

Article

Genetic Diversity and Mating System Analysis in a Second-Generation Seed Orchard of *Chamaecyparis hodginsii*

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Abstract

Plantations of the valuable Chinese timber species, *Chamaecyparis hodginsii*, established by planting, primarily rely on seed orchards for propagation. Therefore, effective management of the genetic composition of these orchards is essential to ensure a sustainable supply of high-quality seeds. However, the mating system and pollen dispersal mechanisms in its high-generation seed orchards remain unclear, limiting precise genetic management. To address this, we analyzed 30 parental clones and 75 of their open-pollinated progeny from a second-generation seed orchard using 15 polymorphic EST-SSR markers. Compared to reported natural populations of *C. hodginsii*, both parental and progeny populations maintained high genetic diversity (mean $uHe = 0.438$ and 0.449 , respectively), with a significant excess of heterozygotes (mean $Fis = -0.084$ and -0.066 , respectively). Population genetic structure analysis indicated weak genetic differentiation among the parental genetic groups (mean $Fst = 0.012$), which was further reduced in the progeny population (mean $Fst = 0.003$) due to open pollination, reflecting a trend toward genetic homogenization. The mating system was characterized exclusively by outcrossing ($tm = 1.000$). However, paternity analysis revealed highly skewed paternal contributions, a low effective number of pollen donors ($Nep = 8.13$), and contributions of S5, S11, and S17 as dominant pollen parents, with 17.33% external pollen flow. These findings elucidate the mechanisms underlying sustained genetic diversity despite unequal paternal contributions and provide a theoretical basis for optimizing parental configuration and pollen management.

Keywords: *Chamaecyparis hodginsii*; seed orchards; EST-SSR; genetic diversity; mating system; paternity analysis; gene flow



Academic Editor: John E. Carlson

Received: 12 November 2025

Revised: 9 January 2026

Accepted: 14 January 2026

Published: 15 January 2026

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1. Introduction

Chamaecyparis hodginsii, an evergreen conifer belonging to the family Cupressaceae, is a valuable timber and ecological species native to southeastern China and northern Vietnam. Its rapid growth, straight trunk, and superior wood quality confer significant economic

value [1–4]. It comprises biologically active secondary metabolites (e.g., diterpenoids) exhibiting antibacterial and anticancer effects. Furthermore, the leaf-derived essential oil possesses mosquito-repellent properties, underscoring its ecological and medicinal importance [5,6]. As a monoecious conifer, *C. hodginsii* exhibits a unique reproductive strategy characterized by two distinct flowering seasons. The spring flowering (April–May) produces non-viable seeds that mature by October, while the autumn flowering (September–October) yields fertile seeds, with cones maturing in October of the following year [7]. Phenological observations on grafted second-generation breeding germplasm indicate that, despite some genotypic variation, the peak anthesis for both male and female strobili is consistently concentrated in early November [8,9]. These specific biological traits—a prolonged seed development cycle and a relatively narrow and synchronized window of peak receptivity—underscore the critical need to understand its mating system for the effective genetic management of seed orchards. Floral synchrony is recognized as the most significant factor affecting the mating system and pollination efficiency in seed orchards [10–12]. However, long-term overexploitation has caused a sharp decline in its natural populations. Consequently, establishing high-quality plantations has become a crucial strategy for the conservation and sustainable utilization of this species [13–15].

Improved seeds are crucial for the establishment of high-quality plantations and promoting the regeneration of natural forests. Currently, the improved seeds of *C. hodginsii* are primarily obtained from selected superior trees. Seed orchards serve as the primary source of genetically improved seeds, and the genetic quality of their seeds directly governs plantation productivity and quality [16]. Advanced-generation seed orchards have a substantial impact globally. For instance, in the United States, over 400,000 ha of *Pinus taeda* L. plantations are established annually using improved seeds derived from seed orchards [17]. In Sweden, up to 85% of *Pinus sylvestris* and *Picea abies* plantations rely on improved seeds derived from seed orchards [18,19]. In China, the breeding program for *C. hodginsii* commenced in the 1970s [20] and has progressed to the second-generation seed orchard stage. These second-generation seed orchards can significantly enhance genetic gain and adaptability in the progeny via grafting of superior clones selected through genetic testing [21–23].

A genetic analysis of the production population is crucial to ensure the sustained production of high-quality seeds in seed orchards. Understanding its genetic diversity, which is the foundation of population adaptation and evolution, is essential for evaluating the orchard's breeding potential and the quality of its progeny stands [24,25]. Torres-Dini et al. [26] evaluated a clonal seed orchard of *Pinus taeda* L. using SSR markers and reported that it could provide up to 17% genetic gain in wood volume for the progeny while maintaining a diverse genetic base. For a clonal seed orchard of *Pinus koraiensis*, Yan et al. [27] reported rich genetic variation in the parental population, with 98% of the variation occurring within populations, and established a DNA fingerprinting system for clone identification.

The mating system is another key factor influencing the genetic composition of seed orchards. Inadequate mating patterns can lead to inbreeding depression, significantly reducing seed viability and seedling performance [28,29]. Paternity analysis can significantly elucidate mating systems, enabling the identification of dominant pollen parents and estimation of external pollen flow levels, guiding optimal parental configuration. A study on *Pinus sylvestris* [30] combined microsatellite-based paternity assignment with a neighborhood mating model to quantitatively partition the effects of male fecundity, inter-tree distance, and anisotropic pollen dispersal on mating success. This approach demonstrated that variation in pollen production alone could explain up to 78% of the variance in male reproductive success, highlighting the power of such analytical frameworks in deciphering

the complex interplay of factors governing mating patterns in wind-pollinated conifer seed orchards. Paternity analysis reported by Torimaru et al. [31] revealed a high level of external pollen flow (pollen contamination) of 52% along with a low selfing rate (2.3%), offering important insights into the orchard's functional dynamics and the potential impact of ex-ternal pollen flow on genetic gain. Previously, another paternity analysis, conducted using SSR markers, revealed highly skewed paternal contributions in a *Picea abies* seed orchard, with an effective population size (N_e) of only 60%–76% of the actual parental number and external pollen flow rates of 20%–28% [32]; this study confirmed the positive role of external pollen flow in maintaining genetic diversity, particularly when the parental number is limited.

Besides genetic diversity and mating patterns, fertility variation among parental individuals is another critical factor profoundly influencing the genetic composition and progeny quality of seed orchards. Within an orchard, significant differences in pollen output and seed set often exist among clones or individuals. This unequal distribution of fertility can lead to a dramatic reduction in effective population size relative to the actual number of parents, thereby exacerbating genetic drift and potentially lowering the predicted gene diversity of the seed crop [33]. For instance, studies in second-generation seed production areas and clonal seed orchards of *Eucalyptus camaldulensis* have demonstrated that high fertility variation resulted in substantially reduced effective population size, which in turn affected the genetic diversity and field survival of the progeny [34]. This indicates that even within a well-designed mating system, failure to manage the reproductive contributions of parents may still prevent the optimal balance between breeding gain and maintenance of genetic diversity. Therefore, a comprehensive genetic evaluation of a second-generation *C. hodginsii* seed orchard should not only clarify its mating system and gene flow, but also elucidate the mating dynamics, including the identity and relative contribution of paternal parents.

Despite these reports on several conifers, the mating system and pollen dispersal mechanisms in second-generation seed orchards of *C. hodginsii* remain unclear, which significantly limits the precise management of the seed production process and further genetic improvement. Therefore, accurate assessment using reliable molecular marker-based techniques is necessary. Simple sequence repeat (SSR) markers, with high polymorphism and co-dominant nature, present an effective tool for assessing genetic diversity and deciphering mating systems [35,36].

Given the utility of these markers, this study was conducted with the following objectives: first, to determine if genetic diversity is effectively maintained from parental to progeny generations within a managed second-generation seed orchard; and second, to characterize the mating system, specifically assessing the degree of outcrossing, the distribution of paternal contributions, and the level of external pollen flow.

Accordingly, we used 15 polymorphic EST-SSR markers to assess the genetic diversity and structure of both parental and progeny populations, identify dominant pollen parents and estimate the external pollen contamination rate through paternity analysis, and elucidate the characteristics of the mating system. By pursuing these objectives, this study aims to provide a scientific basis for optimizing the genetic management of *C. hodginsii* seed orchards and to facilitate the development of advanced-generation breeding strategies.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

The 30 parental clones (S1–S30) used in this study were selected through a multi-stage genetic evaluation. The selection was based on a 12-year-old progeny trial established from phenotypically superior trees across nine natural provenances. These provenances

constitute the nine genetic groups (Group 1 to Group 9) analyzed throughout this study. The group assignment for both parental clones and their open-pollinated progeny is based on the geographic provenance of their maternal parent (The detailed geographic origins corresponding to each group and the specific assignment of each individual are provided in Supplementary File S1). Phenotypic values and predicted genetic gains for stem volume, tree height, and diameter at breast height (DBH) were estimated (Supplementary File S1). The primary selection criterion was a high predicted genetic gain for stem volume ($>20\%$), while provenance diversity was also considered to ensure a broad genetic base.

