Postprint of Genetic Diversity Analysis of Natural Populations of Daming Pine Based on SSR Markers

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Date: 2021-05-27T00:00:00+00:00

Abstract

Pinus damingensis is a unique high-elevation pine species endemic to Guangxi and Guizhou, possessing high economic and ecological value. Its natural populations have long lacked adequate conservation and utilization, which is detrimental to the long-term stable development of this species. To rationally conserve and utilize the natural genetic resources of Pinus damingensis, this study employed 12 SSR molecular markers to investigate the genetic diversity of three natural populations, analyzing inter-population genetic differentiation and gene flow to provide references for conservation strategies of this species. The results indicated that 12 primer pairs detected a total of 37 alleles, with a percentage of polymorphic loci of 100%; the mean observed number of alleles per locus (Na) was 3.08, and the mean effective number of alleles (Ne) was 1.68, with substantial variation in effective number of alleles among different loci. The mean observed heterozygosity (Ho) per locus was 0.35, the mean expected heterozygosity (He) was 0.40, and the mean polymorphic information content (PIC) was 0.31. The Shannon' s diversity index for the three populations ranged from 0.48 to 0.65, and Nei' s gene diversity ranged from 0.27 to 0.39, indicating relatively low genetic diversity compared with other related pine species. The population mean observed heterozygosity was 0.40, the mean expected heterozygosity was 0.33, and the mean effective number of alleles was 1.58. The coefficient of genetic differentiation among populations (Gst) was 0.10, indicating a low level of genetic differentiation among populations, with the majority of variation residing within populations. Gene flow among populations (Nm) was 2.74, suggesting relatively sufficient gene exchange among Pinus damingensis populations. This study can provide important reference basis for the biodiversity conservation of Pinus damingensis and lay a foundation for the scientific utilization of Pinus damingensis resources.

Full Text

Preamble

Genetic Diversity Analysis of Natural Populations of *Pinus taiwanensis* var. *damingshanensis* Based on SSR Markers

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Abstract

Pinus taiwanensis var. *damingshanensis* is an endemic alpine pine species distributed in Guangxi and Guizhou with high economic and ecological value. However, its natural populations have not received adequate protection and utilization for a long time, which is detrimental to the long-term stable development of this species. To rationally protect and exploit the natural genetic resources of *P. taiwanensis* var. *damingshanensis*, this study investigated the genetic diversity of three natural populations using 12 SSR molecular markers, analyzed inter-population genetic differentiation and gene flow, and provided a reference for conservation strategies. The results showed that 37 alleles were detected across the 12 primer pairs, with 100% polymorphic loci. The mean observed number of alleles per locus (Na) was 3.08, and the mean effective number of alleles (Ne) was 1.68, though Ne varied considerably among loci. The mean observed heterozygosity (Ho) was 0.35, mean expected heterozygosity (He) was 0.40, and mean polymorphism information content (PIC) was 0.31. Shannon' s diversity index for the three populations ranged from 0.48 to 0.65, while Nei' s gene diversity ranged from 0.27 to 0.39, indicating relatively low genetic diversity compared with other closely related pine species. At the population level, mean observed heterozygosity was 0.40, mean expected heterozygosity was 0.33, and mean effective number of alleles was 1.58. The coefficient of gene differentiation (Gst) among populations was 0.10, showing low genetic differentiation, with most variation occurring within populations. Gene flow (Nm) among populations was 2.74, indicating relatively sufficient gene exchange. This study provides an important reference for biodiversity conservation of *P. taiwanensis* var. *damingshanensis* and lays a foundation for scientific utilization of its resources.

Keywords: *Pinus taiwanensis* var. *damingshanensis*, natural population, genetic diversity, genetic differentiation, SSR molecular marker

Introduction

Pinus taiwanensis var. *damingshanensis* belongs to the family Pinaceae and genus *Pinus*. Named after its first discovery in Daming Mountain, Guangxi in 1974, it was identified and published in *Acta Phytotaxonomica Sinica* in 1975

by Chinese gymnosperm taxonomists Zheng Wanjun and Fu Liguo (Zheng et al., 1975). It is a variety of *Pinus taiwanensis* (Taiwan pine) distributed in southwestern China (Editorial Committee of Flora of China, Chinese Academy of Sciences, 1978; Liang, 1994), differing from Taiwan pine by having both medial and marginal resin canals in its needles, whereas Taiwan pine has only medial resin canals (Fan and Xue, 1993).

