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Development of gene-SSR molecular markers and analysis of genetic diversity in Phoebe zhennan from Guizhou

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Phoebe zhennan (Nanmu) is a exceptional and precious timber and ornamental tree species native to China and is classified as a nationally protected endangered species. As Guizhou represents a major distribution area for this species, studying its genetic diversity (GD) is vital for developing effective preservation and utilization strategies. Due to the lack of reported reference genomes for the Nanmu, the development of molecular markers has been somewhat limited. In this study, magnetic bead enrichment was employed to develop Simple Sequence Repeats (SSR) molecular markers for the Nanmu genome, resulting in 794,128 SSR loci. From 108 primer pairs, 20 pairs with high polymorphism and good stability were used, and the GD of 174 individuals from 24 populations, including representative Nanmu populations in Guizhou, was analyzed. The results revealed that most SSR loci were of the dinucleotide repeat type (54.31%), with the AG/CT motif occurring most frequently. The Polymorphic Information Content (PIC) values for the 20 polymorphic SSR primer pairs from 0.777 to 0.903, with a mean value of 0.859, indicating rich polymorphisms. The Shannon Information Index for the Guizhou Nanmu population ranged from 1.196 to 1.928, with a mean of 1.518. Nei's GD index (H) varied between 0.661 and 0.832, with a mean of 0.743. The SN population exhibited relatively high GD, while the MT population showed comparatively low diversity. A evaluation of GD between Guizhou Nanmu populations. Sichuan Gulin, Sichuan Zigong, Chongqing Tongnan, and Hunan Baijia revealed that the Guizhou Nanmu populations possessed high and stable GD. Genetic differentiation analysis indicated that the primary source of genetic variation in the Nanmu population in Guizhou is within-population variation, with frequent gene flow and wide distribution of allele loci across populations. Phoebe bournei, Phoebe sheareri, and Phoebe chekiangensis clustered in distinct branches from other regional Nanmu populations. The SSR molecular indicators established in this study confirmed, at the molecular level, that these organisms belong to separate taxa. This research provides useful SSR molecular markers and valuable references for Phoebe germplasm breeding,

species identification, and GD studies.

Development of ~~Gene-~~SSR ~~Molecular~~ Markers and Analysis of Genetic Diversity in Phoebe zhennan from Guizhou

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Abstract

Phoebe zhennan (Nanmu) is a exceptional and precious timber and ornamental tree species native to China and is classified as a nationally protected endangered species. As Guizhou represents a major distribution area for this species, studying its genetic diversity (GD) is vital for developing effective preservation and utilization strategies. Due to the lack of reported reference genomes for the Nanmu, the development of molecular markers ~~has been somewhat~~ limited. In this study, magnetic bead enrichment was employed to develop Simple Sequence Repeats (SSR) molecular markers for the Nanmu genome, resulting in 794,128 SSR loci. From 108 primer pairs, 20 pairs with high polymorphism and good stability were used, and the GD of 174 individuals from 24 populations, including representative Nanmu populations in Guizhou, was analyzed. The results revealed that most SSR loci were of the dinucleotide repeat type (54.31%), with the AG/CT motif occurring most frequently. The Polymorphic Information Content (PIC) values for the 20 polymorphic SSR primer pairs from 0.777 to 0.903, with a mean value of 0.859, indicating rich polymorphisms. The Shannon Information Index for the Guizhou Nanmu population ranged from 1.196 to 1.928, with a mean of 1.518. Nei's GD index (H) varied between 0.661 and 0.832, with a mean of 0.743. The SN population exhibited relatively high GD, while the MT population showed comparatively low diversity. A evaluation of GD between Guizhou Nanmu populations. Sichuan Gulin, Sichuan Zigong, Chongqing Tongnan, and Hunan Baijia revealed that the Guizhou Nanmu populations possessed high and stable GD. Genetic differentiation analysis indicated that the primary source of genetic variation in the Nanmu population in Guizhou is within-population variation, with frequent gene flow and wide distribution of allele loci across populations. Phoebe bournei, Phoebe sheareri, and Phoebe chekiangensis clustered in distinct branches from other regional Nanmu populations. The SSR molecular indicators established in this study confirmed, at the molecular level, that these organisms belong to separate taxa. This research provides useful SSR molecular markers and valuable references for Phoebe germplasm breeding, species identification, and GD studies.

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1 Itroduction

Phoebe zhennan S. K. Lee, belonging to the Phoebe genus of the Lauraceae family, is indigenous to China. It is classified as a rare and endangered species of the second grade and was included in the IUCN Red List of Threatened Species in 1984. Nanmu ~~(as it is commonly known)~~ is primarily distributed in Sichuan, Guizhou, Hubei, and other regions, with notable populations in Zunyi, Tongren, and Qiandongnan in Guizhou Province (Lu et al. 2018). This species is highly valued for its straight trunk, distinctive fragrance, and durable, hard material, making it significant in architecture, furniture~~,~~ and handicrafts (Yue & Runguo 2023; Hanbo et al. 2022). The most important seed disperser of Nanmu is fruit-eating birds. However, wild populations have drastically declined due to slow growth, environmental degradation, and over-harvesting (Jianhua et al. 2020; Lichao et al. 2022). This has also complicated the discrimination between pairs of Phoebe species through minor differences in leaf morphology. Therefore, developing molecular markers and investigating the GD of Phoebe species are essential for enhancing understanding of genetic variation, which plays a significant role in germplasm cultivation, species identification, and conservation.

Here are some common molecular markers that are better than other markers for identification compared to other markers:DNA molecular markers are directly induced into the inherent genetic qualities of species. It is when these are not influenced by extrinsic environmental factors and have benefits such as high co-dominance and abundant availability. Consequently, DNA markers are widely applied in genetic breeding, gene pool construction, and species identification (Christian 2004; Cai 2008). Among these, Simple Sequence Repeats (SSRs) are particularly valuable owing their high polymorphism, stability, repeatability, and low cost, making them ideal for developing genetic markers, conducting species identification, and studying population genetic structures (Changming et al. 2015; Wangcang et al. 2023). Existing studies on SSR marker development and GD in Nanmu have primarily relied on EST(expressed sequence tag-simple repeat,EST-SSR) or transcriptome data. For instance, Xiaodong et al. (2016) employed high-throughput sequencing to develop SSR markers from the transcriptome of Sichuan Nanmu and identified nine highly polymorphic primer pairs. Qun et al. (2023) used 14 pairs of SSR primers to demonstrate the high GD of Nanmu germplasm resources and applied the marker results to construct identity profiles for 60 Nanmu germplasm resources. However, publicly available genomic data for Nanmu are limited, hindering further SSR marker development and their broader application. ~~Additionally, research on the GD of Nanmu in Guizhou is scarce~~. Furthermore, the interspecific relationships between some Phoebe species, such as Phoebe zhennan and Phoebe bournei, remain controversial (Juan et al. 2018;Ding et al. 2018). Therefore, further research on the GD and identification of Phoebe species of these vegetation types are important ~~basis~~ for the development of more effective conservation measures for ~~conserving~~ existing Nanmu resources. In this study, the SSR ~~molecular~~ markers were developed by sequencing an SSR-enriched library from the Nanmu genome, sourced from the ~~mother~~ forest of Nanmu in Changning, Sichuan Province. We also analyzed the GD of Nanmu in its main distribution areas in Guizhou. This work not only contributes to the

enrichment of the Nanmu genomic data but also aids in comprehending the genetic processes underlying the species' vulnerability and the GD of Nanmu populations in Guizhou. The findings provide valuable data ~~and theoretical references~~ for Nanmu's genetic mechanisms, germplasm resource cultivation, wild resource protection, and species identification.

