Effects of Supplementary Clones on Genetic Diversity in Slash Pine Seed Orchards (Postprint)

Authors: Qin Xianyu, Feng Yuanheng, Xie Junkang, Meng Lanyang, Yang Zhangqi

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Abstract

Supplementing clones in seed orchards with few established clones or many missing plants is an important measure for scientific management of seed orchards. This study took the slash pine (Pinus elliottii) clonal seed orchard at the Nanning Forestry Research Institute as the research object, analyzed the impact of clone supplementation on the genetic diversity of the orchard, aiming to provide reference suggestions for scientific management of seed orchards. Using 16 pairs of SSR markers, the changes in genetic diversity before and after clone supplementation in the orchard were analyzed, and the genetic specificity and relatedness of the orchard materials were characterized through constructing fingerprint profiles and genetic cluster analysis. The results showed that: (1) By supplementing the clones from 18 to 50, the polymorphic information content of genetic loci increased by 2.24%, the average number of alleles increased by 14.29%, the average number of effective alleles increased by 1.19%, and the Shannon index increased by 3.84%. (2) Using 11 pairs of SSR markers, all 50 established clones could be identified, and fingerprint profiles for 50 slash pine clones were constructed accordingly. (3) The genetic distance among the 50 clones ranged from 0.018 to 0.670, and the UPGMA clustering diagram, with 0.251 as the threshold, could divide the 50 clones into 7 groups. Based on the above research results, the following conclusions can be drawn: Supplementing clones can improve the genetic diversity of slash pine seed orchards, but the improvement is limited; constructing fingerprint profiles can provide a reliable tool for identification of superior slash pine clones and paternity analysis of seed orchard progeny; the genetic clustering results of established clones can provide a reference for parental selection in slash pine hybridization experiments and can effectively avoid inbreeding.

Full Text

Effects of Supplementary Clones on Genetic Diversity of *Pinus elliottii* Seed Orchard

Qin Xianyu[1],[2], Feng Yuanheng[2], Xie Junkang[2], Meng Lanyang[3], Yang Zhangqi[2]*

[1]College of Life Sciences, Guangxi Normal University, Guilin 541006, Guangxi, China
[2]Timber Forest Research Institute, Guangxi Institute of Forestry Science, Nanning 530002, China
[3]Nanning Institute of Forestry, Nanning 530107, China

Abstract: Supplementing clones in seed orchards with limited or missing clones represents a crucial scientific management strategy. This study examined the clonal seed orchard of *Pinus elliottii* at the Nanning Forestry Research Institute to analyze how supplementary clones influence genetic diversity, providing reference recommendations for scientific orchard management. Using 16 SSR marker pairs, we assessed genetic diversity changes before and after clone augmentation, and analyzed the genetic specificity and relatedness of breeding materials through fingerprinting and clustering analysis. Results showed: (1) Increasing clones from 18 to 50 raised the polymorphic information content by 2.24%, average allele number by 14.29%, average effective allele number by 1.19%, and Shannon's index by 3.84%. (2) Eleven SSR marker pairs could distinguish all 50 clones, enabling construction of a fingerprint map for the 50 *P. elliottii* clones. (3) Genetic distances among the 50 clones ranged from 0.018 to 0.670, with UPGMA clustering at a 0.251 threshold dividing them into seven groups. Based on these findings, we conclude that while clone supplementation can improve genetic diversity in *P. elliottii* seed orchards, the improvement is modest. The constructed fingerprint map provides a reliable tool for superior clone identification and progeny parentage analysis. Genetic clustering results can guide parent selection for hybridization experiments, effectively preventing inbreeding.

