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# Genetic Diversity and Population Structure Analysis of Tree Peony (*Paeonia* Section *Moutan* DC.) Germplasm Using Sixteen Functional SSR Markers

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**Abstract:** Tree peony (*Paeonia* section *Moutan* DC.) is a traditional ornamental flower of China, which has thousands of varieties with different flower colors and types after a long history of natural selection and artificial breeding. However, tree peony is a perennial woody plant with a long breeding, and there are still significant challenges to accelerate the process of genetic improvement of important ornamental traits. In this study, a total of sixteen primer pairs with high polymorphism and good universality were selected from the initial pool of 115 SSR markers. The SSR marker set was derived from published papers on the genetic linkage map and association analysis of tree peony. Furthermore, we conducted a genetic diversity and population structure analysis on 322 tree peony cultivars using molecular markers with functional. These SSRs amplified a total of 391 alleles, the average number of different alleles was 5.113 alleles across all loci. The average Shannon's information index, gene diversity and polymorphism information content were 0.842, 0.532, and 0.503 over all loci, respectively. Population genetic diversity analysis indicated that the average expected heterozygosity of the total population was larger than the observed heterozygosity, showing the presence of a certain degree of heterozygous deletion phenomenon. The Japan varieties had the richest diversity with the highest *H* (0.508) and *PIC* (0.479) values. The Zhongyuan varieties showed the greatest variation may be related to its longstanding cultivation history. Moreover, the STRUCTURE and principal coordinate analyses indicated that 322 tree peony individuals from five populations were grouped into two clusters. An analysis of molecular variance demonstrated significant genetic diversity among different populations. This research may contribute to the sustainable management, conservation, and utilization of tree peony resources.

**Keywords:** tree peony; functional SSR markers; polymorphism; genetic diversity; population structure

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## 1. Introduction

Tree peony (*Paeonia* section *Moutan* DC.), a perennial deciduous shrub belonging to the genus *Paeonia*, has a long history of cultivation in China [1]. It is renowned as the 'king of flower' due to its large and vibrant flowers, rich fragrance, and exquisite appearance [2]. Tree peony has highly ornamental, medicinal, and oil values, and has been widely planted in the world [3–6]. With the wide introduction of peony varieties, different cultivation groups for tree peonies have gradually emerged worldwide. It is well known that germplasm (genetic) resources are the material basis for genetic improvement of plant

varieties and the core of agriculture [7]. Currently, the number of tree peony varieties has exceeded 2200, which poses huge challenges in understanding their genetic relationship and background [8].

Various random molecular marker techniques, including amplified fragment length polymorphism (AFLP) [9], intersimple sequence repeat (ISSR) [10], random amplified polymorphic DNA (RAPD) [11], sequence-related amplified polymorphism (SRAP) [12], conserved DNA-derived polymorphism (CDDP) [12], and SSR markers [13–15] have been utilized for genetic diversity assessment, genetic relationship analysis, and population structure analysis in tree peony cultivars. As an ideal marker, SSRs have been frequently used in studies of many economically important trees due to their ubiquity, reproducibility, high level of polymorphism, codominant inheritance, and high level of transferability [16–19], such as for the assessment of genetic diversity [17,20,21], variety identification [22,23], genetic relationship classification [24,25], pollination [26], genetic-linkage maps [27,28], fingerprints [29], and evaluation of core germplasm resources [30,31]. Furthermore, the utilization of SSR markers with potential functional is of significant importance in the early screening of tree peony varieties with desired characteristics. Cai et al. [32] screened 79 pairs of effective SSR primers from 400 pairs, carried out a linkage analysis on 195 $F_1$ populations, and ultimately constructed a genetic linkage map of tree peony with 72 SSR markers. Subsequently, a QTL (quantitative trait locus) analysis was performed with 27 quantitative traits and located multiple traits related to branches, leaves, flowers, fruits, and flower color. Similarly, the QTLs for the six traits including branch number, leaf length, flower number, pod height, pod diameter, and flower diameter were successfully identified based on an $F_1$ population of 120 full-sibs [33]. These above studies indicated that the marker loci on the genetic linkage map of tree peony had potential functional significance and served as genetic variation tools for a further exploration of target traits.