2 Materials & Methods

2.1 Plant material

The distribution range of wild Nanmu in Guizhou was initially determined through consultation with the Chinese Virtual Herbarium (CVH, http://www.cvh.org.cn/) and the Chinese Local Flora. With guidance and approval from the local Forestry Bureau, representative Nanmu areas were selected for field surveys, where data such as collection sites, latitude and ongitude, were recorded. Fresh, healthy leaves were ~~then~~ collected, flash-frozen in liquid nitrogen, and stored at -80℃ in an ultra-low temperature freezer (Table 1,Table S1). ~~Nanmu is typically located near village settlements rather than in nature reserves. Since we only collect a small amount of branches and seeds, written permits are not required. It is sufficient to contact local villagers or forest rangers to inform them of our purpose.~~

The Nanmu populations from Guizhou were named according to the collection sites and include Daozhen (DZ), Tongzi (TZ), Jianhe (JH), Taijiang (TJ), Yuqing (YQ), Fenggang (FG), Chishui (CS), Dejiang (DJ), Jiangkou (JK), Sinan (SN), Meitan (MT), Shiqian (SQ), Wuchuan (WC), Xishui (XS), Zhengan (ZA), and Yanhe (YH). Additionally, materials from other primary Nanmu-producing regions, such as Zigong (ZG) and Hejiang County (HJ;P. zhennan) in Sichuan Province, Gulin County (GL) in Luzhou City, Tongnan District (TN) in Chongqing, and Baijia Town (BJ) in Hunan Province, were also collected. Three Nanmu germplasm species (Phoebe bournei from Guizhou University, Phoebe sheareri from Guizhou Botanical Garden, and Phoebe chekiangensis from Qingyuan County, Zhejiang Province) were included, bringing the total number of experimental materials to 174. Nanmu samples for SSR enrichment library construction were obtained from Sichuan Changlin Maternal Forest and provided by the Guizhou Forestry Academy of Sciences.

2.2 Methods

2.2.1 Extraction of ~~Nanmu~~ DNA

Genomic DNA of ~~the Nanmu~~ was extracted employing ~~a adapted~~ CTAB technique (Yichen et al. 2021). The quality of the extracted DNA was assessed using 1% agarose gel electrophoresis. The the genomic DNA was diluted to 50 ng/μL, and stored at -20℃ for future use.

2.2.2 Construction and sequencing of SSR-enriched libraries ~~for Nanmu~~

The DNA samples were dispatched to Shanghai Parsonage Biotechnology Co., Ltd. for the construction and sequencing of SSR-enriched libraries. High-quality genomic library was constructed by randomly fragmenting genomic DNA through ultrasonic treatment. The library SSR fragments were enriched using magnetic bead-based enrichment, and were then subjected to high-throughput sequencing. ~~Bioinformatics software was used to process and filter using~~

bioinformatics software to obtain high-quality results.Raw data have been deposited to National Center for Biotechnology Information (NCBI) under the BioProject number PRJNA1224921.

2.2.3 Characterization of SSR loci in Nanmu and primer design

Based on the high-quality sequencing data obtained, MISA software (Microsatellite identification tool) was utilized to detect SSR loci, which were subsequently characterized and statistically analyzed. Primers for SSR loci with more than two polymorphisms were designed using primer3 software, with the target amplification product size spanning from 100 to 400 bp (Andreas et al. 2012)

2.2.4 SSR primer screening and PCR amplification for Nanmu

108 pairs of eligible polymorphic SSR primers were selected from the designed primers and synthesized by Shanghai Bioengineering Co. The selected primers were amplified via PCR using genomic DNA from 16 Nanmu samples as templates. After amplification, they were detected by 2% agarose gel electrophoresis, after further screening. SSR-PCR products were then subjected to capillary electrophoresis (Agilent, USA) to select primers that consistently amplified bands with high polymorphisms.

2.2.5 Data analysis

The results of capillary electrophoresis were scored as 0 or 1, and GD parameters were calculated based on manual band reading. GD indices, including the number of alleles (Na), the effective number of alleles (Ne), Shannon information index (I), genetic differentiation coefficient (Gst), and gene flow (Nm), were computed using GenAlEx 6.51 software. Nei's GD index (H) was determined by Power Marker software. UMPGA clustering of populations was performed based on Nei's genetic distances, and the resulting cluster tree was visualized through the iTOL online website (https://itol.embl.de/). Molecular ANOVA was conducted using Arlequin. Population genetic arrangement was examined using Structure software to categorize the populations. The results folder was then compressed and submitted to Structure Harvester (https://taylor0.biology.ucla. edu/structureHarvester/).

3 Results

3.1 Characterization of SSR loci

A total of 794,128 Nanmu SSR loci were recognized in 1,642,465 sequences of the Nanmu SSR-enriched library using MISA software (Table 2). Characterization of the SSR sites revealed (Figure 1A) that dinucleotide SSRs comprised the highest percentage, with 431,326 occurrences, accounting for 54.31%. This was followed by mononucleotide SSRs and trinucleotide SSRs, with 209,961 and 141,218 occurrences, respectively, representing 26.44% and 17.78%. The occurrences of tetranucleotide, pentanucleotide, and hexanucleotide SSRs were lower, collectively accounting for 1.47%. Among these, the most common mononucleotide SSRs were of the A/T type, constituting 98.81%, while the AG/CT type of dinucleotide SSRs exhibited the highest frequency of occurrence, at 84.73 %. Most trinucleotide SSRs were of the AAG/CTT type, representing 80.08 %.

The analysis further revealed that the number of SSR loci decreased gradually with an rise in the number of repetitive motif repeats (Figure 1B). However, mononucleotide and

dinucleotide SSRs exhibited a trend of decreasing followed by increasing, with mononucleotide SSRs showing a sharper decline. The repetitions of mononucleotide SSRs were primarily concentrated between 10 and 15 repeats, while dinucleotide SSRs were centered between 6 and 10 repeats. Trinucleotide SSR repetitions were primarily concentrated between 5 and 7 repeats, and tetranucleotide, pentanucleotide, and hexanucleotide SSRs were predominantly limited to five repetitions.