Keywords: *Pinus elliottii*, EST-SSR, genetic diversity, clonal expansion, genetic breeding

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Introduction

Pinus elliottii, native to the southeastern coastal regions of North America, Cuba, and Central America, is recognized globally as a premium industrial timber species due to its strong adaptability, drought and barren tolerance, high survival rate, rapid early growth, and high resin yield (Ding et al., 2020). Introduced to China in the 1930s and widely cultivated from the mid-1980s, it has become a key afforestation species in southern China. The United States, as the species' origin, has established a comprehensive breeding system with notable

improvement results (Wang, 2012; Zhang et al., 2017). As an introduced species, China's genetic improvement efforts began later, with the first *P. elliottii* clonal seed orchard established in Guangdong in the 1960s (Zhao et al., 2018). By the early 21st century, China had established six national improved variety bases (State Forestry Administration, 2009, 2017), accompanied by research on flowering phenology (Zou, 2011; Zhang et al., 2022), phenotypic variation (Huang et al., 2015; Wu et al., 2016; Gong et al., 2017; Wu et al., 2019), progeny testing (Liu et al., 2010; Lin, 2015), resin trait improvement (Pan, 2014; Zhang et al., 2017), and clonal genetic diversity evaluation (Yi et al., 2000; Zhao et al., 2001; Deng et al., 2020).

As China increasingly emphasizes intellectual property rights for "seeds," independent innovation in forest germplasm has become critical (Lü, 2015). Localized improvement to create new *P. elliottii* germplasm with higher productivity and better adaptation to China's geographical and climatic conditions is a current breeding priority (Huo et al., 2023; Feng et al., 2023). As a timber and resin dual-purpose species with a 15–20+ year management cycle and complex mountainous/foothill planting sites, *P. elliottii* seedlings require rich genetic diversity for ecological adaptability. This demands seed orchards with both high genetic gain and broad genetic bases. However, as an introduced species without natural germplasm resources in China, its genetic foundation depends entirely on the richness of introduced materials. Early introductions lacked systematic planning, with southern provinces failing to separate breeding and production populations. Instead, they relied on seed orchards for both seed production and breeding research, leading to purposeful introductions that prioritized high yield and stability of target traits (Ling, 2007; Shen and Huang, 2018; Kang, 2023) while neglecting population diversity considerations, thereby constraining localized improvement progress. To support sustainable localized improvement, introduced *P. elliottii* germplasm resources must be surveyed and organized, with new breeding materials collected to supplement existing seed orchards.

This study used clones from the Nanning Forestry Research Institute *P. elliottii* seed orchard as research material. Based on SSR loci information obtained from transcriptome sequencing, primers were designed and screened via PCR amplification and polyacrylamide gel electrophoresis. By analyzing genetic diversity changes before and after clonal supplementation and the post-supplementation genetic structure, we aimed to evaluate the effectiveness of clone supplementation in increasing genetic diversity, providing molecular-level theoretical guidance for localized sustainable improvement and parent selection in controlled pollination breeding.

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1. Materials and Methods

1.1 Study Site Overview The *P. elliottii* clonal seed orchard at Nanning Forestry Research Institute is located in Wuming District, Nanning, Guangxi

(108°00 E, 23°10 N). The region features a south subtropical monsoon climate with an average annual temperature of 21.5°C, precipitation of 1,246 mm, evaporation of 1,613.8 mm, and 358 frost-free days. Distinct wet and dry seasons occur with ~79% average relative humidity. The orchard sits on gentle hill terraces among karst peak forests at ~120 m elevation, with deep lateritic red soil (pH 5.5–6.5) (Zhou, 2020).

Established in 1990 with 18 clones, this orchard serves as Guangxi's primary *P. elliottii* seed production and hybridization test base. Since 2006, Guangxi Forestry Science Academy has conducted intraspecific and interspecific hybridization trials for 16 consecutive years, creating over 1,000 hybrid combinations and selecting one new forest variety and two improved varieties. The limited number of clones restricted both seed genetic diversity and parent selection range for hybridization. To address this, the research team initiated a new round of superior tree selection in 2010 from *P. elliottii* genetic test sites and resin-producing stands across Guangxi, excluding progeny stands to avoid inbreeding. In 2015, 32 new clones were selected from the collection nursery and added to the orchard, expanding the total to 50 clones (Table 1).

1.2 Experimental Materials Fresh, disease-free needles were collected from each of the 50 clones in the seed orchard (Table 2), placed in self-sealing bags labeled by clone number, and stored at -80°C.

1.3 Genomic DNA Extraction and Detection Total DNA was extracted from needles using the Biospin Plant Genomic DNA Extraction Kit (BioFlux, Beijing). Concentration was measured at ~25 ng $\cdot$ L^{-1} using a spectrophotometer, then stored at -20°C.

