Genetic Diversity Analysis of Clones in the Zixi Mountain Armand Pine Seed Orchard Based on SRAP Markers: Postprint

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Abstract

To clarify the genetic background among clones from different provenances in the Zixi Mountain Chinese pine (Pinus armandii) seed orchard in Chuxiong City, Yunnan Province, this study collected needles from 60 individual clones of Chinese pine from six provenances within the orchard, extracted total DNA using a modified CTAB method, and performed genetic diversity analysis using SRAP molecular markers. The experimental results showed that: 15 pairs of polymorphic SRAP primers were selected from 100 primer combinations, and after SRAP-PCR amplification, a total of 194 loci were obtained, with the percentage of polymorphic bands (PPB) being 85.05 %, Nei' s gene diversity index (H) being 0.233 7, Shannon' s information index (I) being 0.341 9, and the genetic differentiation coefficient (Gst) among provenances being 0. 355 5. The six provenances of Chinese pine exhibited high genetic diversity, and genetic variation primarily existed within provenances; among them, the genetic distance between the Huize (HZ) and Weishan (WS) provenances was the smallest (D= 0.050 1), while the genetic distance between Huize (HZ) and Yiliang (YL) provenances was the largest (D = 0.361 8). Cluster analysis revealed that the six Chinese pine provenances were grouped into three clusters: Huize (HZ) and Weishan (WS) provenances clustered into one group; Chuxiong (CX), Nanhua (NH), and Yiliang (YL) provenances clustered into another group; and Tengchong (TC) provenance formed a separate group. This indicates that genetic differentiation among clones in the Zixi Mountain Chinese pine seed orchard is at a relatively high level, and the research results can provide molecular-level theoretical basis and practical guidance for parental selection in Chinese pine hybrid breeding and germplasm resource evaluation.

Full Text

Genetic Diversity Analysis of Clones in a *Pinus armandii* Seed Orchard in Zixi Mountain Based on SRAP Markers

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Abstract

To clarify the genetic background among different provenance clones in the *Pinus armandii* seed orchard of Zixi Mountain, Chuxiong City, Yunnan Province, this study collected needles from 60 clones representing six provenances. Total DNA was extracted using a modified CTAB method and genetic diversity was analyzed using SRAP molecular markers. The results showed that 15 pairs of polymorphic SRAP primers were selected from 100 primer combinations. SRAP-PCR amplification yielded 194 loci, with a polymorphic loci percentage (PPB) of 85.05%, Nei’ s gene diversity index (H) of 0.2337, Shannon’ s information index (I) of 0.3419, and genetic differentiation coefficient among provenances (Gst) of 0.3555. The six provenances exhibited high genetic diversity, with genetic variation primarily existing within provenances. The genetic distance was smallest between Huize (HZ) and Weishan (WS) provenances (D = 0.0501) and largest between Huize (HZ) and Yiliang (YL) provenances (D = 0.3618). Cluster analysis divided the six provenances into three groups: Huize (HZ) and Weishan (WS) formed one group; Chuxiong (CX), Nanhua (NH), and Yiliang (YL) formed another group; and Tengchong (TC) formed a separate group. These results indicate that genetic differentiation among clones in the Zixi Mountain *P. armandii* seed orchard is at a relatively high level, providing molecular-level theoretical basis and practical guidance for parental selection in hybrid breeding and germplasm resource evaluation.