3.2 Polymorphism analysis of the SSR markers

Sixteen Nanmu species from different seed sources in Guizhou were selected as materials, and genomic DNA was extracted using the modified CTAB. This DNA served as a template for the preliminary validation of the synthesized SSR primers. The results revealed that 33 out of 108 primer pairs successfully amplified clear and expected bands, yielding an amplification efficiency of 30.57%. Subsequent capillary electrophoresis of the PCR products from these 33 primer pairs identified 20 pairs of primers with high polymorphism (Table S2),Selected capillary electrophoresis results are shown in Figure S1.

218 alleles were identified across 16 individuals using the 20 selected SSR primers. The number of alleles per locus ranged from 7 to 15, with an average of 10.9. The observed number of effective Ne varied from 1.693 to 2.667, with a mean of 2.260. Shannon's information index (I) ranged from 0.472 to 0.970, with a mean of 0.763. The mean values of observed heterozygosity (Ho) and expected heterozygosity (He) were 0.705 and 0.487. Nei's GD index (H) ranged from 0.801 to 0.910, with a mean of 0.872 The Polymorphic Information Content (PIC) of the SSR loci ranged from 0.777 to 0.903, with an average of 0.859. These results indicated that the 20 ~~Nanmu~~ SSR loci were highly polymorphic and could be effectively used for assessing the GD ~~of Nanmu~~ (Table 3).

3.3 GD Analysis of Guizhou ~~Nanmu~~ Population

Genetic parameters serve as indicators of GD levels within ~~different~~ Nanmu populations (Table 4). The analysis results revealed that, in Guizhou Nanmu populations, the Na value ranged from 3.850 to 8.150, with a mean of 5.581. The highest Na value was observed in the SN population, while the minimum values were found in the JH and MT populations. The Ne value ranged from 3.313 to 6.402, and the I value varied from 1.196 to 1.928, with the maximum and minimum values of Ne and I observed in the SN and MT populations, respectively. The Ho values from 0.406 to 0.823, with the maximum value in the JK group and the minimum in the DZ group. The He values ranged from 0.654 to 0.831, with the maximum in the SN group and the minimum in the MT group. In addition, with the exception of the DJ, JH, and MT groups, the Ho values for the ~~remaining~~ Nanmu populations were smaller than the He values, suggesting the possibility of inbreeding within these populations. The variation in Nei's GD Index (H) from 0.661 to 0.832, with the highest value in the SN population and the lowest in the MT population. Overall, these results suggested that the SN population exhibited higher GD, while the MT population had lower GD.

When comparing the GD parameters of Nanmu from Sichuan, Chongqing, and Hunan to those in Guizhou, the BJ, GL, ZG, and TN populations showed intermediate GD, lower than the

SN population but higher than the MT population. The Nei's GD indices for BJ, GL, ZG, and TN were all greater than 0.7, suggesting that these populations maintain high GD. Additionally, the Ho values for BJ, GL, ZG, and TN were all smaller than their respective He values, indicating a certain degree of inbreeding in these populations.

A comparison of the GD indices between the collected Nanmu (P. zhennan) from Hejiang County, Sichuan Province, and other species such as P. bournei, P. chekiangensis, and P. sheareri showed that the Shannon information index for the P. bournei population was the highest, at 1.917. The Ho value for P. chekiangensis reached a maximum of 0.802, while the maximum He value for P. bournei was 0.821. In general, the Ho values for all populations, except the P. chekiangensis, were lower than the He values, which may indicate inbreeding or could be attributed to sample size or environmental factors. The Nei's GD indices (H) ranked from high to low as P. bournei, P. sheareri, P. zhennan, and P. chekiangensis, with the combined analysis confirming that all four Phoebe species exhibited high GD.

3.4 Analysis of genetic differentiation of Guizhou Nanmu populations

The genetic differentiation of Guizhou Nanmu populations was analyzed based on 20 SSR loci (Table 5). ~~Fis represents the inbreeding coefficient within a population.~~ When Fis < 0, an excess of heterozygotes is observed due to distant crosses, indicating outbreeding. Conversely, when Fis > 0, inbreeding results in an excess of homozygotes, indicating a higher occurrence of pure heterozygotes. The Fis values for the 20 SSR loci ranged from -0.323 to 0.478, with a mean value of 0.059. ~~Notably, the Fis values for SSR11~SSR12 and SSR14~SSR20 were negative, suggesting an excess of heterozygotes at these loci. In contrast, the Fis values for SSR1~SSR10 and SSR13 were positive, indicating an excess of homozygotes at these loci.~~ Fit represents the inbreeding coefficient between populations. The Fit values for the 20 SSR loci ranged from -0.089 to 0.628, with a mean value of 0.218, suggesting the presence of some degree of inbreeding between populations. Fst, the coefficient of differentiation between populations, ranged from 0.114 to 0.288, with a mean value of 0.175, indicating significant genetic differentiation among populations. The mean Fst value suggests a relatively high level of genetic differentiation. Nm, which measures gene flow, ranged from 0.619 to 1.941, with a mean value of 1.263. Since Nm > 1, this indicates frequent gene flow between populations, with alleles from each locus being widely distributed across populations.

An AMOVE (Analysis of Molecular Variance) of the 20 loci in Guizhou Nanmu populations was conducted using Arlequin software (Table 6). The findings showed that 9.43% of the genetic variation was ascribed to disparities among populations, while 90.57% resulted from variation within populations. This implies that the majority of genetic variation in Guizhou Nanmu is found within individual populations.

3.5 Analysis of the genetic structure of Nanmu populations

The genetic structure of the Nanmu populations was analyzed using Structure software (Figure 2). The highest ΔK value was observed when K = 3, indicating that the 24 populations could be classified into three subgroups, which likely originated from three distinct homologous gene pools. The classifications are represented by red, green, and blue colors(Figure 3). The

classification was based on the Q value, where Q ≥ 0.6 indicates a relatively homogeneous genealogy, allowing the individuals to be clustered into a single group (Table S3). The first group (red) includes 54 individuals from 13 populations, accounting for 31.03% of the total. This group consists of all individuals from the BJ, FG, GL, JH, TJ, YQ, and YH groups, and some individuals from the CS (2), JK (1), HJ (1), SN (1), SQ (1), and ZA (1) groups. The second group (green) includes all individuals from three populations: ZIN (P. sheareri), ZJN (P. chekiangensis), and MN (P. bournei), accounting for 17.24% of the total. The third group (blue) consists of 88 individuals from 14 populations, accounting for 50.57% of the total. This group includes all individuals from the DJ, DZ, TN, TZ, WC, XS, and ZG groups, as well as some individuals from the CS (5), GL (1), JK (7), HJ (7), MT (3), SN (7), SQ (10), and ZA (5) groups. This indicates that Guizhou, Sichuan, Chongqing, and Hunan populations share a certain degree of genetic overlap. In addition, MT20-03 and SN20-11 exhibit Q < 0.6, suggesting a more complex genealogy, and ~~therefore,~~ these two individuals were categorized into a fourth group.