These selected clones constitute the second-generation *C. hodginsii* seed orchard (Figure 1), which is located at the Tuditan Work Area of Anxi Fengtian State-Owned Forest Farm ($25^{\circ}16'37''$ N, $118^{\circ}02'05''$ E; Anxi, Fujian, China). This region has a subtropical monsoon climate, with a mean annual temperature of 18.5°C and an annual precipitation of 1600 mm. The seed orchard was established in 2012 by rootstock planting and grafted in 2013; it covers a total area of 3.33 hectares (ha) at an elevation of 410–620 m on a southwestern slope. The soil is mountain red soil, characterized by moderately deep layers, a thin humus layer, and a clay loam to clay texture.



Figure 1. The second-generation *C. hodginsii* seed orchard.

The orchard comprises these 30 parental clones (S1–S30), selected from nine provenances. They are distributed across three production zones, resulting in a total of 2685 grafted ramets in a controlled, completely randomized layout, with a $4\text{ m} \times 4\text{ m}$ spacing and a minimum distance of $\geq 6\text{ m}$ between ramets of the same clone.

Although systematic phenological monitoring was not conducted during the seed collection year (2018) of this study, detailed observations on the grafted breeding population, which constitutes this seed orchard, have been previously carried out by our group [9]. That work confirmed that peak anthesis for both male and female strobili in such clones is consistently concentrated in early November, with male cone pollen shedding preceding

female cone receptivity by approximately three days. Consequently, the mating system analysis in the present study is interpreted within this established phenological framework.

To evaluate the genetic outcome of this orchard, an open-pollinated progeny trial was established in March 2020 using one-year-old bare-root seedlings. These seedlings were grown from seeds collected in October 2018 from the second-generation seed orchard (approximately 5–6 years post-grafting at that time). Field observations confirmed that the majority of grafted ramets had reached reproductive maturity with sufficient flowering, ensuring a diverse and effective pollen pool for open pollination. The seeds were sown in January 2019. The trial was performed at the Xiazhen Work Area of the forest farm (Compartment 04, Subcompartments 15–1, 15–2, and 15–3), which covered an area of 6.87 ha; a randomized complete block design (RCBD) with six replications of five-tree plots at a spacing of 2 m × 2 m was considered.

The plant materials comprised 30 parental clones from the second-generation seed orchard and 75 of their open-pollinated progeny. Additionally, we included 11 candidate paternal parents derived from open-pollinated half-sib families in a nearby 40-year-old *C. hodginsii* plantation (one individual per family), resulting in a total of 41 candidate paternal parents.

Total genomic DNA was extracted from all *C. hodginsii* samples using a plant genomic DNA Kit (DP320, Tiangen, Beijing, China). Extracted DNA was qualitatively and quantitatively assessed using 1% agarose gel electrophoresis (DYY-6C, Liuyi Biotechnology, Beijing, China) and a NanoDrop ONE spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Only qualified DNA samples were processed for subsequent experiments.

2.2. PCR Amplification and EST-SSR Analysis

We used 192 EST-SSR primer pairs in this study, which included primers newly designed using Primer Premier 3.0 [37] based on *C. hodginsii* transcriptome data and those previously developed by our group [38]. The new primers were designed based on the following criteria: (1) primer length of 18–22 bp; (2) expected PCR product length of 100–400 bp; (3) annealing temperature (T_m) of 50–60 °C; and (4) GC content of 40%–60%.

Initially, polymorphism screening of the 192 primer pairs was conducted using DNA samples derived from eight *C. hodginsii* individuals from diverse provenances. PCR amplification was performed using a touchdown PCR method using a 10 µL reaction mixture containing 5.0 µL of 2× Taq PCR Master Mix (GeneTech, Shanghai, China), 1.0 µL of genomic DNA template (~20 ng), 0.5 µL of each forward and reverse primer (10 µM), and 3.0 µL of ddH₂O. The PCR program was set as follows: 95 °C for 5 min; followed by 10 touchdown cycles of 95 °C for 30 s, annealing for 30 s (starting at 62 °C and decreasing by 1 °C per cycle to 52 °C) [39], and 72 °C for 30 s; 25 standard cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s; a final extension at 72 °C for 20 min. Amplification products were resolved through electrophoresis using a 2% agarose gel. Primers yielding clear and polymorphic bands were selected for subsequent high-throughput genotyping.

For this purpose, the selected primers were fluorescently labeled using the TP-M13-SSR method [40]. Specifically, universal M13 primers (5'-TGTAACGACGCGCCAGT-3') with 5' labels of FAM, HEX, ROX, or TAMRA, alongside the corresponding EST-SSR forward primers with a 5' M13 tail and unmodified reverse primers, were synthesized by Fujian Manxiu Biotechnology (Fuzhou, China). Subsequently, fluorescent PCR amplification was performed for all 116 samples using the polymorphic primers under the same conditions described above. The resulting amplification products were separated and detected via fluorescent capillary electrophoresis using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For capillary electrophoresis, 1.0 µL of flu-

orescent PCR product was mixed with 0.5 µL of the GeneScan™ 500 LIZ Size Standard (Applied Biosystems, Foster City, CA, USA) and 8.5 µL of Hi-Di™ Formamide (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommended protocol.

Genotyping was performed based on the original capillary electrophoresis profiles (.fsa). Allele fragment sizing and genotype calling were conducted using GeneMarker software (v2.6.4) [41]. Finally, genotype data across all polymorphic loci for all individuals were collated and formatted using Excel and DataFormater software (Version 2.7) [42] in preparation for subsequent population genetic analyses.

To ensure the robustness of downstream population genetic analyses, we assessed the potential presence of null alleles across all 15 loci. Although EST-SSR markers are generally less susceptible to null alleles than anonymous genomic SSRs due to their origin from conserved coding regions [43], we estimated null allele frequencies using the method implemented in CERVUS 3.0.7 [44], which accounts for deviations from Hardy–Weinberg equilibrium. For subsequent analyses that are sensitive to data quality (e.g., parentage and mating system analyses), loci showing significant deviation from Hardy–Weinberg equilibrium ($p < 0.05$) were excluded to minimize potential bias [45].

2.3. Genetic Diversity and Structure Analysis

After genotyping, we initially evaluated the genetic diversity within the seed orchard. Based on the EST-SSR genotyping results, genetic diversity parameters were assessed for both the parental and progeny populations of the second-generation *C. hodginsii* seed orchard using GenAlEx 6.5 [46]; we analyzed the mean number of alleles per locus (N_a), effective number of alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{is}). Considering that the sample size in our study ($N > 30$) meets the requirement for a “large sample” in population genetic analysis, we used the unbiased estimate of expected heterozygosity (uH_e) to correct for potential bias [47]. The numerical difference between the calculated uH_e and the standard H_e was negligible, justifying direct comparability between the uH_e estimates in our study and the predominantly reported H_e values in the literature.

Subsequently, based on allele frequency data, genetic variation among and within the nine genetic groups (derived from different provenances) of the parental clones was partitioned via an Analysis of Molecular Variance (AMOVA) performed using GenAlEx. This analysis provided global F-statistics, including the fixation index (F_{st} , representing differentiation among groups), the inbreeding coefficient within groups (F_{is}), and the overall inbreeding coefficient (F_{it}). Building on this overall assessment, we then calculated pairwise F_{st} values for all possible combinations among the nine genetic groups, as well as separately among the nine progeny genetic groups, using the same AMOVA framework to compare fine-scale genetic differentiation patterns. Finally, the gene flow (N_m) was estimated using the formula:

$$N_m = (1 - F_{st}) / (4F_{st}). \quad (1)$$

To further elucidate the genetic structure of the seed orchard, the parental and progeny populations were analyzed using the EST-SSR markers. First, a Bayesian clustering analysis was implemented in STRUCTURE (v2.3.4) [48,49]. The number of genetic clusters (K) was tested from 1 to 10. For each run, the burn-in period and the number of Markov Chain Monte Carlo (MCMC) repetitions were both set to 100,000. Each K value was independently run 10 times to assess consistency. The results were evaluated using Structure Harvester (<https://lmme.ac.cn/StructureSelector/>, accessed on 13 January 2026). The optimal K was determined based on the ΔK statistic [50]; individual ancestry coefficients (Q -matrices) across replicate runs were aligned and averaged using CLUMPP (v1.1.2) [51].

Additionally, pairwise genetic distances (Shared Allele Distance, SAS) among all parental and progeny individuals were calculated using GenAlEx 6.5 (Supplementary File S2). Based on the resulting distance matrix, a principal coordinates analysis (PCoA) was conducted. Simultaneously, a neighbor-joining tree was constructed using MEGA 6.0 [52], rooted with Group 2 as the outgroup (the group exhibiting the largest average pairwise genetic distance). The tree was visualized on the Evolview online platform (<https://www.evolgenius.info/>, accessed on 13 January 2026).