The species is primarily distributed in mountainous areas above 1,000 m in Guangxi and Guizhou, including Daming Mountain, Dayao Mountain, and Yinzhu Laoshan in Guangxi, and Leigong Mountain, Yuntai Mountain, Fanjing Mountain, and Dashahe in Guizhou, forming a discontinuous, patchy distribution pattern. *P. taiwanensis* var. *damingshanensis* is light-loving and adapts to cool, humid alpine climates. It grows well in deep, well-drained acidic soils on sunny slopes, tolerates poor soil conditions, but grows slowly. The wood is solid and resin-rich, suitable for furniture, utensils, and boards, as well as for construction, mining timber, and wood fiber industrial raw materials, making it an important timber species for high-altitude regions in southwestern China. Its elegant tree form also makes it valuable for landscaping.

Due to long-term neglect, *P. taiwanensis* var. *damingshanensis* has not been systematically utilized or protected, and there is a lack of systematic and targeted investigation and research on its population distribution, genetic diversity level, and natural regeneration capacity. Current research on this species is scarce. Jia et al. (2019) analyzed the breeding strategies and mating systems of four pine species, including *P. taiwanensis* var. *damingshanensis*, *P. massoniana*, *P. yunnanensis* var. *tenuifolia*, and *P. latteri*, by comparing their cone and seed traits. Feng et al. (2019) investigated the germplasm resources of *P. taiwanensis* var. *damingshanensis* in Guangxi and Guizhou, clarifying the distribution characteristics of its germplasm resources, but did not analyze or evaluate its population genetic diversity level. Therefore, urgent research is needed on the genetic diversity of existing natural populations of this species.

Genetic diversity is the product of long-term evolution in biological populations and the prerequisite for their survival, adaptation, and development. Higher genetic diversity provides richer genetic variation and stronger adaptability to environmental changes, making its study essential for conservation and utilization. Genetic markers are the most commonly used tools for studying genetic diversity, among which SSR (simple sequence repeat) markers are among the most polymorphic and widely used. Zhou et al. (2021) used 23 pairs of SSR polymorphic primers for PCR amplification of 54 avocado germplasm accessions to analyze their genetic diversity and relationships. Zang et al. (2021) analyzed the genetic diversity of natural populations of *Quercus fabri* using SSR markers to guide rational development and conservation of its germplasm resources. Chen et al. (2020) analyzed the genetic diversity level of natural *Quercus mongolica* populations in Liaoning Province using ten newly developed nuclear SSR markers specific to Mongolian oak. Chen et al. (2020) analyzed the genetic diversity of 35 pomelo germplasm resources from different locations using SSR

molecular markers to provide references for conservation, variety identification, and genetic improvement.

In light of this, this study investigates the genetic diversity of natural populations of *P. taiwanensis* var. *damingshanensis* using SSR molecular markers, analyzing its diversity level and genetic differentiation. This will strengthen conservation efforts for this rare tree species, promote biodiversity protection and ecological construction, and lay a solid foundation for scientific utilization of rare pine resources. Therefore, studying the genetic diversity of natural populations of *P. taiwanensis* var. *damingshanensis* in the Guizhou-Guangxi region has important theoretical and practical significance.

Materials and Methods

1.1 Materials

The experimental materials included three natural populations: Guangxi Daming Mountain, Guizhou Fanjing Mountain, and Guizhou Dashahe (Table 1). The Daming Mountain population (DM) was collected from Xianrentai in Daming Mountain National Nature Reserve (108°25 54 -108°25 58 E, 23°30 12 - 23°30 13 N), which has a south subtropical humid mountain monsoon climate. The frost-free period is 292–312 days, with long sunshine hours and abundant light and heat. The climate features humid summers and dry winters, with dry-cold and hot-humid periods occurring simultaneously, and obvious vertical climate variation (Feng et al., 2019).

The Fanjing Mountain population (FJ) was collected from Mianxuling in Fanjing Mountain National Nature Reserve (108°39 46 -108°39 52 E, 27°54 45 - 27°54 46 N), which has a mid-subtropical humid mountain monsoon climate. The frost-free period is 270–278 days, with average relative humidity reaching 80% (Feng et al., 2019).

The Dashahe population (DS) was collected from Zengziyan in Dashahe National Nature Reserve (107°35 04 -107°35 09 E, 29°08 13 -29°08 22 N), which has a north subtropical humid monsoon climate. The terrain slopes from high in the north to low in the south, with maximum elevation of 1,939.9 m, minimum of 560 m, and average of 1,400 m, and relative humidity of 88% (Feng et al., 2019).

More than 30 individuals were sampled from each population at 30 m intervals. Due to the small spatial extent of the Daming Mountain population, only 26 individuals were collected. Needles were placed in centrifuge tubes and dried with silica gel for preservation.

