_{p(f)}$] was higher than that of male parents [$N_{p(m)}$], except for the poor year (2017), when it reached 25.6 (99 %) in 2017 to 23.9 (92 %) in 2016. These results showed that $N_{p(f)}$ and $N_{p(m)}$ values hardly matched the abundance of strobilus production in the clonal seed orchard of *P. thunbergii*.

The clonal fertility variation (Ψ, sibling coefficient) was close to one with the lowest in 2017 and peaked in 2015 (Table 5). On the contrary, the effective parent number (N_p) was the lowest in 2015 and the highest in 2017. The loss of gene diversity (*GD*) in seed crops was estimated to range from 1.98 (2017) to 2.10 (2015) based on clonal fertility variation (Table 5). When four years were pooled, the variation of clonal fertility and the effective number of parents were improved.

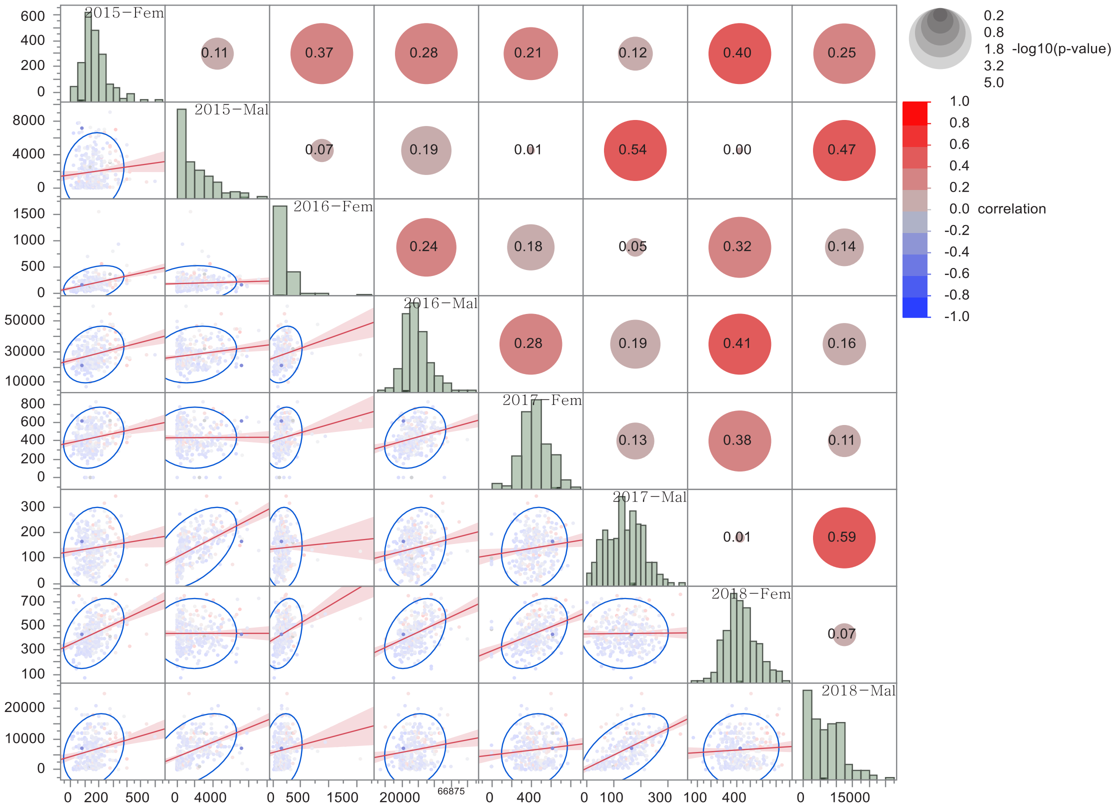

Fig 1
Scattered plot matrix of female and male strobilus production. The diagonal graphs represent the distribution of strobilus production, below the diagonal is the disposition of strobilus production, and above the diagonal shows the correlation among annual production of female and male strobilus production.