2.4. Paternity Analysis and Mating System Estimation

Paternity analysis was conducted focusing on the 75 open-pollinated progeny and 41 candidate paternal parents using CERVUS 3.0.7 [44]. Based on the known maternal identity, a simulation of 10,000 progeny was run with the proportion of sampled candidate fathers and the loci mistyping rate set to 0.8 and 0.01, respectively [32]. A 70% confidence level [53] was adopted for paternity assignment to ensure biologically meaningful results, considering the typically lower assignment rates at higher stringency levels. At this level, the most likely father was identified for each progeny based on the Logarithm of Odds (LOD) score for each progeny-candidate father pair.

Subsequently, the mating system parameters of the second-generation seed orchard were estimated using the Expectation-Maximization (EM) algorithm in the MLTR software (v3.4) [54], based on the genotypes of the 75 open-pollinated progeny and their known mothers. The analysis was initiated with default parameters ($t = 0.9$, $rt = 0.1$, $rp = 0.1$, $F = 0.1$) [55]. To assess the statistical significance of the parameter estimates, 1000 bootstrap replicates were performed, with resampling conducted at the maternal family level (i.e., each bootstrap sample randomly resampled 30 maternal families with all their respective progeny) to reflect the true data structure. The estimated parameters included the single-locus outcrossing rate (ts), the multilocus outcrossing rate (tm), the maternal inbreeding coefficient (Fis), the single-locus correlation of paternity ($rp[s]$), and the multilocus correlation of paternity ($rp[m]$). The biparental inbreeding coefficient ($tm - ts$) was calculated as the difference between the multilocus and single-locus outcrossing rates; a larger value for this difference indicates a closer genetic relationship between outcrossing paternal and maternal parents. The $rp(m)$ estimates the probability that two randomly chosen progeny from the same mother share the same father. Moreover, a higher value suggests that a greater proportion of progeny is sired by a few fathers, indicating more concentrated paternal contribution. The effective number of pollen donors (Nep) was further calculated using the formula [56]:

$$Nep = 1/rp(m). \quad (2)$$

3. Results

3.1. EST-SSR Primer Screening and Validation

Based on capillary electrophoresis detection, 15 EST-SSR primer pairs were successfully screened from the candidate primers (Supplementary File S3). These primers exhibited stable amplification, clear bands, and polymorphism. The results of polymorphism analysis of these 15 loci are presented in Table 1.

Across the 15 loci, a total of 34 alleles were detected. The number of alleles per locus (Na) ranged from 2 to 4, with an average of 2.267 per locus. The effective number of alleles (Ne) varied from 1.439 to 2.170, with an average of 1.789. The average Shannon's information index (I) ranged from 0.483 to 0.985, with a mean of 0.657. The mean observed heterozygosity (Ho) and expected heterozygosity (He) were 0.425 and 0.432, respectively. The average inbreeding coefficient (Fis) was 0.011, indicating a slight overall deviation from the Hardy–Weinberg equilibrium. Notably, heterozygote excess ($Ho > He$) was detected for

seven loci. The polymorphism information content (PIC) for individual loci was 0.259–0.483, with a mean of 0.349. All screened loci were classified as moderately polymorphic markers according to Botstein’s criteria [57]. Among them, FJBSSR124 (PIC = 0.483) and FJBSSR113 (PIC = 0.468) exhibited a high polymorphism.

Overall, the 15 EST-SSR loci, exhibiting satisfactory polymorphism, were considered suitable for all downstream genetic analyses.

Table 1. Polymorphism analysis of the 15 EST-SSR loci.

Locus	Na	Ne	I	Ho	He	Fis	PIC
FJBSSR006	2.000	1.439	0.483	0.125	0.305	0.591	0.259
FJBSSR031	2.000	1.752	0.621	0.375	0.429	0.127	0.337
FJBSSR037	2.000	1.439	0.483	0.375	0.305	−0.228	0.259
FJBSSR042	2.000	1.970	0.685	0.625	0.492	−0.270	0.371
FJBSSR043	2.000	1.752	0.621	0.625	0.429	−0.456	0.337
FJBSSR049	2.000	1.752	0.621	0.375	0.429	0.127	0.337
FJBSSR080	2.000	1.752	0.621	0.625	0.429	−0.456	0.337
FJBSSR088	2.000	1.970	0.685	0.375	0.492	0.238	0.371
FJBSSR090	2.000	1.970	0.685	0.125	0.492	0.746	0.371
FJBSSR113	3.000	2.133	0.900	0.625	0.531	−0.176	0.468
FJBSSR122	2.000	1.439	0.483	0.375	0.305	−0.228	0.259
FJBSSR124	4.000	2.170	0.985	0.500	0.539	0.073	0.483
FJBSSR147	3.000	1.662	0.702	0.375	0.398	0.059	0.354
FJBSSR159	2.000	1.752	0.621	0.625	0.429	−0.456	0.337
FJB91-1	2.000	1.882	0.662	0.250	0.469	0.467	0.358
Mean	2.267	1.789	0.657	0.425	0.432	0.011	0.349

3.2. Genetic Diversity and Structure

3.2.1. Genetic Diversity in Parental and Progeny Populations

Genetic diversity of both parental and progeny populations in the second-generation *C. hodginsii* seed orchard was comparatively assessed using 15 EST-SSR loci (Table 2); the parental population exhibited a relatively high level of genetic diversity. A total of 35 alleles were detected across all loci in the parents. The mean Na and Ne were 2.333 and 1.855, respectively. The average I was 0.666. The mean Ho and unbiased expected heterozygosity (uHe) were 0.467 and 0.438, respectively. The average Fis of −0.084 indicated a general excess of heterozygotes in the parental population. The mean PIC was 0.349.

Table 2. Genetic diversity at 15 EST-SSR loci in parental and progeny populations.

Locus	Parental Population							Progeny Population						
	Na	Ne	I	Ho	uHe	Fis	PIC	Na	Ne	I	Ho	uHe	Fis	PIC
FJBSSR006	2.000	1.301	0.393	0.267	0.235	−0.154	0.204	2.000	1.301	0.393	0.267	0.233	−0.154	0.204
FJBSSR031	2.000	1.998	0.693	0.433	0.508	0.132	0.373	2.000	1.997	0.692	0.560	0.503	−0.122	0.373
FJBSSR037	2.000	1.342	0.423	0.167	0.259	0.346	0.222	2.000	1.488	0.510	0.333	0.330	−0.017	0.272
FJBSSR042	2.000	1.991	0.691	0.667	0.506	−0.339	0.373	2.000	1.942	0.678	0.533	0.488	−0.100	0.365
FJBSSR043	2.000	1.471	0.500	0.400	0.325	−0.250	0.267	2.000	1.642	0.580	0.533	0.394	−0.364	0.312
FJBSSR049	2.000	1.867	0.657	0.533	0.472	−0.148	0.351	2.000	1.933	0.676	0.520	0.486	−0.078	0.363
FJBSSR080	2.000	1.835	0.647	0.700	0.463	−0.538	0.343	2.000	1.692	0.599	0.573	0.412	−0.402	0.324
FJBSSR088	3.000	1.784	0.768	0.500	0.447	−0.138	0.379	3.000	2.008	0.796	0.413	0.505	0.177	0.428
FJBSSR090	2.000	1.965	0.684	0.467	0.499	0.050	0.368	2.000	1.987	0.690	0.413	0.500	0.168	0.373
FJBSSR113	3.000	2.113	0.898	0.500	0.536	0.051	0.444	4.000	1.851	0.821	0.440	0.463	0.043	0.409
FJBSSR122	2.000	1.557	0.543	0.333	0.364	0.068	0.293	2.000	1.750	0.620	0.432	0.431	−0.009	0.337
FJBSSR124	4.000	3.352	1.275	0.667	0.714	0.050	0.621	4.000	2.902	1.189	0.667	0.660	−0.017	0.582
FJBSSR147	2.000	1.923	0.673	0.533	0.488	−0.111	0.361	3.000	1.897	0.694	0.533	0.476	−0.128	0.407
FJBSSR159	2.000	1.301	0.393	0.267	0.235	−0.154	0.204	2.000	1.523	0.527	0.333	0.346	0.029	0.285
FJB91-1	3.000	2.020	0.755	0.567	0.514	−0.122	0.431	3.000	2.048	0.753	0.520	0.515	−0.016	0.436
Mean	2.333	1.855	0.666	0.467	0.438	−0.084	0.349	2.467	1.864	0.681	0.471	0.449	−0.066	0.365

Compared with the parental population, the progeny population maintained stable and even slightly increased genetic diversity. A total of 37 alleles were detected in the progeny population. The values of Na and Ne increased to 2.467 and 1.864, respectively, compared with the parental counterparts; the average I was 0.681, and Ho and uHe slightly rose to 0.471 and 0.449, respectively. The mean Fis was -0.066 , indicating heterozygote excess persisting in the progeny population. The mean PIC also increased to 0.365.

These results demonstrate that the progeny population effectively inherited the genetic diversity from the parents, and the observed heterozygote advantage in both generations facilitates the management of the seed orchard.