3.6 Cluster analysis and principal component analysis of Nanmu populations

Genetic distance reflects the kinship between Nanmu populations, with a larger genetic distance indicating greater divergence. UPGMA clustering analysis was performed based on Nei's genetic distance among the groups (Figure 4). When the groups were clustered into three categories: the first category comprised the YH group; the second category included three groups—P. bournei, P. sheareri, and P. chekiangensis; and the third category included the DZ, JH, GL, BJ, FG, YQ, TJ, MT, XS, TZ, P. zhennan, ZG, TN, WC, JK, CS, DJ, ZA, SQ, and SN Nanmu groups. When the 24 groups were clustered into five categories, the first category remained the same as in the previous classification, while the third category comprised the DZ Nanmu group; the fourth category included the JH, GL, BJ, FG, YQ, and TJ Nanmu groups; and the fifth category included the MT, XS, TZ, P. zhennan, ZG, TN, WC, JC, JK, CS, DJ, ZA, SQ, and SN Nanmu groups. In the comprehensive analysis, this study successfully distinguishes P. bournei from the other Nanmu species. It was found that P. bournei is more closely related to P. sheareri. P. bournei, P. chekiangensis, and P. sheareri form a distinct branch, while the remaining groups cluster into another branch. Notably, P. zhennan is more closely related to the Nanmu groups from ZG, TN, and TZ, indicating closer affinities between them. The YH Nanmu group from Guizhou formed a separate branch, suggesting that it may represent a different species of Phoebe.

In a principal component analysis of 174 samples based on Nei's genetic distance (Figure 5), all individuals from the three groups—P. bournei, P. sheareri, and P. chekiangensis—were clustered into one group in the upper right corner (red), with no overlap between them. The YH individuals formed a distinct group on their own (blue), while the remaining Nanmu groups were distributed in a cross pattern, in agreementwith the findings of the cluster analysis.

4 Discussion

4.1 Development of SSR molecular markers for the Nanmu genome

The morphological differences between species of Phoebe are minimal, and their distribution is wide. Based on traditional morphological classifications and the currently

developed EST and transcriptomic molecular markers, it is challenging to distinguish the interspecific relationships among some Phoebe species and to accurately identify species within the genus. P. zhennan and P. bournei are often considered controversial species due to small differences in leaf traits, with environmental factors also potentially influencing leaf phenotypes. Juan et al. (2018) failed to distinguish P. zhennan from P. bournei using ISSR markers and suggested that these two Nanmu species should be combined. In contrast, Ding et al. (2018) integrated morphology data with RAD-seq sequencing to distinguish P. zhennan from P. bournei through SNP detection. Compared to SNP detection technology, SSR markers, as a second-generation molecular marker technology, offer low-cost, accurate detection, and were developed and applied earlier. For example, Jian (2016) developed SSR molecular markers based on the whole genome of Camphora officinarum and demonstrated the applicability of 27 pairs of markers across the genera Phoebe and Machilus. Yingchun et al. (2023) used SSR marker technology to confirm that Pinus yunnanensis is a typical outcrossing plant and maintains medium to high GD.

In this study, we constructed a Nanmu SSR enrichment library using the magnetic bead ~~enrichment~~ method to identify SSR loci within the Nanmu genome. More than 794,128 SSR loci were identified, which are more than those found in the transcriptome. ~~According to the statistical analysis of identified SSR loci,~~ the most common SSR types were dinucleotide repeats (54.31%), succeeded by mononucleotide and trinucleotide repeat types. Tetranucleotide, pentanucleotide, and hexanucleotide repeat types appeared least frequently. Out of these dinucleotide SSRs, the AG/CT repeat motif was the ~~most common~~, consistent with the repeat motifs found in many plants, such as Pseudotsuga menziesii and Camellia oleifera (VindhyaAmarasinghe & ECarlson 2002; Lang et al. 2024).

From the genomic DNA of 16 Nanmu materials, 20 pairs of SSR polymorphic primers were screened. Of these, 90 % were dinucleotide and trinucleotide repeat types, and the polymorphic information content ranged from 0.777 to 0.903. This indicates a high level of polymorphism for the selected primers, and their suitability for analyzing the GD of Nanmu. These primers further provided more SSR molecular markers for the identification of Nanmu germplasm resources. Principal Component Analysis (PCoA) revealed that individuals from the P. bournei, P. chekiangensis, and P. sheareri groups were dispersed in one principal component without overlap, while the YH group was distinct from these groups. This however changes as the remaining Nanmu had a more spread out distribution in the other components. Similarly, the results of UPGMA cluster analysis confirmed this, indicating that the developed molecular markers were able to separate P. zhennan, P. bournei, P. sheareri, and P. chekiangensis on the basis of their genotypes.However, the suitability of these molecular markers for identifying germplasm resources in other Phoebe species requires further verification.

Furthermore, based on previous studies, the higher occurrence rate of a short nucleotide repeat motifs in the SSR loci revealed the higher evolutionary level of a species, while the greater frequency of long nucleotide repeats implied a lower evolutionary level or mutation frequency (Gábor et al. 2000) . The SSR loci discovered in the current study, which consisted

mainly of 80.75% mononucleotide and dinucleotide repeats, indicate a high evolutionary background of Nanmu. GD is a crucial index for assessing the stability of a species' development. Molecular marker technologies, such as SSR, help mitigate the impact of environmental factors and provide insights into the GD of a species at the molecular level (Dharminder & Gagandeep Singh 2022 ; Weichang 2024). The 20 pairs of polymorphic primers identified in this study can be used for the subsequent analyses of GD in Nanmu.

GD of Guizhou Nanmu Populations Nanmu is one of the main forest resources in Guizhou with excellent material performance, high economic value and ornamental value. However,Nanmu resources have been decreasing steadily due to environmental changes, human activities, and other reasons. Despite this, there is an absence of studies on the GD of Nanmu populations in Guizhou. Understanding the GD of Nanmu is crucial for developing effective conservation strategies to protect the ~~remaining~~ resources.

4.2Genetic Diversity of Guizhou Nanmu Populations

Nanmu were studied using 20 ~~pairs of~~ SSR ~~primers~~ in this work. The observed Ho value of 0.701 was ~~smaller~~ than the expected He value of 0.737, indicating a certain degree of inbreeding within the Guizhou Nanmu population. The GD levels of the Guizhou population, in descending order, were as follows: SN > SQ > YQ > ZA > JK > WC > CS > TJ > DJ > XS > FG > DZ > TZ > JH > MT. When compared to ~~the congener~~ Phoebe chekiangensis (H=0.3206) and other endangered woody plants (Liriodendron chinense, H=0.740; Senegalia pennata, H=0.573; Dalbergia nigra, H=0.740) (Kangqin 2013; Gao Jie 2008; Renata Santiago de Oliveira et al. 2012), the GD of Guizhou Nanmu populations (H=0.743) was found to be relatively high. This indicates that even though the Guizhou Nanmu population is fragmented, it may maintain high GD due to its earlier origin, longer lifespan, and the accumulation of genetic variation. Among the populations, MT, JH, TZ, and DZ exhibited GD indices (H) between 0.6 and 0.7, which are lower compared to the rest of Guizhou populations. These populations require special conservation attention to maintain GD. On the other hand, the GD indices (H) of FG, YH, XS, DJ, TJ, CS, WC, JK, and ZA ranged between 0.7 and 0.8, suggesting they are relatively stable and can be conserved under the current protection measures. The populations of SN, SQ, and YQ, with GD indices (H) greater than 0.8, demonstrate high GD and should be prioritized for protection. These populations could also serve as sources for screening superior Nanmu plants. Furthermore, a comparison of the GD of the Guizhou Nanmu population with those from Sichuan, Hunan, and Chongqing revealed that the populations in these regions also possess high GD. In summary, despite the significant decline of Nanmu resources in the past due to natural disasters and anthropogenic factors, the overall GD of the Nanmu population remains high. This indicates that Nanmu is genetically stable, though continued human intervention is necessary for its protection and breeding.