Keywords: *Pinus armandii*, seed orchard, clone, SRAP, genetic diversity

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Pinus armandii Franch. is a species in the genus *Pinus*, subgenus *Strobus*, family Pinaceae, and is a distinctive five-needle pine tree endemic to China. It serves as an important afforestation species in high-altitude regions. Since the 1970s, genetic improvement programs for *P. armandii* have been implemented in Yunnan and Guizhou provinces, establishing several first-generation clonal seed orchards with superior clones, followed by genetic testing, superior family selection, and supplementary pollination. The Zixi Mountain clonal seed orchard of *P. armandii* is located in Chuxiong City and serves as a national forest improved variety base. However, due to the lack of continuous germplasm improvement, this seed orchard remains in the initial stage of establishment, resulting in severe disease and pest problems and low productivity. Consequently, germplasm improvement and innovation are urgently needed. Hybrid breeding has proven to be one of the most effective methods for genetically improving undesirable traits in plants. However, hybrid breeding of *P. armandii* has lagged behind, with parental selection primarily based on morphology and provenance origin, without clear understanding of the genetic diversity richness and genetic relationships among collected germplasm resources, thereby limiting the degree and effectiveness of improvement. Therefore, it is particularly important to characterize the genetic diversity level and genetic relationships among germplasm resources in the seed orchard for targeted hybrid breeding.

With advances in molecular biology research and related technological capabilities, detection methods for genetic diversity in *P. armandii* have evolved from morphological observation to molecular marker analysis. For example, Zhao et al. (2012) analyzed genetic diversity among 85 clones in a *P. armandii* clonal seed orchard in Pingba, Guizhou using ISSR markers and found that genetic variation existed primarily within provenances. Zhu (2016) analyzed genetic diversity in 52 natural populations of *P. armandii* using SSR markers, revealing that genetic variation existed mainly within individuals with high genetic diversity levels. Zhu (2006) previously studied genetic diversity of some clones in the Zixi Mountain *P. armandii* seed orchard using RAPD markers, finding significant genetic differences among provenances with rich genetic diversity. However, RAPD (Random Amplified Polymorphic DNA) markers are relatively early technology and have certain limitations compared to newer molecular markers (Ruan et al., 2016), potentially limiting the reliability of these results. These studies have provided important theoretical foundations for genetic improvement and molecular evaluation of *P. armandii* germplasm resources.

Among various molecular markers, SRAP (Sequence-Related Amplified Polymorphism) technology has been widely applied in forest tree genetic diversity analysis due to its high polymorphism, simple operation, ease of sequencing, and relatively uniform distribution of most markers in the genome of experimental samples (Tilman et al., 2006; Li et al., 2001). Applications include studies on *Castanea henryi* (Xiang et al., 2016), *Dalbergia odorifera* (Meng et al., 2018), *Actinidia* germplasm (Zhang et al., 2018), *Gardenia jasminoides* (Jiang et al., 2019), and *Myrica rubra* (Chen et al., 2017). Zhao et al. (2012) optimized the SRAP-PCR reaction system for *P. armandii* clones in the Pingba seed orchard but did not conduct specific genetic diversity analysis. Currently, few studies have reported on genetic diversity analysis of *P. armandii* using SRAP markers.

Given the production status of the *P. armandii* seed orchard in Zixi Mountain, Chuxiong City, Yunnan Province, this study selected 60 superior clones from six provenances to conduct SRAP marker analysis aimed at evaluating genetic diversity and genetic relationships for hybrid breeding and germplasm resource assessment. The results provide molecular-level theoretical and practical guidance for germplasm resource evaluation and parental selection in hybrid breeding within this seed orchard.

1.1 Plant Materials

Fresh, young needles were collected from 60 disease-free clones in the *P. armandii* seed orchard of Zixi Mountain, Chuxiong City, Yunnan Province. Needles from individual clones were numbered, placed in ziplock bags, transported to Southwest Forestry University, and stored at -80°C. All materials originated from six provenances within Yunnan Province (Table 1).

1.2 Total DNA Extraction

Genomic DNA was extracted from 4-5 needles per sample using a modified CTAB method (Xie et al., 2014). Extracted DNA was diluted to 50 ng · L^{1} with 50 L TE buffer, detected using 0.8% agarose gel electrophoresis, and stored at -20°C.

1.3 SRAP-PCR Amplification

Primer sequences were obtained from published literature (Li et al., 2011; Cai et al., 2014). Ten forward and ten reverse primers were selected and randomly combined to generate 100 primer pairs (Table 2). After primary and secondary screening, 15 polymorphic primer pairs with clear bands were successfully selected (Table 3). The 25 L SRAP-PCR reaction system for *P. armandii* contained: 1 L DNA template, 12.5 L Green Taq Mix, 9.5 L ddH O, and 1 L each of forward and reverse primers. The PCR amplification program was: 94°C for 5 min; 5 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 1 min; 30 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min; final extension at 72°C for 10 min; and storage at 4°C.