1.4 SSR-PCR Amplification and Product Detection SSR loci were identified from transcriptome-assembled EST sequences with the following criteria: SSR length 12–30 bp, di-nucleotide repeats $ $6, tri- and tetra-nucleotide repeats $ $5 and $ $3, respectively. Primers were designed for 50–500 bp amplicons, 18–22 bp length, and $ $3°C annealing temperature difference between forward and reverse primers. A total of 136 EST-SSR primer pairs were developed and synthesized by Guangzhou Aiji Biotechnology.

SSR-PCR used a 10 L reaction: 2 L DNA (25 ng $\cdot$ L^{-1}), 1 L PCR Buffer (10×), 0.2 L dNTPs (10 mmol $\cdot$ L^{-1} Mg^{2+}) (Shanghai Jierui), 0.25 L each primer (10 mmol $\cdot$ L^{-1}), 0.07 L Taq DNA Polymerase (5 U $\cdot$ L^{-1}) (Shanghai Jierui), and 6.23 L ddH_2O. Amplification was performed on a TAdvanced 96G gradient PCR with: 94°C for 4 min; 30 cycles of 94°C for 15 s, 56°C for 15 s, 72°C for 30 s; final extension at 72°C for 20 min; hold at 12°C.

Products were separated on 8% polyacrylamide gels. For each 10 L PCR product, 7 L loading buffer was added, and 7 L was loaded per well. Electrophoresis ran at 220 V until the indicator exceeded 2/3 of the gel length. Gels were then

fixed, silver-stained, developed until clear bands appeared, and photographed (Li et al., 2023).

1.5 Data Statistics and Analysis Amplified bands were manually scored and numbered by size. CERVUS 3.07 was used to calculate polymorphism information content (PIC) per locus. POPGENE 1.32 calculated observed allele number (Na), effective allele number (Ne), observed heterozygosity (Ho), and Shannon's index (I). NTSYS 2.10 constructed UPGMA dendrograms. PIC_{CALC} computed PIC values (Botstein et al., 1980). PIC values classify SSR polymorphism: PIC $ $0.25 (low), 0.25<PIC<0.5 (moderate), PIC>0.5 (high). Effective amplification rate = (amplified primers)/(total primers). Polymorphism rate = (polymorphic amplified primers)/(total primers).

1.6 DNA Fingerprinting Construction Following methods for poplar (Liu et al., 2021) and *Pinus armandii* (Cao, 2024), a fingerprint map was constructed. SSR primers for identification were sequentially labeled A, B, C…E (excluding I due to similarity with 1). Allele types per clone for each primer pair were recorded with "/" separators, using only numeric values; absent loci were coded as 000. Each clone received a unique alphanumeric SSR fingerprint code.

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2. Results

2.1 Polymorphic Primer Screening Eight *P. elliottii* samples were used for initial screening. Of 136 primer pairs, 98 successfully amplified target fragments (72.06% effective amplification rate). Rescreening with 18 samples yielded 16 primer pairs with clear bands, good repeatability, and high polymorphism (Table 3), giving a 16.33% polymorphism rate. These 16 primer pairs were used to detect polymorphism in 50 clones (Figure 1 [Figure 1: see original paper]) and analyzed with Popgene 1.32.

Polymorphism analysis (Table 4) showed pre-supplementation Na ranged 2.00–5.00 (mean 2.63), with PIC values 0.10–0.59 (highest: PQ111; lowest: PQ1, PQ26, PQ585). Two loci were highly polymorphic (PIC>0.5), seven moderately (PIC>0.25), and seven low (PIC<0.25). Post-supplementation, Na ranged 2.00–5.00 (mean 3.00), PIC values 0.06–0.58 (highest: PQ128, PQ593; lowest: PQ585). Eight primers (PQ26, PQ31, PQ90, PQ111, PQ319, PQ585, PQ606) showed decreased PIC after supplementation. Two loci remained highly polymorphic, six moderate, and eight low. Overall, the 16 loci showed low polymorphism, with supplementation decreasing PIC at eight loci.