3.2.2. Genetic Diversity Among Genetic Groups

We further investigated the genetic diversity among the nine genetic groups (derived from different provenances) within the parental and progeny populations of the second-generation *C. hodginsii* seed orchard. The nine parental genetic groups exhibited moderate and comparable levels of genetic diversity (Table 3). Group 8 displayed the highest genetic diversity, with its Na (2.067), I (0.643), and uHe (0.529) representing the highest values, whereas Group 5 showed the lowest genetic diversity (Na = 1.800, I = 0.441, uHe = 0.336). The observed heterozygosity (Ho) was consistently high across all groups and, in most cases, it exceeded the corresponding uHe values. The Fis ranged from -0.491 to 0.021 , except for Group 5, which showed a slightly positive Fis value (0.021), suggesting potential mild inbreeding. The remaining eight groups had negative Fis values, indicating pronounced heterozygote excess.

Table 3. Genetic diversity of 15 EST-SSR loci among nine genetic groups.

Group	Parental Groups						Progeny Groups					
	Na	Ne	I	Ho	uHe	Fis	Na	Ne	I	Ho	uHe	Fis
Group 1	2.000	1.737	0.572	0.517	0.436	-0.358	2.267	1.824	0.651	0.467	0.451	-0.096
Group 2	2.067	1.778	0.583	0.533	0.500	-0.405	2.267	1.852	0.674	0.511	0.483	-0.129
Group 3	1.867	1.712	0.529	0.433	0.414	-0.202	2.200	1.826	0.645	0.440	0.443	-0.037
Group 4	1.933	1.676	0.531	0.467	0.467	-0.356	2.200	1.811	0.645	0.500	0.460	-0.167
Group 5	1.800	1.524	0.441	0.283	0.336	0.021	2.333	1.887	0.680	0.531	0.468	-0.191
Group 6	2.000	1.782	0.601	0.444	0.493	-0.073	2.267	1.743	0.625	0.433	0.421	-0.071
Group 7	1.933	1.720	0.531	0.400	0.467	-0.167	2.000	1.700	0.568	0.422	0.458	-0.105
Group 8	2.067	1.835	0.643	0.667	0.529	-0.491	2.067	1.851	0.646	0.644	0.529	-0.471
Group 9	2.200	1.809	0.634	0.489	0.452	-0.187	2.200	1.731	0.617	0.410	0.435	-0.029
Mean	1.985	1.730	0.563	0.470	0.455	-0.246	2.256	1.824	0.653	0.480	0.454	-0.115

Notes: The groups (Group 1 to Group 9) correspond to the maternal provenance. Parental values are based on clones from each provenance; progeny values are based on individuals whose mother originated from the respective provenance.

The average genetic diversity across the progeny groups (mean Na = 2.256, Ne = 1.824, I = 0.653) was higher than the parental counterparts, indicating that effective hybridization among parental clones derived from different provenances within the seed orchard had broadened the genetic base of the progeny. Furthermore, the progeny Group 5 exhibited the most significant improvement in diversity, while Group 8 successfully maintained the highest diversity level (uHe = 0.529) and the strongest heterozygote excess (Fis = -0.471). The mean observed heterozygosity (Ho = 0.480) remained higher than the unbiased expected heterozygosity (uHe = 0.454) across the progeny groups, and the average Fis value was negative (-0.115), which validates that heterozygote excess persisted in the progeny, albeit to a lesser extent than in the parents (mean Fis = -0.246).

In summary, the genetic diversity of the progeny in the second-generation seed orchard was generally superior to that of the parents, demonstrating the effectiveness of the seed orchard in enhancing genetic diversity.

3.2.3. Genetic Differentiation Among Genetic Groups

We assessed the degree of genetic differentiation among parental genetic groups based on 15 EST-SSR markers using an AMOVA. As shown in Table 4, the analysis revealed that the genetic variation was entirely attributed to differences within groups (100%), with no significant contribution from variation among groups. This absence of population structure was statistically confirmed by a non-significant overall fixation index (Global $F_{st} = -0.012$, $p = 0.621$; Table 5), suggesting a low level of genetic differentiation among the parental clones.

Table 4. AMOVA of parental clones by genetic group in *C. hodginsii*.

Source of Variation	df	SS	MS	Est. Var.	Percentage of Variation (%)
Among Groups	8	23.000	2.875	0.000	0%
Within Groups	51	170.667	6.627	3.500	100%
Total	59	193.667		3.500	100%

Note: Degrees of freedom (df) and sum of squares (SS) were calculated based on allele data ($n = 60$ haplotypes), whereas the variance components reflect the genetic variance structure at the diploid individual level. The “Within Groups” variance component comprises variation both among individuals and within individuals.

Table 5. Global F-statistics and associated significance tests derived from AMOVA.

Statistic	Value	<i>p</i> -Value	Interpretation
F_{st} (Differentiation)	−0.012	0.621	Degree of genetic differentiation among groups
F_{is} (Inbreeding)	−0.056	0.786	Inbreeding coefficient of individuals relative to their group
F_{it} (Total inbreeding)	−0.069	0.861	Inbreeding coefficient of individuals relative to the total

Notes: *p*-values were obtained based on 999 permutations. Negative values for F-statistics, arising from negative variance component estimates, are conventionally interpreted as zero in population genetics, indicating no differentiation or an excess of heterozygosity.

For further pairwise analysis, we compared fixation index (F_{st}) and gene flow (N_m) between parental and progeny groups. The complete values are provided in Supplementary File S4 and visualized in the heatmap (Figure 2). Permutation tests (999 permutations) showed that none of the pairwise F_{st} comparisons among parental groups were statistically significant (all p -value > 0.05). The observed F_{st} values were consistently low, ranging from 0.000 to 0.077 with a mean of 0.012 (Figure 2A, lower triangle). Consequently, estimates of gene flow (N_m) were high, with several values approaching infinity (∞) due to F_{st} values tending toward zero (Figure 2B, lower triangle), which is mathematically expected under negligible genetic differentiation.

Compared to this undifferentiated parental baseline, the progeny generation exhibited a clear trend toward further genetic homogenization. The pairwise F_{st} values among progeny groups were even lower on average (mean $F_{st} = 0.003$) and likewise non-significant (all p -value > 0.05; Figure 2A, upper triangle). This further reduction in differentiation was accompanied by a pronounced increase in estimated gene flow, with more pairwise comparisons yielding undefined (∞) N_m values (Figure 2B, upper triangle).

These results demonstrate that sufficient interspecific hybridization within the seed orchard effectively promoted genetic homogenization in the progeny, even starting from an initial state of very low genetic structure.

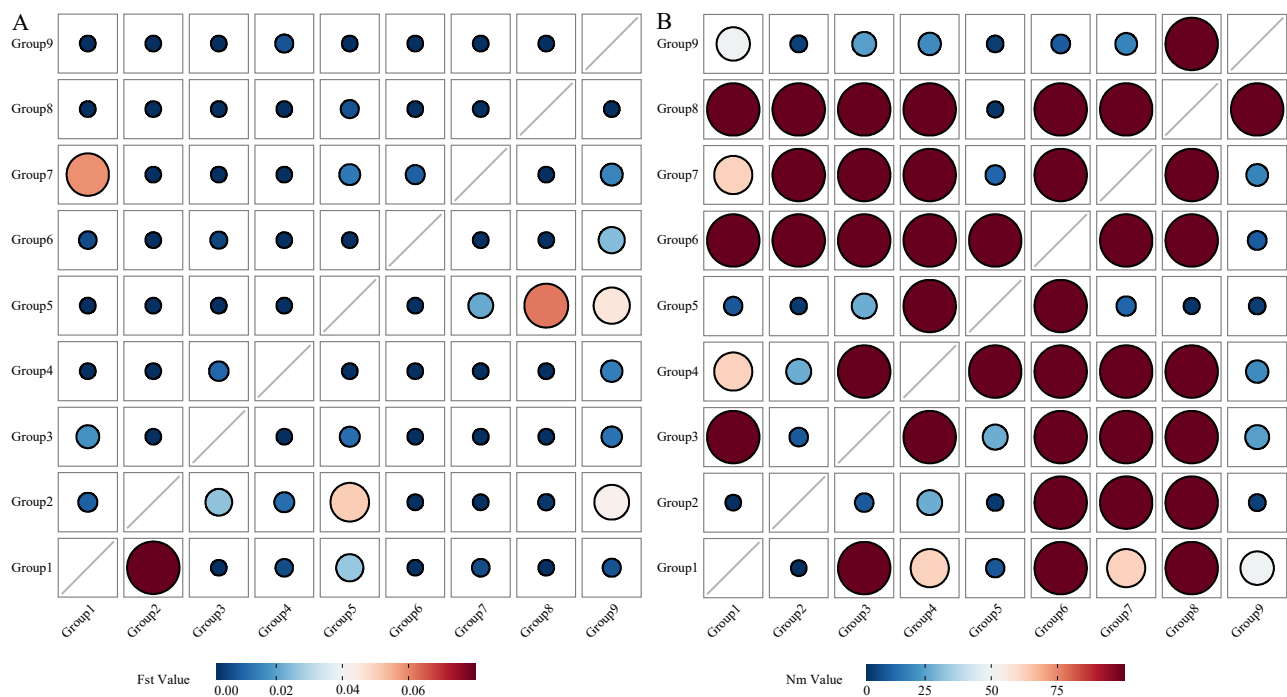


Figure 2. Genetic differentiation and gene flow among genetic groups. Notes: (A,B) represent pairwise F_{st} and N_m among parental (lower triangle) and progeny (upper triangle) genetic groups, respectively. The groups (Group 1 to Group 9) correspond to the maternal provenance. Parental values are based on clones from each provenance; progeny values are based on individuals whose mother originated from the respective provenance. All pairwise F_{st} comparisons were not statistically significant ($p > 0.05$). In (B), for visualization purposes, undefined N_m values (∞) resulting from $F_{st} \rightarrow 0$ have been represented with a value of 100 in the heatmap. The complete data are available in Supplementary File S4.