1.4 Polyacrylamide Gel Electrophoresis

Six microliters of PCR product were mixed with 2 L of 6×Loading buffer and loaded into wells of 8% polyacrylamide gel for electrophoresis at 230 V for 150 min. After electrophoresis, gels were fixed, stained, developed, and photographed.

1.5 Data Analysis

Clear and distinguishable polymorphic bands were used as reference patterns. Based on the polyacrylamide gel electrophoresis results, clear bands at the same locus were scored as "1" and absent or weak bands as "0," with ambiguous bands excluded, to generate a binary matrix formatted for software analysis. Polymorphic loci numbers and polymorphism percentages for the 60 *P. armandii* samples were calculated using Excel. Genetic diversity parameters (observed alleles (Na), effective alleles (Ne), gene diversity (H)) were analyzed using POPGENE version 1.32. NTSYS 2.10 software was used for cluster analysis of the six provenances to generate a dendrogram.

2.1 Genomic DNA Extraction Results

Pinus armandii genomic DNA was detected using 1% agarose gel electrophoresis. Representative results are shown in Figure 1 [Figure 1: see original paper]. DNA bands were clear without smearing or diffusion, meeting requirements for subsequent experiments.

2.2 SRAP-PCR Amplification Results

Fifteen polymorphic primer pairs were used for PCR amplification of 60 clones from six provenances, yielding 165 polymorphic loci (Table 4). The PPB (percentage of polymorphic loci) was 85.05% across all provenances. Within provenances, PPB ranged from 41.24% to 81.96%, with an average of 59.37%. The lowest PPB was observed in Yiliang (YL) provenance (41.24%), while the highest was in Tengchong (TC) provenance (81.96%). The six provenances ranked by PPB were: Tengchong (TC) > Huize (HZ) > Weishan (WS) > Chuxiong (CX) > Nanhua (NH) > Yiliang (YL).

2.3 Genetic Diversity Analysis

Nei's gene diversity (H) among the six provenances ranged from 0.1873 to 0.3234, with the lowest in Chuxiong (CX) and highest in Tengchong (TC). Shannon' s information index (I) ranged from 0.2770 to 0.4716, lowest in Chuxiong (CX) and highest in Tengchong (TC). The highest number of alleles (Na) was observed in Tengchong (TC) (1.8196) and the lowest in Yiliang (YL) (1.1424). The highest effective number of alleles (Ne) was in Tengchong (TC) (1.5809) and the lowest in Yiliang (YL) (1.2691). Both H and I indicated that genetic variation among provenances, from highest to lowest, was: Tengchong (TC) > Huize (HZ) > Weishan (WS) > Nanhua (NH) > Yiliang (YL) > Chuxiong (CX) (Table 5).

2.4 Genetic Differentiation Analysis

Nei' s index estimation revealed total gene diversity (Ht) of 0.3420 and within-provenance gene diversity (Hs) of 0.2204 across the six provenances. The genetic differentiation coefficient (Gst) was 0.3555, indicating that 35.55% of genetic variation occurred among provenances while 64.45% existed within provenances, demonstrating substantially greater genetic differentiation within than among provenances. Gene flow (Nm) among provenances was 0.9065 (<1), indicating relatively low gene exchange among the six *P. armandii* provenances.

2.5 Genetic Similarity and Distance Analysis

Genetic similarity among the six provenances ranged from 0.6964 to 0.9511, with an average of 0.8141. Genetic distance ranged from 0.0501 to 0.3618, with an average of 0.2123 (Table 6). The largest genetic distance was between Yiliang (YL) and Huize (HZ) (D = 0.3618), with relatively low genetic similarity (Gs = 0.6964), indicating the most distant genetic relationship. The smallest genetic distance was between Huize (HZ) and Weishan (WS) (D = 0.0501), with the highest genetic similarity (Gs = 0.9511), indicating the closest genetic relationship.