2.2 Genetic Diversity Analysis of *P. elliottii* Seed Orchard Popgene 1.32 analysis revealed that pre-supplementation, 16 primer pairs detected 42 alleles across 18 clones, averaging 2.62 alleles per primer. Ne ranged 1.12–2.79 (mean 1.63); I ranged 0.22–1.23 (mean 0.57); Ho ranged 0.28–0.89 (mean 0.67).

Post-supplementation (50 clones), 48 alleles were detected, averaging 3.00 alleles per primer (14.29% increase). Mean Ne was 1.65 (1.19% increase). Ho ranged 0.28–0.94 (mean 0.69, 2.19% increase). I ranged 0.14–1.13 (mean 0.59, 3.84% increase).

In summary, genetic diversity increased slightly after supplementation. The greatest improvement was in allele number, while Ne, I, and Ho increased by $ $5%, indicating that supplementation introduced some low-frequency alleles but minimally altered overall gene frequencies and genotype complexity.

2.3 Fingerprinting of *P. elliottii* Seed Orchard DNA fingerprinting should maximize germplasm discrimination with minimal primers for efficiency and cost reduction (Sun et al., 2023). Analysis showed that 11 SSR markers (PQ111, PQ128, PQ593, PQ125, PQ19, PQ1, PQ319, PQ4, PQ90, PQ606, PQ429) could distinguish all clones, labeled sequentially A–L (excluding I). Allele base pair lengths were recorded for each clone, generating unique SSR fingerprint codes (Table 6). For example, Clone 1's code: A112/112B80/90C435/435D000/000E255/267F286/286G374/386H204/204J257/257K407/407L370/378, where A112/112 indicates PQ111 alleles at 91 bp/91 bp, and D000/000 indicates no amplification for primer PQ125.

2.4 Genetic Clustering Analysis of *P. elliottii* Seed Orchard Popgene 1.32 estimated Nei's genetic distances (GD) among clones. Original clones showed GD = 0.018–0.600, with 81.70% of pairwise combinations exceeding the mean (GD=0.260). The maximum GD occurred between clones 5 and 7; the minimum between clones 10 and 18. Supplementary clones showed GD = 0.055–0.670, with 44.76% exceeding the mean (GD=0.251). Maximum GD occurred between clones 43 and 49; minimum between clones 29 and 33. GD between original and supplementary clones ranged 0.018–0.670, with 45.66% exceeding the mean (GD=0.251). Maximum GD occurred between clones 5 and 32; minimum between clones 17 and 33.

NTSYS 2.10 clustering based on Nei's GD for 50 clones (Figure 2 [Figure 2: see original paper]) identified seven breeding groups (G1–G7) at GD=0.251 (Table 7). Clones did not cluster by geographic origin. Further clustering of the seven breeding groups (Figure 3 [Figure 3: see original paper]) clarified inter-group genetic relationships.

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3. Discussion

3.1 SSR Primer Polymorphism and Seed Orchard Genetic Diversity SSR primer utility and polymorphism richness can be assessed via PIC and Na. Yi et al. (2017) developed 24 *P. elliottii* SSR primers with high, moderate, and low polymorphism rates of 25.00%, 25.00%, and 50.00%, respectively, averaging

3.38 alleles among 113 families. In comparison, our 16 primer pairs showed pre-supplementation rates of 12.50% (high), 43.50% (moderate), and 43.75% (low), with post-supplementation rates of 12.5%, 37.5%, and 50%. Mean Na was 2.63 pre-supplementation and 3.00 post-supplementation, both lower than Yi's study. This discrepancy arises because Na heavily depends on sample size; our supplementation increased Na, but Yi's larger sample (113 families) naturally yielded higher values. These findings confirm our SSR markers are suitable for *P. elliottii* genetic diversity research.