Population genetic differentiation is an indicator that reflects the genetic structure of a population. The main factors influencing genetic differentiation include genetic drift, gene flow, and evolutionary history. Previous studies have shown that gene flow between populations can effectively prevent genetic differentiation due to genetic drift when the value of Nm > 1 (Suqi et

al. 1998). The results of genetic differentiation in this study indicated that the majority of genetic differentiation occurred within populations, with 82.5% of the genetic variation observed within populations and 17.5% between populations. Specifically, 9.43% of the total genetic variation was attributed to differences between populations, while 90.57% was within populations. This suggests that the Guizhou Nanmu populations exhibit a high degree of geographic coherence, with minimal genetic differentiation resulting from geographic isolation. The gene flow (Nm=1.354) was greater than 1, indicating that there is sufficient gene exchange between the Guizhou Nanmu populations to prevent genetic differentiation due to genetic drift. This also suggests that wild Nanmu populations can remain stable in the absence of anthropogenic ~~factors~~. Additionally, the Nanmu populations in this study were mainly came from mountainous regions, while the dispersal of Lauraceae seeds rely largely on birds, gravity, or human activities, which may further contribute to genetic exchange among Guizhou Nanmu populations .

A total of 174 individuals from 24 Nanmu populations were ~~analyzed using Structure software to study the~~ genetic structure. The results revealed that the populations could be roughly classified into three groups: the first group, the second group, and the third group, which accounted for 31.03%, 17.24%, and 50.57% of the total number of populations, respectively. Two individuals, MT20-03 and SN20-11, showed a Q-value of less than 0.6 and exhibited more complex genetic backgrounds, so they were categorized into a fourth group. UPGMA clustering analysis based on Nei's genetic distance revealed that samples collected from HJ were clustered with the ZG, TN, and TZ populations. This suggests that the ZG, TN, and TZ populations were more closely related to the Hejiang samples from Luzhou. Additionally, Nanmu samples from Gulin County, Sichuan Province, were clustered with the BJ, FG, YQ, and TJ groups, indicating that these groups of Nanmu were more closely related to one another. Both cluster and principal component analyses grouped the YH population separately, indicating that YH was genetically more distant from the other groups. The YH group was morphologically identified as Lindera megaphylla. Taken together, the genetic distance between the Nanmu populations was not found to correlate with geographical distance. This finding suggests that factors other than geography may influence the genetic structure of the populations, and the underlying reasons for this need further investigation at a deeper level (Ami & Byoung-Un 2022).

In addition, during the collection of Nanmu samples, it was observed that the Nanmu at the collection site were seriously infested with pests and diseases, and were not adequately treated. Meanwhile, Phoebe bournei in Guizhou was found to be mixed with Phoebe zhennan, and accurate data on Nanmu populations was not properly recorded during the listing process, which has caused some challenges in the conservation of Nanmu species. It is recommended to strengthen the prevention and control of pests and diseases affecting Nanmu, conduct a comprehensive census of Nanmu resources in Guizhou, and rectify the listing information.

5 Conclusions

In this study, Nanmu genomic SSR molecular markers were developed based on the sequencing of ~~Nanmu~~ genomic SSR-enriched libraries. Analysis using 20 highly polymorphic primer pairs demonstrated that all Guizhou Nanmu populations exhibited high GD, with the SN

Nanmu population showing the highest GD, while the MT population exhibited lower diversity. The genetic differentiation within the Guizhou Nanmu populations was found to be primarily intra-population. Cluster analysis revealed that the Nanmu populations from ZG (Zigong, Sichuan), TN (Tongnan District, Chongqing), and TZ (Tongzi County, Guizhou) were more closely related. Furthermore, the polymorphic primers identified in this study effectively distinguished Nanmu from Phoebe bournei, Phoebe sheareri, and Phoebe chekiangensis.