1.2.1 DNA Extraction

DNA was extracted from needle samples using a modified CTAB lysis-silica bead adsorption method (Doyle & Doyle, 1990) and purified. Sample DNA quality was tested by agarose gel electrophoresis (1% concentration), and DNA concen-

tration was measured using a UV spectrophotometer. Samples were diluted to a uniform concentration and stored at -20 °C for later use.

1.2.2 SSR Primer Sources and PCR Reaction Conditions

The SSR primers used in this study were designed and developed based on sequences from the *P. massoniana* genome (Feng et al., 2016). Twelve polymorphic primer pairs with clear main bands were selected after screening with eight *P. taiwanensis* var. *damingshanensis* DNA samples. Primers were synthesized by Shanghai Generay Biotech Co., Ltd., which also supplied Taq polymerase and dNTPs.

The PCR reaction system was 10 L: 10 mmol·L^{-1} Tris-HCl (pH 8.0), 50 mmol·L^{-1} KCl, 2.5 mmol·L^{-1} Mg^{2+}, 0.2 mmol·L^{-1} dNTPs, 2.5 pmol primers, 0.08 U Taq polymerase, and 10–20 ng DNA. The amplification program was: 94 °C for 4 min; 25 cycles of 94 °C for 15 s, 55–60 °C for 15 s, 72 °C for 30 s; final extension at 72 °C for 20 min (Feng et al., 2016).

PCR products were subjected to 8% polyacrylamide gel electrophoresis with silver staining detection, following the method of Yang et al. (2014).

1.2.3 Genetic Diversity Analysis

SSR band interpretation followed the method of Yang et al. (2014). After obtaining SSR genotyping data, POPGENE32 software (Yeh et al., 1997) was used to calculate the following genetic diversity parameters for the 12 loci: percentage of polymorphic loci (PPL), polymorphic information content (PIC), observed number of alleles (Na), effective number of alleles (Ne) (Hartl et al., 1989), Shannon's information index (I) (Shannon et al., 1949), observed heterozygosity (Ho), expected heterozygosity (He) (Nei et al., 1973), and Nei' s gene diversity (h). Based on these parameters, the fixation index (F), coefficient of gene differentiation (Gst), gene flow (Nm), and genetic distance (GD) of *P. taiwanensis* var. *damingshanensis* populations were further calculated.

Results and Analysis

2.1 SSR Primer Screening Based on Interspecific Transferability

Twelve SSR primer pairs were obtained for genetic diversity detection in *P. taiwanensis* var. *damingshanensis*. Comparing the observed number of alleles for these 12 primer pairs between *P. taiwanensis* var. *damingshanensis* and *P. massoniana* (Feng et al., 2016) revealed that six primer pairs had fewer alleles in *P. taiwanensis* var. *damingshanensis*, three had the same number, and only three had more alleles. The mean observed number of alleles was 3.08 for *P. taiwanensis* var. *damingshanensis* and 3.50 for *P. massoniana*, indicating that *P. massoniana* has richer genetic variation at these loci.

2.2 Genetic Diversity Analysis of Natural Populations

The mean observed number of alleles (Na) across the 12 SSR loci was 3.08. The effective number of alleles (Ne) ranged from 1.16 to 2.87, with an average of 1.68, showing considerable variation among loci. Observed heterozygosity ranged from 0.14 to 0.66, with a mean of 0.35, while expected heterozygosity ranged from 0.12 to 0.92, with a mean of 0.40, also showing large differences among loci. The mean Shannon' s index (I) was 0.62, and mean Nei' s diversity index (h) was 0.35. PIC values ranged from 0.13 to 0.58, with a mean of 0.31; the highest was at locus PF695 and the lowest at PF464.

Genetic diversity parameters were calculated separately for the three *P. taiwanensis* var. *damingshanensis* populations to compare diversity levels among them. The ranking of observed number of alleles from high to low was: Dashahe population > Fanjing Mountain population > Daming Mountain population. However, the rankings for effective number of alleles (Ne), expected heterozygosity (He), Shannon' s diversity index, and Nei' s gene diversity were: Dashahe population > Daming Mountain population > Fanjing Mountain population. This indicates that the Dashahe population has the richest genetic diversity, followed by the Daming Mountain population, with the Fanjing Mountain population having the lowest.

2.3 Genetic Structure and Gene Flow Analysis

The mean Fit value for *P. taiwanensis* var. *damingshanensis* populations was -0.16, and mean Fis was -0.26, indicating deviation from Hardy-Weinberg equilibrium and excess heterozygosity. The mean fixation index (Fst) was 0.08, showing no significant genetic differentiation among populations. Gene flow (Nm) among populations, calculated based on Fst values, ranged from 0.43 to 31.68 across loci, with an average of 2.74, indicating relatively sufficient gene exchange among the three populations.