2.6 Cluster Analysis of Genetic Relationships

Cluster analysis based on genetic similarity among the six provenances is shown in Figure 2 [Figure 2: see original paper]. At a threshold of 0.87, the six provenances were divided into three groups: Huize (HZ) and Weishan (WS) formed one group; Chuxiong (CX), Nanhua (NH), and Yiliang (YL) formed a second group; and Tengchong (TC) formed a separate group.

3 Discussion and Conclusion

The genetic differentiation coefficient (Gst) is an important indicator for measuring population genetic differentiation and effectively reflects population genetic structure. Based on Gst values, four scenarios are recognized: Gst = 0–0.05 indicates negligible genetic differentiation; Gst = 0.05–0.15 indicates moderate differentiation; Gst = 0.15–0.25 indicates high differentiation; and Gst > 0.25 indicates very high differentiation (Sun et al., 2016; Jiang et al., 2018). This study found total gene diversity (Ht) of 0.3420, within-provenance gene diversity (Hs) of 0.2204, and Gst of 0.3555 across six *P. armandii* provenances, with 35.55% genetic variation among provenances and 64.45% within provenances. This demonstrates substantially greater genetic differentiation within than among provenances, indicating very high genetic differentiation among these six provenances. This aligns with Wheeler et al. (1992), who noted that geneticists need not be concerned that seed orchards with limited population sizes might have lower genetic diversity than natural populations. Zhu (2006) previously analyzed RAPD genetic diversity among four provenances in the Chuxiong *P. armandii* seed orchard, reporting PPB of 71.42%, Ne of 1.3649, H of 0.1404, and I of 0.2193, indicating large genetic differences and rich genetic diversity among 64 individuals. Using material from the same seed orchard, this SRAP analysis yielded PPB of 85.05%, Ne of 1.3866, H of 0.2337, and I of 0.3419, with all parameters significantly improved and main genetic variation existing within provenances. Because RAPD markers have limitations including low repeatability, unstable amplification products, and poor specificity, previous results may have been constrained. SRAP markers offer strong polymorphism, uniform distribution, and greater stability (Zhou, 2008), thus providing richer and more reliable genetic variation information. Moreover, varying degrees of genetic variation among individuals within the seed orchard facilitate more convenient in-situ genetic improvement of *P. armandii*.

In biological evolution, gene flow enhances genetic diversity and prevents population differentiation (Ellstrand and Elam, 1993). Widen and Svensson (1992) analyzed gene flow (Nm) in 124 plant species and concluded that genetic drift significantly influences population differentiation. Wright (1950) proposed that when $\mathrm{Nm} < 1$, genetic drift is the main cause of obvious genetic differentiation among populations. Other researchers have suggested that if gene flow ($\mathrm{Nm} > 0.5$) declines substantially from previously high levels, possible causes include environmental fragmentation, reduced pollination, and intensified natural selection (Yang et al., 2014). This study found $\mathrm{Nm} = 0.9065$ (<1) among clones in the Zixi Mountain seed orchard, indicating relatively low gene exchange among the six populations and that genetic variation in *P. armandii* exists primarily within provenances—that is, among different superior individuals within provenances. Therefore, parental selection for hybrid breeding can be conducted within provenances, which not only improves cross-compatibility but also enriches genotypes in hybrid offspring, facilitating artificial selection.