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Figure 1

Analysis of SSRs Site Characteristics

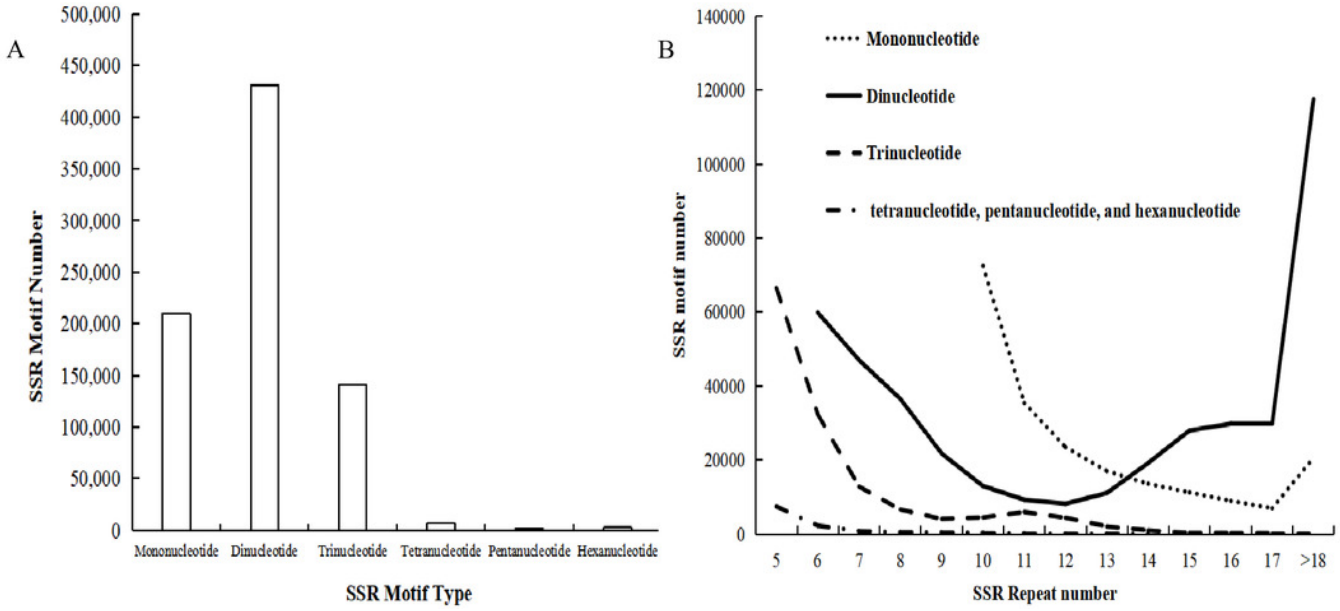

Figure 2

Selection of optimal K value

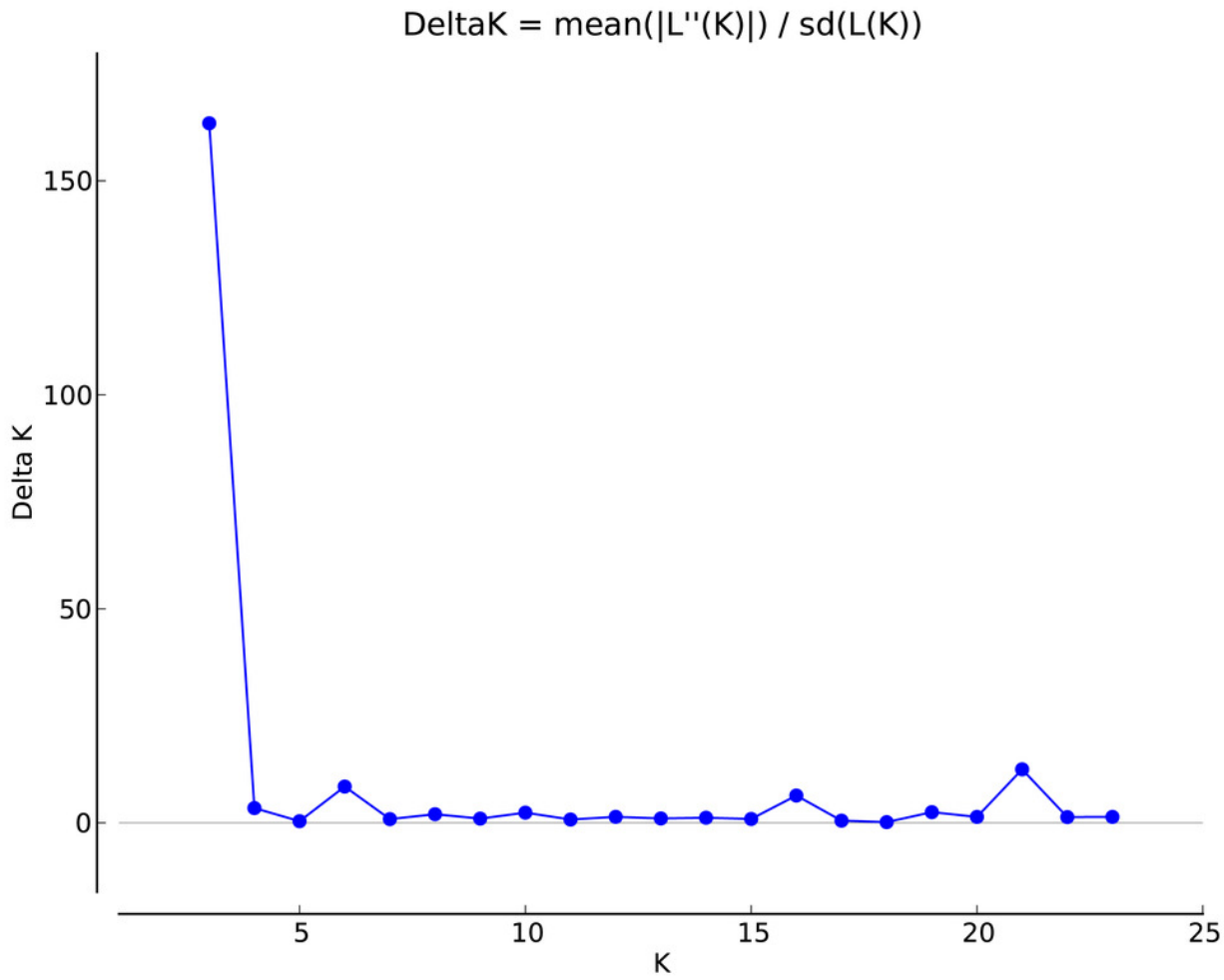

Figure 3