3.2.4. Genetic Structure of the Seed Orchard

Considering the minimal genetic differentiation, we further investigated the genetic structure of the second-generation *C. hodginsii* seed orchard using a combined dataset of parents and progenies (105 individuals) genotyped with 15 EST-SSR loci to detect potential subpopulation differentiation. The STRUCTURE analysis revealed that as K increased from 1 to 10, the ΔK peaked at $K = 2$, the potential number of genetic clusters (Figure 3A,B). However, further inspection of the individual ancestry coefficients (Q-matrix) at $K = 2$ (Supplementary File S5) revealed that all 105 individuals were highly admixed, with Q-values of 0.35–0.65, and no clear clustering pattern (Figure 3C). As K continued to increase ($K = 3$ –10), no significant substructure was detected, indicating frequent gene flow among these genetic clusters and a highly similar genetic composition among all individuals.

The absence of discrete population structure was further validated through Principal Coordinates Analysis (PCoA). The PCoA plot (Figure 4) revealed a continuous distribution of all individuals, with Principal Coordinate 1 (PC1) and Principal Coordinate 2 (PC2) explaining 12.42% and 10.15% of the total variation, respectively. Parental and progeny individuals from different genetic groups were uniformly mixed in the genetic space, showing no group-related clustering pattern. Despite the overall continuous distribution, two progeny individuals (S1-1-23 and S1-2-26) were positioned distinctly outside the main cluster in the PCoA plot, reflecting their unique genetic backgrounds.

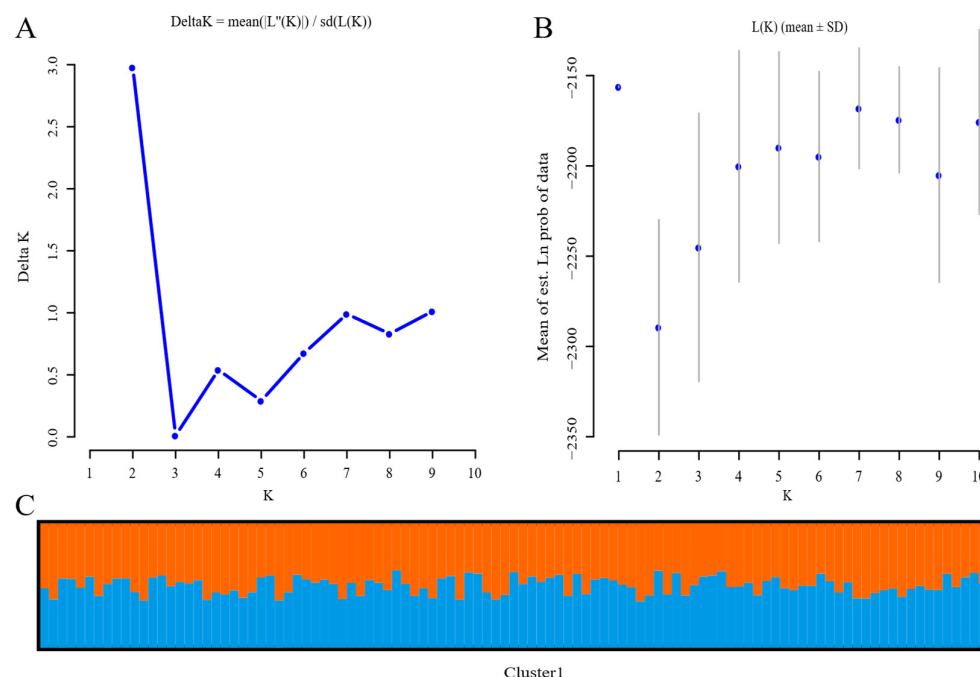


Figure 3. Structure analysis of the *C. hodginsii* seed orchard. Notes: (A) Relations between the optional number of cluster K and Delta K; (B) Calculation of population structure using Mean Ln P(K); (C) Population genetic structure of *C. hodginsii* seed orchard (K = 2).

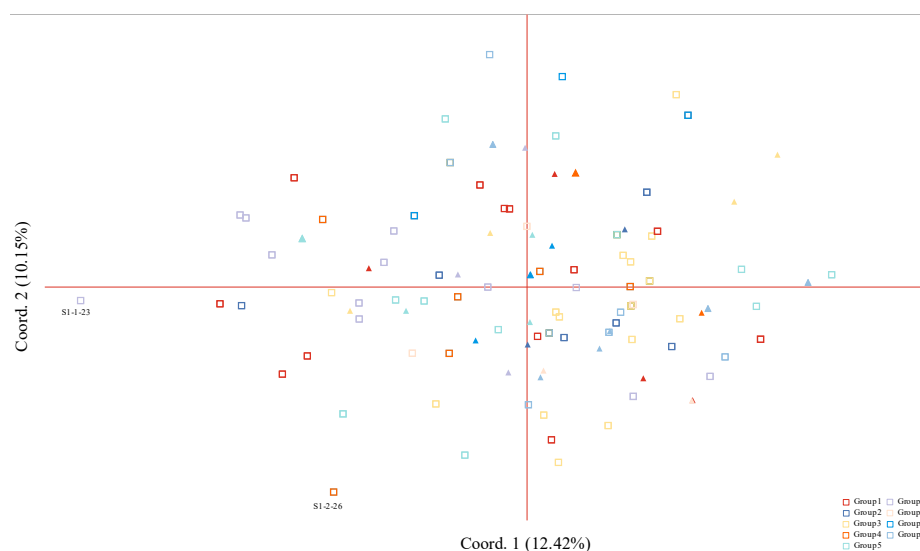


Figure 4. Principal coordinates analysis (PCoA) of the *C. hodginsii* seed orchard. Notes: Solid triangles represent the 30 parental clones; hollow squares represent the 75 open-pollinated progeny. The groups (Group 1 to Group 9) correspond to the maternal provenance. Parental values are based on clones from each provenance; progeny values are based on individuals whose mother originated from the respective provenance.

Subsequently, neighbor-joining trees were constructed at both the genetic group level (based on 9 groups) and the individual (based on 105 individuals) levels. The group-level tree (Figure 5A), rooted with Group 2 as the outgroup, showed that different genetic groups did not form distinct, separate branches. The individual-level tree (Figure 5B) revealed that each cluster contained individuals derived from multiple groups; parents and progeny were scattered across different branches, indicating no correlation of the clustering pattern with group origin.

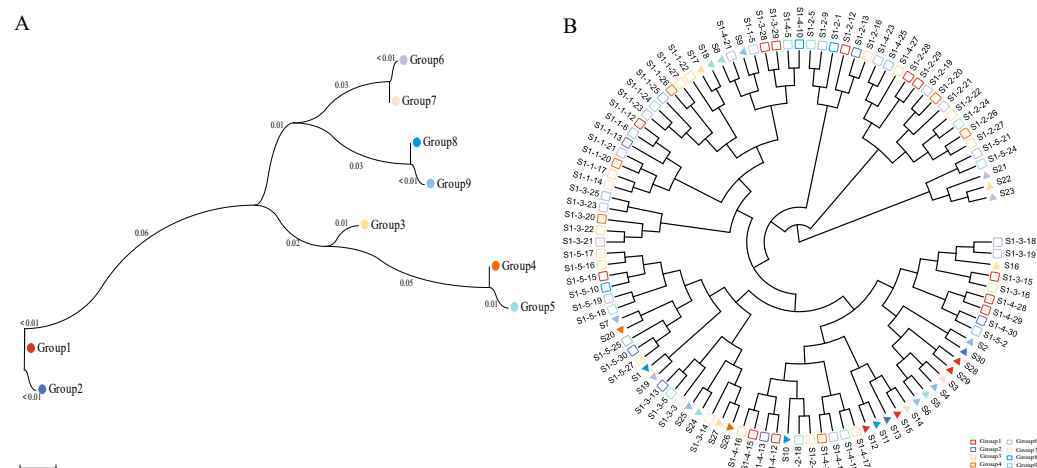


Figure 5. Neighbor-joining tree of the *C. hodginsii* seed orchard. Notes: (A) NJ-tree of nine parental genetic groups, rooted using Group 2 as the outgroup. (B) NJ-tree of 105 individuals (parental and progeny). Solid triangles and squares represent the 30 parental clones and the 75 open-pollinated progeny, respectively. The group labels (Group 1 to Group 9) correspond to the nine maternal provenances. Parental values are based on clones from each provenance; progeny values are based on individuals whose mother originated from the respective provenance.