Table 1
Strobilus production (mean), range (min and max), coefficient of variation (*CV*), and correlation (*r*) between females and males

Year	2015		2016		2017		2018	
Strobilus	Female	Male	Female	Male	Female	Male	Female	Male
Mean	167.5	2,243.2	189.5	28,325.8	443.2	139.4	439.3	7,231.9
Min	94.5	155.0	81.1	22,181.8	349.6	52.0	289.4	1,300.0
Max	272.0	4,960.0	337.8	39,333.3	548.3	210.6	593.3	14,000.0
CV	0.251	0.615	0.294	0.160	0.122	0.312	0.158	0.497
r	0.233*		0.159*		0.042		0.072	

* represents statistical significance at a 0.5 % probability level.

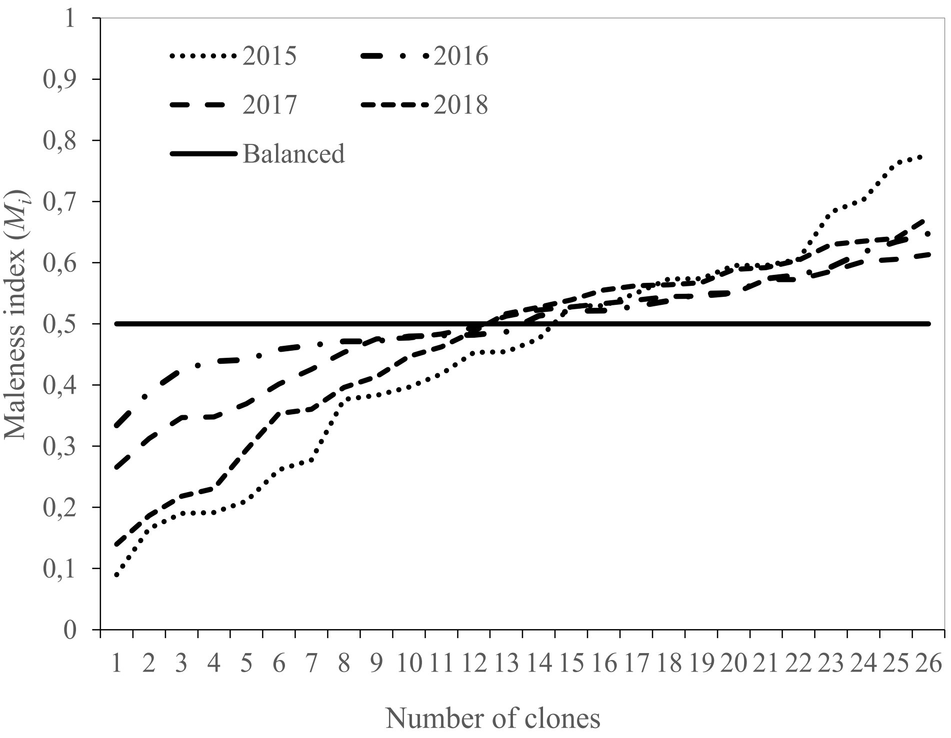

Fig. 2
Maleness index in the second-generation clonal seed orchard of *P. thunbergii*. The horizontal line represents the balanced contribution of female and male parents

Table 2
The variance components of additive (clone, σ^2_c) and environment (error, σ^2_e), and the narrow-sense heritability (h^2)

Year	2015		2016		2017		2018	
Strobilus	Female	Male	Female	Male	Female	Male	Female	Male
σ2c	845*	799,882*	1,255*	13,792,940*	1,775*	1,396*	3,043*	6,909,521*
σ2e	6,863	2902,599	17,682	44,305,311	16,984	2,892	10,829	16,702,594
h^2	0.110	0.216	0.066	0.237	0.095	0.326	0.219	0.293

* and ** represent the significant difference at the probability levels of 0.5 and 0.01, respectively.