Genetic diversity is fundamental for population adaptation and disease resistance. Pre-supplementation analysis of 18 clones showed mean Shannon's index of 0.57, higher than Yi et al. (2000) but lower than other pine seed orchards (Chen et al., 2024; Cao et al., 2024; Yan et al., 2024), indicating a relatively narrow genetic base. As an introduced species, *P. elliottii*'s genetic foundation depends on introduced germplasm richness. The Nanning orchard, established in 1990 with limited surviving clones due to technical constraints, had far fewer clones than other orchards. Supplementing 32 superior clones increased Shannon's index by <5%, below expectations. This likely reflects limited genetic background diversity, as most new clones originated from Guangxi (same 1990s introduction batch) or Hunan (28.12% from a single forest farm). Thus, while introducing clones can broaden genetic bases, selection must consider genetic background, superior traits, and adaptability through wide geographic and genetic background sampling.

3.2 Molecular Markers and Breeding Material Relationship Management Molecular markers in forestry are applied to study genetic variation patterns from mating systems or geographic isolation (Zhang, 2008), conduct genetic classification and phylogenetic research (Ajatd et al., 2018; Everaert et al., 2020), evaluate genetic diversity (Ruis et al., 2019; Yang et al., 2021), identify germplasm (Mei et al., 2017), construct genetic linkage maps (Dong and Shi, 2004), and enable marker-assisted breeding (Huang, 2006). In seed orchard management, they primarily assess genetic diversity, mating system stability, superior clone identification, and breeding material genetic management.

In this study, 16 SSR markers evaluated genetic diversity while constructing an 11 × 50 DNA fingerprint map, clarifying DNA marker characteristics for two improved varieties (including one new variety). These SSR markers can also analyze mating systems, verify hybrid offspring, and conduct parentage analysis of open-pollinated progeny, forming a complete SSR-based marker-assisted breeding technology for *P. elliottii*.

Clustering showed 60% of clones grouped together, indicating small genetic background differences. Most clones from Miluo Taolin State Forest Farm (Hunan) and Guangxi test stands (Bobai, Qipo, Gaofeng, Liuzhou, Nanning) clustered together, suggesting close genetic relationships. Original clone 5 formed a separate group, indicating distant relatedness. Inbreeding causes viability and reproductive declines, weak growth, and developmental defects (Zhao et

al., 2019). As both production and breeding populations, seed orchards must avoid inbreeding. However, few studies correlate *P. elliottii* inbreeding levels with growth performance. Based on our results, we propose two schemes for controlled pollination:

1. **Seven-Group Structure:** At GD=0.251, divide 50 parents into seven breeding groups (G1–G7) containing 1–29 clones each. Select hybrid parents from different groups, ensuring inter-group GD $ $0.251 to prevent inbreeding. The drawback is uneven group sizes complicating genetic design, with BG2 containing ~60% of clones, preventing intra-group crosses among 29 clones.
2. **Inbreeding-Group Structure:** Clones within GD<0.200 on the same dendrogram branch are considered high-similarity inbreeding groups (15 groups, 1–15 clones each). Parents cannot come from the same inbreeding group. This approach maximizes crossing opportunities while preventing obvious inbreeding, though more research is needed to confirm GD=0.20 as the critical non-inbreeding threshold.

3.3 Clone Expansion and Sustainable Seed Orchard Renewal Expanding and adjusting clones to improve genetic quality represents active orchard management. China's forest improvement program has established numerous primary and advanced-generation seed orchards. With age, these orchards become tall, low-yielding, costly to harvest, and genetically outdated, leading to abandonment or reconstruction. However, reconstruction requires substantial resources and years to become productive. Early-flowering species like *Pinus massoniana* need 4–5 years to produce seeds and 7 years for full production; late-flowering species like *P. elliottii* require even longer. With breeding cycles shortening, superior materials may emerge within 10 years of orchard establishment, necessitating updates without complete reconstruction.

Orchard renovation primarily uses trunk cutting or crown grafting (Huang et al., 2017). Trunk cutting only rejuvenates without genetic improvement, while grafting is technically demanding and costly. Natural mortality continuously reduces orchard stocking, especially during senescence. The Nanning orchard had <60% survival before supplementation. Planting new clones in empty spaces increases clone numbers, improves genetic diversity, and mitigates pollen shortage and yield decline from missing trees. Subsequently, low-combining-ability clones can be systematically removed based on progeny test results while supplementing new superior clones, enabling gradual orchard renewal without complete reconstruction—saving costs and avoiding production interruption.

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Note: Figure translations are in progress. See original paper for figures.