Analysis of genetic differentiation revealed that genetic diversity within populations accounted for 87.64% of total diversity, while diversity among populations accounted for 12.36% (Table 5). The coefficient of gene differentiation (Gst) calculated from H was 0.10, with the ratio of within-population gene diversity being 0.90. This indicates low genetic differentiation among *P. taiwanensis* var. *damingshanensis* populations, with most variation existing within populations.

2.4 Genetic Distance Analysis

Genetic distances (GD) among natural populations of *P. taiwanensis* var. *damingshanensis* were relatively small (GD < 0.0770), with the genetic distance between Daming Mountain and Dashahe populations being particularly close (GD = 0.0269). Genetic identity (GI) among populations was high (GI > 0.9259) (Table 6).

Discussion

3.1 Overall Genetic Diversity Assessment

Based on genetic diversity results from 37 alleles at 12 loci, the observed number of alleles in natural populations of *P. taiwanensis* var. *damingshanensis* ranged from 2.33 to 2.83, with a mean of 2.61. Observed and expected heterozygosity ranged from 0.32 to 0.49 and 0.28 to 0.40, respectively, with means of 0.40 and 0.33. Shannon' s information index was 0.54, and Nei' s gene diversity was 0.32. In comparison, Al-Rabab' ah & Williams (2002) reported observed number of alleles, observed heterozygosity, and expected heterozygosity of 7.75, 0.52, and 0.65, respectively, for loblolly pine (*Pinus taeda*). Mehes et al. (2009) reported values of 6.50, 0.72, and 0.81 for western white pine (*Pinus monticola*). Karhu et al. (2006) reported observed number of alleles and observed heterozygosity of 8.19 and 0.73 for radiata pine (*Pinus radiata*). Compared with these congeneric species, all indices for *P. taiwanensis* var. *damingshanensis* were lower, indicating relatively low genetic diversity.

Additionally, European spruce (*Picea abies*) had heterozygosity of 0.79 (Pfeiffer et al., 1997), Douglas fir (*Pseudotsuga menziesii*) had heterozygosity of 0.67 (Amarasinghe & Carlson, 2002), and cork oak (*Quercus suber*) had heterozygosity of 0.65 (Hornero et al., 2001). Compared with these different genera, heterozygosity indices for *P. taiwanensis* var. *damingshanensis* were also relatively low. This may be related to its fragmented geographic distribution. The three populations surveyed in this study were all relatively small in size with narrow distribution ranges and large geographic distances between different populations. Consequently, its genetic variation level is lower than that of pine species with large, continuously distributed populations such as *P. taeda* and *P. radiata*. On the other hand, the SSR primers used in this study were developed from the *P. massoniana* genome, and molecular markers with good interspecific transferability are often located in relatively conserved sequences, resulting in lower polymorphism.

3.2 Genetic Structure Characteristics

This study found that genetic variation within *P. taiwanensis* var. *damingshanensis* populations (87.64%) was far greater than that among populations (12.36%), with low genetic differentiation among populations and most variation existing within populations. Mean gene flow among populations was 2.74, indicating relatively sufficient gene exchange among the three populations. As pines are wind-pollinated with pollen having strong long-distance dispersal capability, long-distance geographic isolation has some limiting effect on gene exchange among *P. taiwanensis* var. *damingshanensis* populations but has not yet led to obvious genetic differentiation. Similar conclusions were reached in studies on genetic diversity of natural populations of *Pinus yunnanensis* (Xu et al., 2016) and *Pinus tabuliformis* (Wu et al., 2018). It can therefore be inferred that phenotypic diversity differences among different populations of *P. taiwa-*

nensis var. *damingshanensis* are largely formed by environmental differences, with no significant differentiation at the genetic level. Therefore, strategies for selection, collection, and conservation of *P. taiwanensis* var. *damingshanensis* germplasm resources should focus on selecting and preserving individuals with superior traits within each population.

The study also found that observed heterozygosity was greater than expected heterozygosity in *P. taiwanensis* var. *damingshanensis* populations, showing excess heterozygosity and suggesting possible immigration or introgression of exotic genes. Like Taiwan pine, *P. taiwanensis* var. *damingshanensis* is a vertical replacement species for *P. massoniana* (Feng et al., 2019). Since gene introgression occurs between Taiwan pine and *P. massoniana* (Luo et al., 2001; Zhai et al., 2018), it is highly probable that *P. taiwanensis* var. *damingshanensis* has experienced gene introgression with *P. massoniana* in this region.

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