Table 3
The Pearson's product-moment (below the diagonal) and Kendall's rank (above the diagonal) correlation coefficients between female and male strobilus production over four years

		2015		2016		2017		2018	
		Female	Male	Female	Male	Female	Male	Female	Male
2015	Female	-	0.150**	0.365**	0.210**	0.145**	0.083*	0.288**	0.188**
	Male	0.117*	-	0.117**	0.096*	-0.009	0.440**	-0.027	0.438**
2016	Female	0.374**	0.072	-	0.219**	0.147**	0.053	0.277**	0.165**
	Male	0.275**	0.196**	0.237**	-	0.234**	0.112**	0.300**	0.111**
2017	Female	0.211**	0.011	0.179**	0.276**	-	0.087*	0.267**	0.046
	Male	0.121*	0.541**	0.048	0.192**	0.135*	-	0.004	0.481**
2018	Female	0.403**	0.008	0.324**	0.408**	0.381**	0.012	-	0.028
	Male	0.246**	0.475**	0.139*	0.160**	0.111	0.592**	0.067	-

* and ** represent the statistical significance at the probability levels of 0.5 and 0.01, respectively.

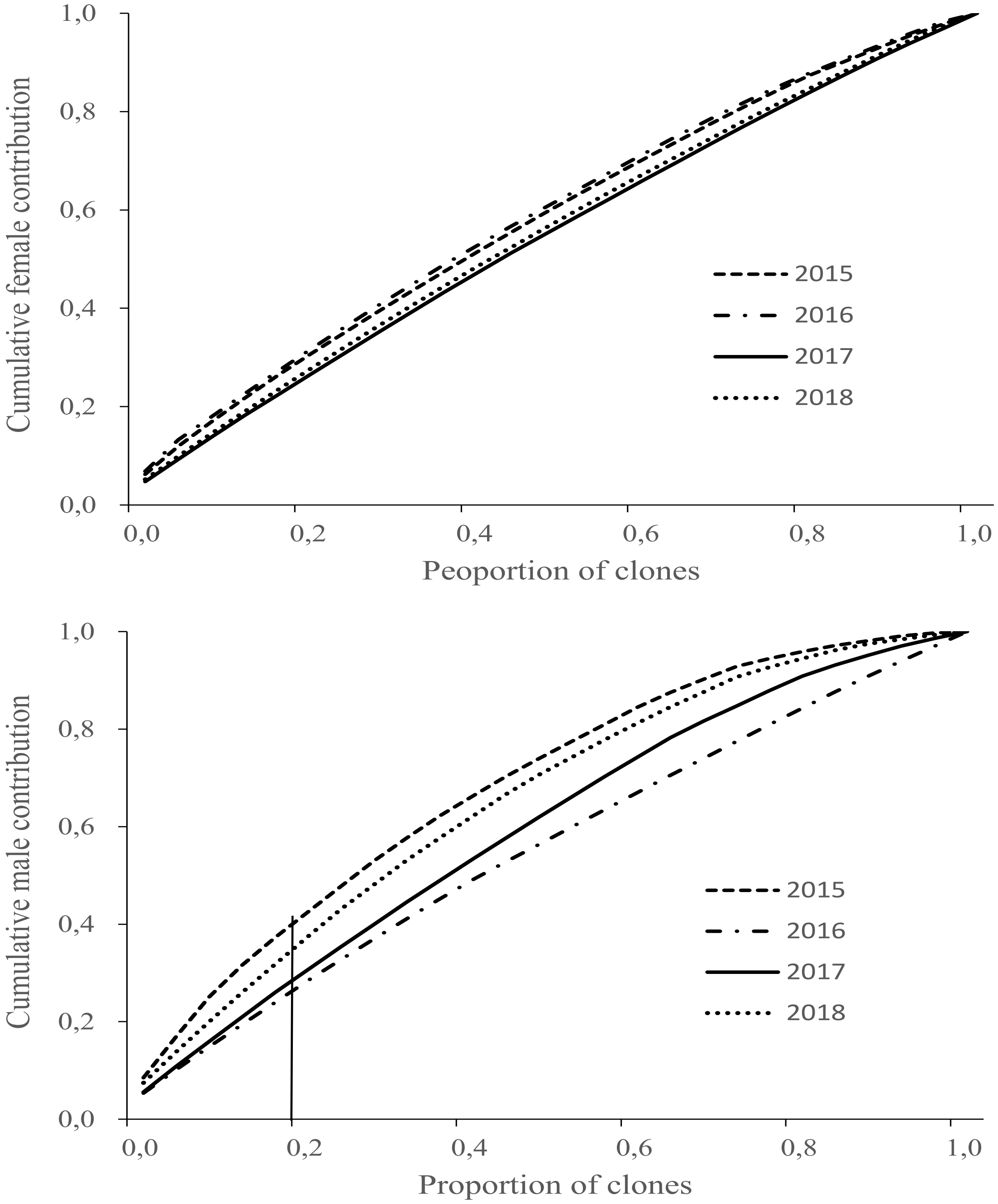

Fig. 3
Parental balance curve for cumulative contribution of female (top) and male (bottom) over a four-year period in the clonal seed orchard of *P. thunbergii*

Table 4
Female (ψ_f), male (ψ_m) fertility variation, effective number of female ($N_{p(f)}$) and male ($N_{p(m)}$) parents, and relative effective number of female [$N_{r(f)}$] and male [$N_{r(m)}$] parents

Year	2015		2016		2017		2018	
Strobilus	Female	Male	Female	Male	Female	Male	Female	Male
ψ_f & ψ_m	1.063	1.379	1.086	1.026	1.015	1.098	1.025	1.247
$N_{p(f)}$ & $N_{p(m)}$	24.5	18.9	23.9	25.3	25.6	23.7	25.4	20.8
$N_{r(f)}$ & $N_{r(m)}$	0.94	0.73	0.92	0.97	0.99	0.91	0.98	0.80

Table 5
Clonal fertility variation (Ψ), effective parent number (N_p), relative effective parent number (N_r) and loss of gene diversity (GD loss) during surveyed years

Year	2015	2016	2017	2018	Pooled