Genetic distance is an important indicator reflecting genetic variation levels and differentiation degree among species and provenances, providing basic genetic parameters for heterosis (Sui et al., 2017). SRAP-based cluster analysis of 60 individuals from six provenances revealed that at a threshold of 0.95, five groups were formed: Huize (HZ) and Weishan (WS) clustered first, while Chuxiong (CX), Nanhua (NH), Tengchong (TC), and Yiliang (YL) each formed separate groups. At a threshold of 0.87, six provenances formed three groups, with Tengchong (TC) separate, Huize (HZ) and Weishan (WS) together, and Nanhua (NH), Yiliang (YL), and Chuxiong (CX) together, indicating close genetic relationship between Huize (HZ) and Weishan (WS). Genetic relationships among provenances correlate with geographic distribution, with geographically close provenances generally having similar genetic backgrounds. However, this study found that Huize (HZ) from Qujing and Weishan (WS) from Dali clustered together, which does not correspond to geographic distribution. Similar phenomena were observed in studies on *Elymus sibiricus* (Yan et al., 2009) and *Gymnocarpos przewalskii* using SCoT markers (Di, 2018), where geographic distance was not significantly correlated with genetic distance. The current genetic composition of plant populations relates not only to current geographic distribution but also to evolutionary history and anthropogenic disturbance (Di, 2018; Liu, 2012; Song, 2014). Therefore, using geographic distance as the primary criterion for parental selection in plant hybrid breeding has significant limitations. Future breeding work should primarily rely on DNA-level analysis results, combined with morphological and geographic information for comprehensive evaluation to determine optimal parental combinations. Through hybridization and subsequent selection, more superior individuals with enhanced traits (such as improved disease/pest resistance and rapid growth) can be obtained, and these superior traits can be fixed through vegetative propagation to ultimately produce improved varieties for large-scale production.

References

CAI YB, YANG XY, CHEN HJ, et al., 2014. Analysis of genetic diversity of Papaya germplasm by SRAP combined with SCoT marker[J]. J Plant Gene Resour, 15(2): 292-298.

CHEN H, YAN HL, HUANG YH, et al., 2017. Genetic diversity analysis of 66 Chinese Bayberry germplasm resources with SRAP[J]. S Chin Fruits, 46(4): 64-67.

CHEN YM, 1990. The Landscape Dendrology[M]. Beijing: China Forestry Publishing House.

DI LN, 2018. Genetic diversity of *Gymnocarpos przewalskii* based on SCoT markers[J]. Bull Bot Res, 38(5): 725-732.

ELLSTRAND NC, ELAM DR, 1993. Population genetic consequences of small population size: implications for plant conservation[J]. Ann Rev Ecol Sys, 24(1): 217-242.

JIANG QC, TAN ZJ, LIAO HB, et al., 2018. Genetic diversity of *Aquilaria sinensis* in Wuguishan, Zhongshan City, Guangdong Province[J]. Guihaia, 38(6): 804-811.

JIANG W, WU ZG, TAO ZM, et al., 2019. Genetic diversity analysis of *Gardenia jasminoides* based on ISSR and SRAP molecular markers[J]. Chin Trad and Herb Drugs, 50(2): 510-516.

LI G, QUIROS CF, 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica[J]. Theoretical & Appl Gene, 103(2-3): 455-461.

LI ZP, LI TC, FAN HY, et al., 2011. Genetic diversity analysis of Papaya Resources by SRAP and SCoT combination[J]. Acta Laser Biol Sin, 20(2): 236-244.

LIU L, 2012. Population evolution history and speciation study of *Pinus armandii* and its three affinities[D]. Beijing: Graduate University of Chinese Academy of Sciences.

MENG H, CHEN B, YANG Y, 2018. Analysis of genetic diversity of *Dalbergia odorifera* growing in Hainan by SRAP[J]. Chin J Trop Agric, 38(12): 37-42.

RUAN ZY, WANG BY, OUYANG ZQ, et al., 2016. Characterization of microsatellites in genome of *Pinus squamata*, a critically endangered species in the world[J]. Bull Bot Res, 36(5): 775-781.

SONG CF, LIU QX, ZHOU YF, et al., 2014. Genetic diversity analysis of *Glehnia littoralis* (Apiaceae) revealed by SRAP[J]. Guihaia, 34(1): 15-18.

SUN WT, YU DD, DONG ML, et al., 2016. Genetic diversity of control-pollinated progenies in seed orchard of *Larix gmelinii* var. *principis-rupprechtii* Mayr[J]. Acta Bot Boreal-Occident Sin, 36(8): 1662-1670.