Genetic structure analysis results

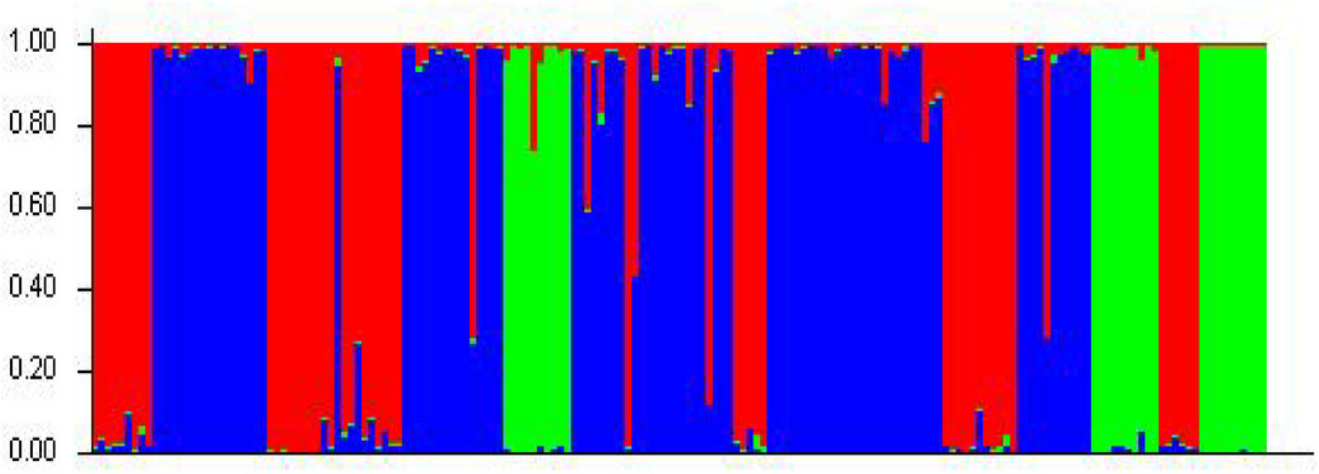

Figure 4

Cluster analysis

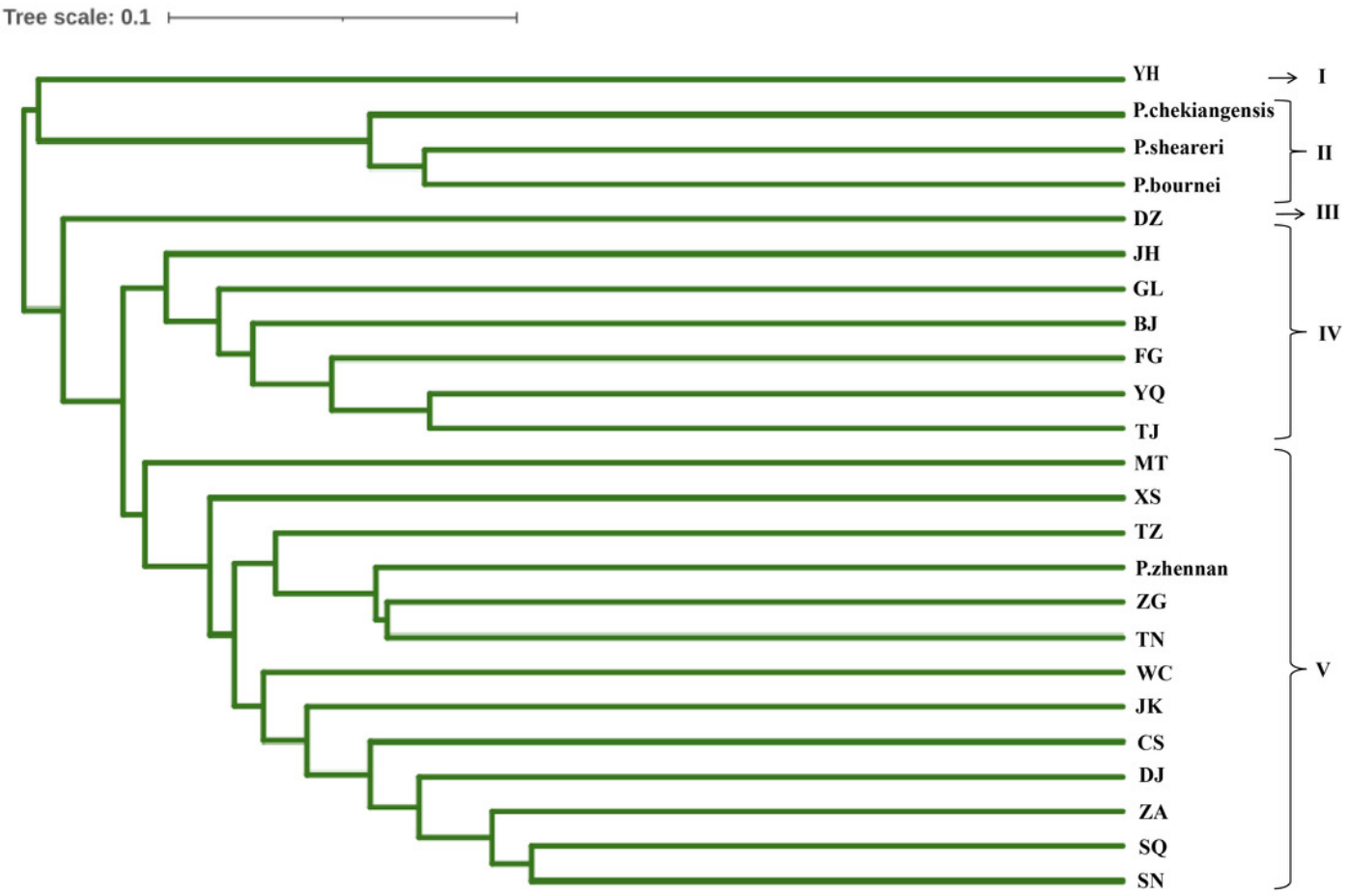

Figure 5

Principal component analysis

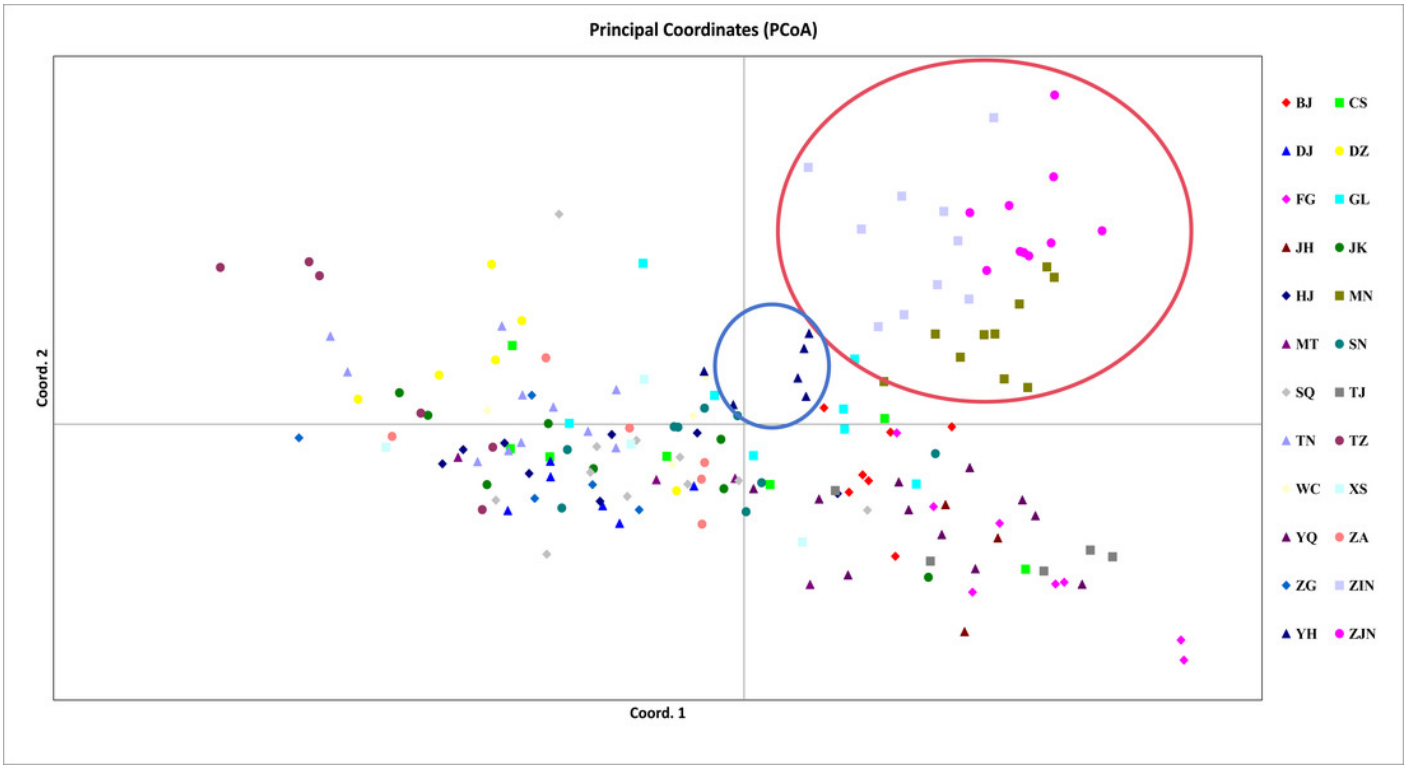

Table 1(on next page)