Ψ	1.092	1.032	1.029	1.071	1.034
N_p	23.8	25.3	25.3	24.3	25.1
N_r (%)	91.5	96.9	97.2	93.4	96.7
GD loss (%)	2.10	1.98	1.98	2.06	1.99

Discussion

Clonal fertility variation is the difference in the degree of fertility among clones. Seed orchard programs could not give the production of genetically superior seeds if the clones are not fertile enough. Also, one of the prerequisites in seed orchard programs is equal contribution among clones. However, these assumptions could not live up to the expectations in the actual situation of seed orchards. Thus, the variation of clonal fertility will affect genetic improvement and gene diversity in orchard seeds (Bilir and Kang, 2021; Nikkanen and Ruotsalainen, 2000). The sibling coefficient (Ψ) can be interpreted as the probability that two random gametes are identical by descent in a series of gametes in the population under consideration due to the variation of clonal fertility (Kang, 2001). Therefore, Ψ=1 means the equal contribution of clones to the gamete gene pool in the seed orchard.

In this study, clone fertility could be divided into female and male fertility (equations 3 and 4), with the condition that the production of female and male strobili is an indicator of the contribution of female and male gametes among clones. However, this condition can be influenced by many other factors, such as reproductive phenology, pollen dispersal, pollen vigor, self-incompatibility, and reproductive success (Eriksson et al.1973; Apsit et al. 1989; El-Kassaby and Reynolds 1990; El-Kassaby and Ritland 1992). On the other hand, Gregorius (1989) pointed out that reproductive energy (i.e., strobilus production) can be used to estimate the potential gamete contribution and thus clonal fertility (Kang and Lindgren 1998).