SUI XZ, ZHENG Y, QIU YP, et al., 2017. Genetic diversity of 4 *Betula alnoides*[J]. Journal of SW For Univ (Nat Sci Ed), 37(3): 21-25.

TILMAN D, REICH PB, KNOPS JM, 2006. Biodiversity and ecosystem stability in a decade-long grassland experiment[J]. Nature, 441(4): 629-632.

WANG XR, ZHAO Y, LI TS, 2007. Preliminary report on artificial supplementary pollination experiment in clonal seed orchard of *Pinus armandii*[J]. J SW For Univ, (3): 25-28.

WHEELER NC, JECH KS, 1992. The use of electrophoretic markers in seed orchard research[J]. New For, 6(1): 311-328.

WIDEN B, SVENSSON L, 1992. Conservation of genetic variation in plants: the importance of population size and gene flow. In: Hansson L (ed.), Ecological principles of nature conservation: Application in temperate and boreal environments[M]. New York: Elsevier Science Publishers Ltd: 113-161.

WRIGHT S, 1950. Genetical structure of populations[J]. Nature, 166(4215): 247-249.

XIANG H, YUAN DY, FAN XM, et al., 2016. The analysis of genetic diversity of *Castanea henryi* (Skan) Rehder & E.H.Wilson by using SRAP[J]. J Plant Gene Resour, 17(6): 1072-1081.

XIE DM, CHEN GM, HE D, et al., 2014. Comparative study on the different organization of genomic DNA extraction in *Pinus armandii* Franch[J]. J Hubei Inst Natl (Nat Sci Ed), 32(3): 255-261.

XIN PY, ZHOU J, DUAN AA, et al., 2010. Research advance of genetic improvement on *Pinus armandii* in China[J]. N Hortic, (19): 210-214.

YANG ZQ, FENG YH, WU DS, 2014. Analysis of genetic diversity of *Pinus yunnanensis* var. *tenuifolia* nature populations by SSR marker[J]. Guihaia, 34(1): 10-14.

YUAN HN, CHEN WH, 2010. Development and utilization status and protection measures of *Pinus armandii*[J]. For Shaanxi, (4): 39.

ZHU J, 2016. Population genetics and speciation study of *Pinus armandii* and its three affinities[D]. Xi' an: Northwestern University.

ZHU XD, 2006. Study on the genetic diversity and seed production in clone seed orchard of *Pinus armandii* Franch.[D]. Kunming: Southwest Forestry College.

ZHAI JJ, BAI SH, ZHANG XQ, et al., 2010. Genetic diversity of wild *Elymus sibiricus* germplasm from the Qinghai-Tibetan Plateau in China detected by SRAP markers[J]. Acta Pratac Sin, 19(1): 173-183.

ZHAI SW, CHEN QG, DAI Y, et al., 2007. Heritability analysis and superior family selection for half-sib test of *Pinus armandii*[J]. Seed, 26(12): 5-8.

ZHAO Y, DAI Y, LI YZ, 2012. Genetic diversity for clonal seed orchard of *Pinus armandii*[J]. J NE For Univ, 40(10): 4-6, 11.

ZHAO Y, LI YX, DAI Y, 2012. Optimization of SRAP-PCR system for *Pinus armandii*[J]. J NW For Univ, 27(5): 87-90, 173.

ZHAO Y, LI TS, DUAN AA, 2003. A research advance in genetic improvement and development strategy of *Pinus armandii* in China[J]. J SW For Univ, 23(1): 91-95.

ZHOU YQ, 2008. Biological genetic marking and application[M]. Beijing: Chemical Industry Publishing House: 135-137, 206.

ZHANG AS, SI QL, QI XJ, et al., 2018. Genetic diversity and fingerprints of *Actinidia* germplasm resource based on SRAP markers[J]. Jiangsu J Agric Sci, 34(1): 138-144.

ZHANG DD, 2008. Study on genetic structure in different populations of *Pinus koraiensis* by SRAP[D]. Harbin: Northeast Forestry University.

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