Geographic coordinates of the sampling site for Phoebe zhennan

1 Table 1 Collection information of Nanmu samples

2

sites	X	Y
DJ	107.88167	28.22694
SN	107.93250	27.83694
SQ	108.13917	27.44778
JK	108.78278	27.63722
WC	107.85972	28.37028
XS	106.58917	28.37000
ZA	107.43250	28.53722
MD	107.43833	27.44500
CS	106.48250	28.42278
YQ	107.97389	27.62278
FG	107.86417	27.94972
TZ	106.64861	28.08028
DZ	107.58083	29.08000
JH	108.62083	26.54139
TJ	108.34194	26.62694
TN	105.73667	29.84194
ZG	104.40972	29.43083
LZ	105.65472	28.65778
GL	105.72361	28.18806
ZJ	108.54057	28.48476
BJ	113.23450	28.04894

Table 2(on next page)

SSRs site search results statistics

1 Table 2. SSR site search results statistics

Sample	Numbers
The total number of sequences examined	1,642,465
The total size of examined sequences (bp)	551,443,009
The total number of identified SSRs	794,128
Number of SSR containing sequences	578,177
Number of sequences containing more than 1 SSR	162,869
The number of SSRs present in compound formation	178,046

2

Table 3(on next page)

SSR primer screening results

1 Table 3 SSR primer screening results

Prime	Allele	Number of effective alleles (Ne)	Shannon Information Index (I)	Observational heterozygosity (Ho)	Expected heterozygosity (He)	Nei's GD index (H)	Polymorphic information content (PIC)
SSR-1	7	2.360	0.784	0.650	0.475	0.830	0.808
SSR-2	9	2.127	0.749	0.600	0.488	0.816	0.793
SSR-3	12	2.127	0.645	0.400	0.400	0.896	0.888
SSR-4	11	1.867	0.589	0.450	0.388	0.881	0.869
SSR-5	10	2.127	0.645	0.550	0.400	0.865	0.851
SSR-6	9	2.133	0.624	0.400	0.375	0.854	0.837
SSR-7	10	1.967	0.624	0.300	0.400	0.857	0.842
SSR-8	8	1.693	0.472	0.350	0.313	0.826	0.804
SSR-9	8	1.967	0.624	0.350	0.400	0.844	0.825
SSR-10	8	2.433	0.832	0.700	0.525	0.801	0.777
SSR-11	12	2.033	0.693	0.850	0.475	0.881	0.869
SSR-12	13	2.400	0.866	0.950	0.563	0.906	0.899
SSR-13	15	2.600	0.936	0.900	0.588	0.910	0.903
SSR-14	12	2.600	0.936	0.850	0.588	0.885	0.874
SSR-15	13	2.567	0.866	0.900	0.538	0.910	0.903
SSR-16	11	2.467	0.866	1.000	0.563	0.879	0.867
SSR-17	12	2.267	0.832	1.000	0.550	0.893	0.883
SSR-18	14	2.333	0.832	0.950	0.550	0.908	0.901
SSR-19	11	2.667	0.970	0.950	0.600	0.889	0.878
SSR-20	13	2.467	0.866	1.000	0.563	0.906	0.899
Mean	10.9	2.260	0.763	0.705	0.487	0.872	0.858

2

Table 4(on next page)

Genetic diversity analysis of Nanmu population

Table 4. GD analysis of Nanmu population

POP	Na	Ne	I	Ho	He	H
CS	6.250	4.832	1.621	0.727	0.750	0.751
DJ	5.000	4.094	1.456	0.798	0.732	0.737
DZ	4.550	3.784	1.335	0.406	0.680	0.701
FG	5.050	3.854	1.398	0.650	0.711	0.714
JH	3.850	3.423	1.223	0.750	0.663	0.664
JK	6.100	4.668	1.608	0.823	0.765	0.773
MT	3.850	3.313	1.196	0.675	0.654	0.661
SN	8.150	6.402	1.928	0.788	0.831	0.832
SQ	7.750	5.292	1.807	0.738	0.799	0.811
TJ	5.500	4.597	1.540	0.740	0.744	0.744
TZ	4.200	3.315	1.273	0.577	0.676	0.685
WC	5.050	4.339	1.515	0.668	0.758	0.762
XS	4.800	4.227	1.442	0.725	0.731	0.736
YQ	8.000	6.015	1.856	0.705	0.807	0.809
ZA	6.350	5.152	1.691	0.732	0.784	0.785
YH	4.850	3.872	1.395	0.720	0.710	0.719
GL#	5.900	4.513	1.553	0.637	0.741	0.763
BJ#	6.200	4.507	1.579	0.652	0.733	0.739
TN#	6.800	4.536	1.650	0.725	0.764	0.779
ZG#	4.700	3.992	1.400	0.590	0.713	0.713
*P. bournei**	8.500	6.087	1.917	0.795	0.821	0.821
P. zhennan *	6.500	5.067	1.699	0.690	0.789	0.793
*P. sheareri**	8.000	5.704	1.863	0.790	0.811	0.816
*P. chekiangensis**	6.800	4.578	1.648	0.802	0.759	0.767

Notes: #Nanmu groups originating from Hunan, Sichuan, and Chongqing; *other species of the *Phoebe* genus.

Table 5(on next page)

Genetic differentiation analysis of Nanmu population

Table 5. Genetic differentiation analysis of the Nanmu population

Locus	Fis	Fit	Fst	Nm
SSR-1	0.049	0.158	0.114	1.941
SSR-2	0.212	0.383	0.217	0.900
SSR-3	0.441	0.524	0.148	1.444
SSR-4	0.290	0.433	0.202	0.985
SSR-5	0.167	0.382	0.258	0.718
SSR-6	0.478	0.628	0.288	0.619
SSR-7	0.341	0.464	0.186	1.097
SSR-8	0.238	0.390	0.199	1.007
SSR-9	0.255	0.421	0.223	0.871
SSR-10	0.367	0.447	0.125	1.745
SSR-11	-0.123	0.051	0.156	1.357
SSR-12	-0.137	0.030	0.146	1.457
SSR-13	0.165	0.289	0.148	1.437
SSR-14	-0.101	0.080	0.164	1.273
SSR-15	-0.228	-0.054	0.141	1.518
SSR-16	-0.323	-0.089	0.177	1.162
SSR-17	-0.223	-0.024	0.163	1.287
SSR-18	-0.228	-0.045	0.149	1.433
SSR-19	-0.267	-0.063	0.161	1.300
SSR-20	-0.196	-0.044	0.128	1.711
Mean	0.059	0.218	0.175	1.263

Table 6(on next page)

Analysis of Molecular Variance of Nanmu Population

Table 6. Analysis of molecular variance of the Nanmu population

Source of mutation	Degrees of freedom	Square sum	Variance component	Variance component ratio
Intergroup	15	260.086	0.76630	9.43%
Intragroup	194	1428.061	7.36114	90.57%
Total	209	1688.148	8.12744	
		Fst：0.09429		