Overall, consistent with the non-significant pairwise F_{ST} comparisons, no significant genetic differentiation was observed between parents and progeny in the second-generation seed orchard. A genetically homogeneous population that included all individuals exhibited no apparent substructure, which is consistent with the findings from the previous AMOVA and genetic differentiation analyses.

3.2.5. Assessment of Null Allele Frequency

Null allele frequencies were estimated for all 15 EST-SSR loci using the method of Dakin et al. [45]. As shown in Table 6, frequencies were low (mean $F(\text{Null}) = 0.001$), with no locus exceeding the 0.100 impact threshold [45]. The low estimated null allele frequencies, and the overall negative inbreeding coefficients (F_{is}) observed in both parental and progeny populations (Table 2, mean $F_{is} = -0.084$ and -0.066 , respectively), indicate that null alleles did not substantially bias our genetic diversity estimates.

Table 6. Genetic Diversity and NE-2P of the 13 EST-SSR Loci.

Locus	k	HExp	PIC	NE-2P	HW	F (Null)
FJBSSR124	4	0.667	0.607	0.596	NS	0.006
FJBSSR113	4	0.483	0.429	0.749	NS	0.012
FJBSSR088	3	0.491	0.407	0.775	NS	0.059
FJB91-1	3	0.513	0.393	0.797	NS	-0.006
FJBSSR031	2	0.500	0.374	0.813	NS	-0.019
FJBSSR090	2	0.498	0.373	0.814	NS	0.100
FJBSSR042	2	0.491	0.370	0.815	NS	-0.083
FJBSSR147	3	0.475	0.369	0.811	NS	-0.053
FJBSSR049	2	0.479	0.363	0.818	NS	-0.032
FJBSSR122	2	0.407	0.323	0.838	NS	0.018
FJBSSR037	2	0.324	0.271	0.865	ND	0.064
FJBSSR159	2	0.319	0.267	0.866	ND	-0.015
FJBSSR006	2	0.233	0.205	0.898	ND	-0.038
Mean	3	0.452	0.365	0.804	-	0.001

Notes: k, number of alleles (assessed across the 75 progeny and 41 candidate parents); HExp, expected heterozygosity; NE-2P, Paternity exclusion probability given the maternal genotype; HW, Hardy–Weinberg equilibrium test (NS, not significant; ND, not determined); F (Null), estimated frequency of null alleles.

3.3. Paternity Analysis

Initially, the genetic background and efficacy of the marker system were evaluated for paternity analysis. Given the overall low null allele frequencies estimated across loci, and to ensure accurate analysis, two loci (FJBSSR043 and FJBSSR080) that deviated from the Hardy–Weinberg equilibrium were excluded. Subsequently, the remaining 13 EST-SSR loci were used to analyze 75 progeny and 41 candidate paternal parents. As summarized in Table 6, the mean PIC of the 13 loci was 0.365, and the combined non-exclusion probability based on the known maternal genotypes (NE-2P) was 0.804, indicating high diversity and exclusion power. Furthermore, the cumulative paternity exclusion probability exceeded 0.999 with only the first five loci and reached an extremely high value (surpassing $1 - 1 \times 10^{-9}$) when all 13 loci were used (Figure 6), demonstrating the high effectiveness of the markers for paternity analysis in open-pollinated progeny of *C. hodginsii*.

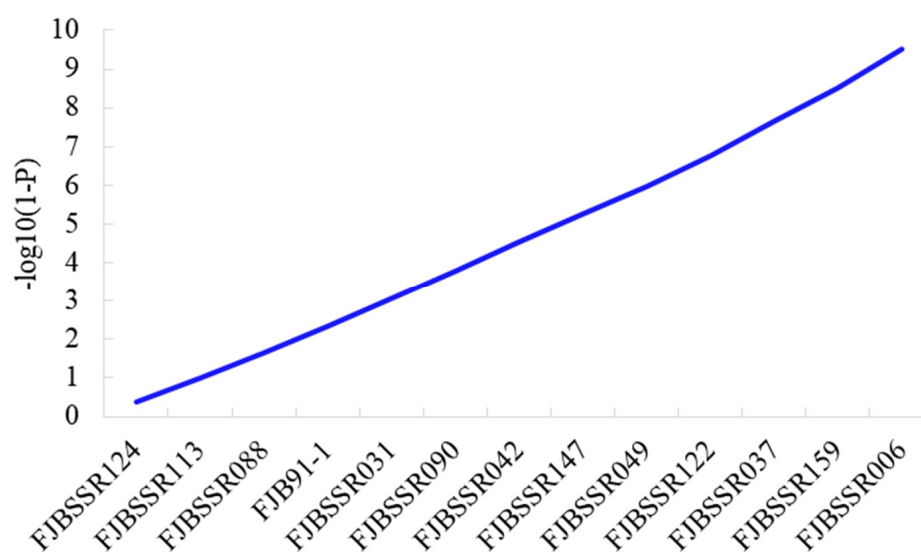


Figure 6. Cumulative paternity exclusion probability for the 13 EST-SSR loci. Note: The y -axis represents the cumulative paternity exclusion probability (P) on a transformed scale $-\log_{10}(1 - P)$. Integer values on the y -axis correspond to $P = 1 - 10^{-y}$; for example, $y = 1, 2$, and 3 correspond to $P = 0.9, 0.99$, and 0.999 , respectively.

Using this marker set, paternity was assigned for 32 out of 75 progeny (42.67%) at a 70% confidence level (Table 7). All these father-progeny pairs exhibited a positive LOD score; sixteen showed a perfect match across all loci. Notably, the paternity assignments for progeny S1-2-9 and S1-3-16 were statistically significant even at the more stringent 95% confidence level.

Analysis of pollination dynamics revealed that all assigned progeny resulted from outcrossing between different clones, with no self-pollination detected. Clones S5, S11, and S17 were identified as the most frequent pollen donors (each appearing three times), which were potentially competitive pollen donors within the orchard. To determine external pollen flow, we further analyzed the paternity assignment results. Nine progeny (12.00%) were related to pollen donors located outside the seed orchard. Furthermore, four other progeny (5.33%) were found to carry alien alleles (i.e., alleles not present in any known parent), indicating that their paternal origin was from unknown pollen donors outside the orchard. Thus, the minimum external pollen flow rate was estimated to be 17.33%.

Table 7. Paternity analysis results for open-pollinated progeny of *C. hodginsii*.

Progeny	Mother	Candidate Father	Trio Loci Mismatching	Trio LOD	Trio Confidence
S1-2-9	S9	S11	0	8.08	95%
S1-3-16	S16	S11	0	8.00	95%
S1-5-10	S10	S12	0	4.67	80%
S1-2-29	S29	S27	1	4.64	80%
S1-4-21	S21	S26	2	4.59	80%
S1-4-10	S10	S9	0	4.28	80%
S1-3-22	S22	S347	2	4.23	80%
S1-2-1	S1	S398	0	4.22	80%
S1-4-5	S5	S11	0	4.08	80%
S1-4-25	S25	S10	0	4.04	80%
S1-2-22	S22	S384	2	3.99	80%
S1-2-24	S24	S5	0	3.97	80%
S1-5-17	S17	S25	2	3.80	80%
S1-3-23	S23	S13	0	3.60	70%
S1-4-30	S30	S437	0	3.55	70%
S1-2-18	S18	S7	2	3.40	70%
S1-4-16	S16	S413	2	3.33	70%
S1-4-20	S20	S343	4	3.32	70%
S1-5-21	S21	S5	2	3.32	70%
S1-1-6	S6	S27	3	3.27	70%
S1-1-21	S21	S17	0	3.23	70%
S1-4-23	S23	S17	0	3.12	70%
S1-2-16	S16	S10	2	3.08	70%
S1-1-5	S5	S415	0	3.03	70%
S1-5-25	S25	S7	0	2.98	70%
S1-4-17	S17	S398	2	2.95	70%
S1-3-28	S28	S17	2	2.81	70%
S1-1-12	S12	S5	0	2.77	70%
S1-5-15	S15	S343	2	2.76	70%
S1-4-15	S15	S19	1	2.75	70%
S1-4-27	S27	S26	1	2.73	70%
S1-3-20	S20	S25	0	2.68	70%

Note: Trio LOD score, logarithm of the odds. Some father-progeny pairs showed 1–4 locus mismatches, potentially due to mutation or genotyping errors; this possibility has been accounted for in the LOD score calculation.

3.4. Mating System Estimation

Furthermore, the mating system of the second-generation *C. hodginsii* seed orchard was analyzed using 15 EST-SSR loci. The 75 progeny were categorized into 26 maternal families. To ensure statistical reliability, single-progeny families were excluded, yielding a final dataset of 21 families comprising 70 progeny for further analysis.

The results (Table 8) confirmed an entirely outcrossing mating system, with both the multilocus (t_m) and the single-locus (t_s) outcrossing rates estimated at 1.000. This validates the absence of self-pollination detected through the paternity analysis. Moreover, the values of $t_m - t_s$ and the maternal inbreeding coefficient (F_{is}) were exactly zero and statistically non-significant, indicating no significant biparental inbreeding or maternal inbreeding, which was comparable to the previously indicated excess of heterozygosity in the parental population.