The maleness index is equal to one (M_i=1) when a clone contributes 100 % as a male parent and does not generate female strobili. If the M_i has a value of 0, then the clone contributes as a female parent without producing male strobili. These cases are in complete sexual imbalance (Lloyd, 1979). On the other hand, if all clones contribute equally (e.g., 1/*N*) to both female and male strobili production, then this is perfect sexual equilibrium. However, the complete sexual balance is rarely met in real conditions of seed orchards (Kang et al., 2005; Na et al., 2014).

Parental balance is displayed through a cumulative gamete contribution curve. The cumulative contribution curve changes in female and male strobilus production among clones as proportional contributions (see Figure 3). The observation of female and male strobilus production was found to be slightly different from what was expected (diagonal straight line in Fig. 3). This means that several clones are contributing significantly to the production of female and male strobilus in the clonal seed orchard of *P. thunbergii*. This imbalance affects the reduction of effective parent numbers and consequently the loss of gene diversity in seed crops.

The difference in female and male strobilus production among clones was statistically significant at the 0.01 probability level for four consecutive years (see Table 2). In addition, the degree of variation in strobilus productions was larger among clones (additive effect) than within a clone (environment effect), suggesting that high variation exists among clones. On an individual ramet basis, the narrow-sense heritability values for female strobilus production ranged from 0.066 in the poor flower year (2016) to 0.219 in the good year (2018). In all years surveyed, the heritability values for female production were lower than those for male production. The narrow-sense heritability indicates that male strobilus production is under moderate genetic control, whereas female strobilus production is under strong environmental effects (Park et al., 2020).

In this study, it was confirmed that the clones with good production tended to produce more strobilus in other years (see Table 3). There was a positive correlation between female and male strobilus production among clones and years, meaning clones with good strobilus provided more strobilus production in other years. The correlation between female and male strobilus production in each year was significantly correlated in all years, indicating that clones producing more female strobili are also producing more male strobili.

The effective parent number (N_p) is equal to the status number (N_s) if the seed orchard clones are unrelated and non-inbred to each other. The concept of N_p is also the same as previous studies in the first-generation seed orchards (Chaisurisri and El-Kassaby, 1993; Fries, 1994; Park et al., 2020), which was formulated as the concept of N_s and considered a characteristic of seed orchard seeds (Lindgren and Mullin, 1998). The N_p does not depend on the relatedness of clones but takes into account fertility variation among clones. Parents in the second-generation seed orchard were selected backward in their progeny tests and propagated by grafting. Thus, genetic relatedness among clones may exist in the seed orchard, and we found that some clones shared the same female parents. These associations will reduce the effective parent number and increase the accumulation of group coancestry (i.e., inbreeding coefficient).

In the present study, the $N_{p(f)}$ was higher than the $N_{p(m)}$ except in 2016. At the clonal level, the values of N_p were high, ranging from 23.8 in 2015 to 25.3 in 2016. The N_p could be improved when four years were pooled, meaning that mixing orchard seeds from different years can catch high gene diversity. Loss of gene diversity is inversely proportional to the effective parent number following random mating [i.e., $1/(2N_p)$] (Kang and Lindgren, 1999, Park et al., 2020). The loss of gene diversity in seed crops ranged from 2.10% in 2015 to 1.98 % in 2016 and 2017. Therefore, some loss of gene diversity is expected in the second-generation seed orchard of *P. thunbergii*.

Conclusion

The study found clonal variation in fertility among clones in the second-generation seed orchard of *P. thunbergii*. Although decreases in gene diversity in seed crops from the orchard were observed compared to wild reference populations, this was not unexpected. Notably, the N_p equation was found to be a useful indicator for determining orchard composition based on expected relatedness and group coancestry of seed crops, reflecting the loss of gene diversity due to fertility variation among clones in seed orchards. Therefore, it provides orchard managers with a valuable tool for maintaining the gene diversity of seed crops in orchard management. It is recommended that orchard managers consider sexual imbalance and variation in clonal fertility, and implement strategies such as equal cone harvesting to ensure effective orchard management.

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