Furthermore, the effective number of pollen donors (N_{ep}) was estimated to be 8.13, which is substantially lower than the number of candidate paternal clones (41). Hence, a highly uneven pollen contribution is suggested, which has been substantiated by the paternity analysis, with identified clones S5, S11, and S17 considered as the most frequent fathers and superior, competitive pollen donors within the orchard.

Table 8. Mating system parameters of *C. hodginsii* second-generation seed orchards.

Parameter	Estimate (SE)
Multilocus outcrossing rate (t_m)	1.000 (0.024)
Single-locus outcrossing rate (t_s)	1.000 (0.001)
Biparental inbreeding ($t_m - t_s$)	0.000 (0.023)
Maternal inbreeding coefficient (F_{is})	0.023 (0.046)
Single-locus correlation of paternity ($r_p(s)$)	−0.200 (0.001)
Multilocus correlation of paternity ($r_p(m)$)	0.123 (0.113)
$r_p(s) - r_p(m)$	−0.323 (0.113)
Effective number of pollen donors (N_{ep})	8.130

Note: The effective number of pollen donors (N_{ep}) was calculated as $1/r_p(m)$ and thus has no associated standard error.

4. Discussion

Reliable molecular markers are crucial for accurately assessing genetic diversity and population structure, and optimizing breeding strategies. The large and complex genome of *C. hodginsii* [58] presents significant challenges for developing genomic SSRs or SNPs. In this study, we utilized EST-SSR markers derived from transcriptome sequencing; this approach circumvents large genome-associated interference and offers stable amplification, clear results, and high cross-species transferability [35,36,43,59], which ensure high genotyping accuracy in applications, including phylogenetics and paternity analysis. Notably, EST-SSR markers are less prone to null alleles than anonymous genomic SSRs due to their origin in conserved coding regions [59]. Our empirical data support this advantage: the estimated null allele frequencies across all 15 loci were negligible (mean F (Null) = 0.001), and the overall negative inbreeding coefficients (F_{is}) observed in both parental and progeny populations (Table 3) are inconsistent with the heterozygote deficiency typically caused by null alleles [60]. The analysis of the second-generation *C. hodginsii* seed orchard using 15 polymorphic EST-SSR markers revealed 34 alleles, with a mean N_a of 2.267. Moreover, the mean H_o (0.425) and uH_e (0.432) closely aligned with values reported by Ding et al. [61] for *C. hodginsii* (mean H_o = 0.540, mean H_e = 0.480), further validating the efficacy of these markers.

Notably, 7 of the 15 loci (e.g., FJBSSR042, FJBSSR043) exhibited a pronounced heterozygote excess (i.e., $H_o > H_e$, negative F_{is}), which is potentially attributed to natural selection (e.g., heterozygote advantage) acting on these genic markers [62,63]. All loci were moderately polymorphic, with FJBSSR124 and FJBSSR113 approaching high polymorphism ($PIC > 0.45$) according to Botstein's criteria [57]. The observed mean PIC values (0.349–0.365) fall within the moderate range, which is a common characteristic of EST-SSR markers due to their origin in conserved genic regions [43,59]. Importantly, for the objectives of our study, the combined discriminatory power of the marker set was high, as demonstrated by the exceptionally high cumulative exclusion probability for paternity analysis ($>1 - 1 \times 10^{-9}$). The markers also successfully resolved the population structure and mating dynamics, confirming their overall adequacy and effectiveness for genetic analysis in this managed breeding population. Therefore, this marker set is considered highly suitable for population genetic analyses.

Considering the reliability of these markers, we assessed the core genetic resource of the seed orchard. Genetic diversity is the foundation for the long-term evolutionary potential and stable productivity of a seed orchard [16]. The 15 EST-SSR marker-based analysis indicated that both the parental (uH_e = 0.438, PIC = 0.349, I = 0.666) and progeny (uH_e = 0.449, PIC = 0.365, I = 0.681) populations of the second-generation *C. hodginsii* seed orchard maintained considerable levels of genetic diversity. The observed diversity is notably higher than that of natural populations which have suffered severe bottlenecks.

Our estimates ($uH_e = 0.438\text{--}0.449$) significantly exceeded values reported for genetically depleted populations (e.g., $H_e = 0.0701$ in Vietnam) [64] and surpassed the average for coniferous tree species ($H_e = 0.207$) [65]. This demonstrates the orchard's success in conserving a broad genetic base relative to degraded natural stands. It remained, however, below the species' highest inherent potential ($H_e = 0.635$) estimated from range-wide studies [64], an expected outcome for a selectively bred population that captures only a subset of the total genetic variation. This achieved level of diversity can be primarily attributed to the fact that the parental clones for our advanced-generation seed orchard were superior genotypes selected through genetic testing from multiple provenances. Although genetic differentiation among these parental genetic groups was non-significant, this artificial selection effectively broke down the genetic isolation characteristic of natural populations and integrated a wider range of favorable alleles into the seed orchard, thereby enhancing its genetic diversity.

Notably, all genetic diversity indices were slightly higher in the progeny than in the parents. This can be considerably attributed to the scientific mating design of the orchard. Although AMOVA indicated non-significant genetic differentiation among the nine parental genetic groups, the selected clones collectively formed a large, outcrossing population. This structure effectively minimized genetic drift, increased the effective population size, and promoted the full recombination of parental alleles in the progeny, maximizing the utilization of standing genetic variation. Other conifers exhibit a similar pattern. For instance, Pakull et al. [66] demonstrated that scientific orchard management can maintain diversity even with limited clones (e.g., 25) by reducing selfing and promoting cross-pollination in *Pseudotsuga menziesii*. Furthermore, the external pollen flow (17.33%) potentially introduced novel alleles, broadening the genetic base of the progeny [67]. Overall, these results demonstrate that advanced-generation seed orchards, obtained through scientific parental selection, optimized mating systems, and the incorporation of moderate external gene flow, can effectively maintain or enrich genetic diversity and achieve genetic gains.

Assessing genetic structure is pivotal for evaluating the functional efficacy of a seed orchard. Our analysis demonstrates the establishment of high genetic homogeneity within the second-generation *C. hodginsii* seed orchard. Although the parental clones originated from different genetic groups, AMOVA revealed that genetic variation was primarily associated with intra-group differences, with negligible inter-group variations (parental $F_{st} = 0.012$); it predominantly reflects variations at the individual level. The differentiation further decreased among progeny groups ($F_{st} = 0.003$) after open pollination. Moreover, individual-based analyses consistently indicated the absence of significant population substructure, which confirm that the design of the orchard effectively dissolved the initial weak genetic patterning, promoted pervasive gene flow among all parents, resulting in a genetically homogeneous population.

However, for wind-pollinated species such as *C. hodginsii* studied here, external pollen contamination remains a key potential risk to the genetic purity of seed orchard breeding outcomes and long-term genetic gain. Gömöry et al. [68] argued that external pollen contributions exceeding 10% can cause substantial contamination of a seed orchard's gene pool, and any limited increase in genetic diversity is unlikely to compensate for the dilution of genetic purity and expected genetic gain. Based on this criterion, the external pollen flow rate observed in our study (17.33%) has exceeded this threshold. This level of contamination is within the range (10.1%–33.7%) reported for an advanced-generation *Cunninghamia lanceolata* seed orchard, highlighting that external pollen ingress is a prevalent issue even in well-managed orchards [69]. This confirms the necessity and urgency of controlling pollen contamination in this seed orchard.

To address this challenge, the seed orchard in our study was established with management strategies centered on flowering synchrony and spatial configuration. Our previous phenological studies [8,9] provide the theoretical basis for this approach, demonstrating that selecting superior parental clones with concentrated and synchronized peak flowering (e.g., belonging to the same late-flowering phenotype) and arranging them in an optimized spatial layout can effectively narrow the population's flowering window, reduce temporal overlap with external, genetically inferior pollen sources, and thereby suppress contamination. The data obtained here establish a baseline for evaluating the practical effectiveness of this strategy.

Therefore, to further enhance genetic gain, future management must aim to reduce the contamination rate to a lower level. From a long-term perspective, continuous pollen flow monitoring, along with dynamic parental deployment and strategy optimization based on flowering phenology and spatial data, constitutes a key strategy for maintaining the genetic integrity of advanced-generation seed orchards and achieving sustainable genetic improvement.

An entirely outcrossing mating system, indicated by the uniformly high outcrossing rates ($t_m = t_s = 1.000$) and the absence of detectable biparental inbreeding ($t_m - t_s = 0.000$, $F_{is} = 0.023$), was observed in the second-generation seed orchard, representing an idealized mating pattern. Such an outcome is plausible within an intensively managed seed orchard of a predominantly outcrossing species. Regarding the statistical power to detect low levels of selfing or inbreeding events, our analysis was based on 70 progeny from 21 maternal families genotyped with 15 polymorphic EST-SSR markers. The collectively extremely high cumulative paternity exclusion probability ($>1 - 1 \times 10^{-9}$; Figure 5) reflects the high polymorphism and discriminatory power of the marker system, providing a necessary genetic-informational foundation for reliably detecting inbreeding events. The observed absence of such genetic signals is likely attributable to the strong self-incompatibility inherent to *C. hodginsii*. Furthermore, the highly homogeneous genetic background among parental clones (parental $F_{st} = 0.012$), coupled with an orchard design (e.g., flowering synchrony) that effectively promotes widespread random mating, fundamentally reduced opportunities for biparental inbreeding. Therefore, considering the methodological power, species biology, and management practices together, the completely outcrossing system observed in this study is reliable. Nevertheless, future studies employing larger progeny arrays or higher-throughput genomic markers (e.g., SNPs) would enable more precise quantification of exceedingly rare mating events. This highly homogeneous genetic background facilitated an efficient outcrossing system. Mating system analysis confirmed an entirely outcrossing system ($t_m = t_s = 1.000$) in the second-generation seed orchard, with no selfing or biparental inbreeding detected. This result verifies that *C. hodginsii*, a typical outcrossing species, retains an effective self-incompatibility mechanism within the orchard. Moreover, the parental configuration, including the spacing of clones and the design for flowering synchrony, was rational and successfully promoted cross-pollination.

Furthermore, both parental and progeny populations exhibited a general heterozygote excess (negative mean F_{is}). The selection history potentially contributed to heterozygote excess in the parental population, wherein superior phenotypes chosen from wild populations may have fixed heterozygous loci conferring adaptive advantages. This phenomenon, known as heterozygote advantage, is a desirable trait in forest tree breeding and is frequently associated with greater adaptability and growth vigor [70]. The maintenance of this heterozygote advantage in the progeny (mean $F_{is} = -0.066$) demonstrates that the mating system in the orchard effectively promoted complete outcrossing, avoided inbreeding depression, and successfully transmitted this beneficial genetic feature to the next generation [71]. This can potentially improve progeny viability and adaptability, and provide

a genetic foundation for high-quality seedling production. Nevertheless, further genetic analysis of the potential influence of this dominance effect on estimates of quantitative genetic parameters is required.

However, we further investigated whether complete outcrossing ensured equitable paternal contributions; we conducted a paternity analysis to gain deeper insights into the mating dynamics. At a 70% confidence level, paternity was successfully assigned to a portion of the progeny. The failure to assign paternity to the remaining portion is likely attributable to the limited genetic resolution of the molecular markers in discriminating among the closely related parents. Although the cumulative exclusion probability of the EST-SSR markers was high, the genetic similarity across the parental clones constrained their practical discriminatory power. Genotyping errors or pollen contamination derived from external, unsampled sources may have also contributed to these assignment failures. Nevertheless, the achieved assignment rate is within a forest tree-specific acceptable range [53].

A significant inequality in paternal contribution was detected. The effective number of pollen donors ($N_{ep} = 8.13$) was considerably lower than that of candidate fathers (41), and reproductive success concentrated on a few dominant clones, including S5, S11, and S17. This pattern is consistent with the completely outcrossing system detected, and is often driven by the advantages of a few clones in flowering phenology, pollen quantity, or dispersal ability. This phenomenon represents a common challenge in seed orchard management, as similarly documented in a third-generation *Cunninghamia lanceolata* seed orchard, where 44% of the paternal clones sired 80% of the offspring [69]. This inequality in paternal contribution is one of the most significant findings of our study, as it directly reveals that reproductive success (e.g., pollen dispersal and siring ability) is a key fitness component that may be independent of the vegetative growth traits upon which the initial clonal selection was based. Consequently, to ensure both high genetic gain and sustained diversity in advanced-generation orchards, the reproductive performance of candidate genotypes must be given paramount consideration during parental selection.

Fortunately, this pollen inequality did not diminish the genetic diversity of the progeny. The concept of effective parent number (N_{ep}), which accounts for fertility variation among clones, provides a quantitative framework to assess this phenomenon [72]. In our orchard, the genetic homogeneity among parents meant that even a pollen pool dominated by a few clones effectively captures the majority of the population's genetic variation. This mechanism, coupled with the introduced external alleles, maintained a broad genetic base in the progeny. Studies in other species, such as *Pinus thunbergii* [72], have similarly shown that despite clonal fertility variation, the resultant loss of gene diversity in seed crops can be relatively low (e.g., ~2%), supporting our observation that skewed paternal contribution does not necessarily equate to a severe loss of overall genetic diversity when the parental pool is diverse and outcrossing is prevalent.

In conclusion, our study demonstrates that the second-generation *C. hodginsii* seed orchard, characterized by complete outcrossing and high genetic homogeneity, constitutes a highly efficient breeding population. Accordingly, the insights from this study offer a clear roadmap for future seed orchard management and breeding. Immediate management strategies should include adjusting the spatial configuration of clones or employing selective pruning to modulate the pollen output of dominant fathers like S5, S11, and S17, thereby promoting a more balanced paternal contribution. Such interventions are crucial as studies show that paternal success is strongly correlated with male fecundity (e.g., strobilus production), which is under moderate genetic control [72], and that a significant portion of effective pollination occurs within short distances [69]. Therefore, spatially separating dominant pollen donors or managing their fecundity can directly influence mating patterns.

More importantly, for the long-term genetic composition of advanced-generation seed orchards, our results unequivocally argue for the integration of reproductive trait assessment into the selection index. Genotypes demonstrating superior siring ability and compatible flowering phenology should be prioritized as core parents in subsequent breeding cycles, provided their economic traits remain favorable. This shift towards combined selection for both growth performance and reproductive fitness is essential for developing seed orchards that are not only productive but also genetically robust and sustainable over multiple generations.

However, our genetic analyses relied on 15 EST-SSR markers which, while sufficient for assessing overall diversity and structure, constrained the resolution of paternity analysis. Furthermore, the parental population consisted of 30 selected clones, a robust but finite sample size that defines the genetic base of this specific orchard. Consequently, the findings and management recommendations of this study are most directly applicable to similarly managed, advanced-generation seed orchards rather than to natural populations. Potential technical challenges, such as undetected genotyping errors, also represent inherent challenges in parentage analysis. The recent availability of the *C. hodginsii* genome [58] enables further development of high-quality SNP markers for higher-resolution genetic analyses and implementation of genotype-phenotype selection in evaluating the actual breeding value of different mating combinations.

In our study, the sustained genetic diversity demonstrates the success of the mating design within a single generation; however, long-term, multi-generational breeding faces challenges in sustaining this diversity should be considered. Yang et al. [73] analyzed successive generations of *Pinus tabulaeformis* seed orchards and found a noticeable decline in genetic diversity parameters from the first to the second generation, primarily due to increased genetic drift resulting from intensive selection. Thus, despite the positive outcomes observed in our second-generation *C. hodginsii* orchard, future advanced-generation breeding should focus on proactive management of coancestry through the continuous introduction of new superior germplasm and optimized parental selection to mitigate the risk of diversity erosion.

5. Conclusions

In this study, we analyzed the genetic composition and mating system in a second-generation *C. hodginsii* seed orchard using 15 EST-SSR markers. The results reflected a highly homogeneous, entirely outcrossing system characterized by a prevalent heterozygote advantage, which provides a genetic basis for high-quality seed production. Although a limited number of superior parents (e.g., S5, S11, S17) were predominantly associated with pollen contributions, genetic diversity in progeny was maintained by the inherent low genetic differentiation ($F_{st} = 0.012$) among parents, coupled with external pollen flow (17.33%). These findings elucidate the mechanisms underlying the sustained genetic diversity under the outcrossing conditions in seed orchards, and provide a theoretical basis for optimizing parental configuration, managing pollen flow, and developing long-term breeding strategies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f17010118/s1>, File S1: Phenotypic and provenance of the parental clones; File S2: SAS matrix for parents and progeny; File S3: Sequence information of the 15 EST-SSR primers; File S4: The F_{st} and N_m among parental and progeny populations; File S5: Individuals Q-matrix ($K = 2$, 10 replicates).

Author Contributions: Conceptualization, L.Y., S.S. and R.Z.; methodology, L.Y., L.Z. and Z.L.; software, L.Y. and Z.W.; validation, L.Y.; formal analysis, L.Y., Z.W. and Z.Z.; investigation, L.Y., Z.W.,

W.G., J.Z., B.Z., G.W., Z.C. and Z.Z.; resources, W.G., J.Z., B.Z., G.W., Z.C. and S.S.; data curation, L.Y. and Z.W.; writing—original draft preparation, L.Y.; writing—review and editing, L.Z., Z.L., S.S. and R.Z.; visualization, L.Y. and Z.W.; supervision, L.Z., Z.L., S.S. and R.Z.; project administration, W.G., J.Z., B.Z., G.W., Z.C., S.S. and R.Z.; funding acquisition, S.S. and R.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the “Eagle Program” of Fujian Province from the Department of Human Resources and Social Security, and the project “Fujian Cypress 1st Generation Core Breeding Population Construction Research” (2021R1010004) from the Department of Science and Technology of Fujian Province.

Data Availability Statement: All data and materials are available upon request from the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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