Genetic resources research plays a crucial role in sustainable management, conservation, and utilization [34–36]. Maintaining genetic diversity is essential for effective conservation efforts [37]. Today, faced with many tree peony germplasm resources, how to explore excellent genetic germplasm and improve breeding efficiency quickly and accurately has become an important problem to be resolved in breeding research. Moreover, genetic diversity research based on functional markers offers a new approach to solve this problem. However, research on genetic diversity evaluation of peony based on functional markers is limited. Hence, this study was initiated with the aim of finding the SSR markers on genetic linkage maps and association mapping of tree peony to evaluate the genetic diversity and population structure in tree peony germplasm. The findings could ultimately provide a valuable foundation for genetic enhancement and the sustainable conservation of tree peony resources.

## 2. Materials and Methods

### 2.1. Materials and Genomic DNA Extraction

In total, 322 samples of tree peony representing 5 populations were randomly collected from the tree peony germplasm resources nursery of Henan University of Science and Technology (112°28′36.34″ E, 34°39′30.34″ N) (the geographical location of the 5 populations is seen in Supplementary Figure S1). In late March 2021, tender and disease-free leaves were collected from all samples and stored at −80 °C after rapid freezing in liquid nitrogen for further use. These varieties included five tree peony variety groups: Japan group ($n$ = 12), Jiangnan group ($n$ = 11), Northwest group ($n$ = 54), Southwest group ($n$ = 8), and Zhongyuan group ($n$ = 237).

Total genomic DNA was extracted from 100 mg of tender leaves using the Super Plant Genomic DNA Kit (Polysaccharides & Polyphenolics-rich) (Tiangen, Beijing, China) according to the manufacturer's instructions. The samples were assessed using a gel imaging analysis system (GelDoc XR, Bio-Rad laboratories Inc., Hercules, CA, USA) with electrophoresis on a 1% agarose gel, and their concentration was determined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE,

USA). Then, samples were diluted to a final concentration of 20 ng·μL$^{-1}$ with a TB elution buffer (Tiangen) and stored at −20 °C until they were used.

### 2.2. Source of Functional SSR Markers

The functional SSR markers used in this study were sourced from previously published papers on the genetic linkage map and association analysis of tree peony. Among them, the genetic linkage map of tree peony included 35 pairs from Guo et al. [33] and 68 pairs from Cai et al. [32], and a total of 95 markers remained after removing 8 duplicate primers. In addition, there were 20 pairs of association analysis primers based on *Paeonia rockii* populations, which contained 2–6 repeats of motifs, and these primers were developed based on transcriptome data [38]. Finally, a group of 115-pair primers was synthesized by Ruibio BioTech Co., Ltd. (Beijing, China) (Supplementary Tables S1 and S2).

### 2.3. Screening of Functional SSR Markers and Genotyping

One hundred and fifteen pairs of SSR primers were employed to screen mixing samples from nine different tree peony varieties with diverse flower types, colors, and origins. PCR amplification was carried out in a 10 μL reaction mixture including 2 μL (20 ng·μL$^{-1}$) of genomic DNA, 0.2 μL (10 μM) of each reverse and forward primer, 5 μL of 2 × Taq Plus Master Mix II (Vazyme Biotech Co., Ltd., Nanjing, China), and 2.6 μL of dd$H_2O$. PCR amplification was performed using a touchdown protocol by Guo et al. [39]. Subsequently, the amplified products were analyzed individually using 1% agarose gel electrophoresis, and the primers exhibiting polymorphism were chosen based on these results.

Then, a second screening was performed using capillary electrophoresis [40], with 36 different peony varieties with varied flower types, colors, and origins (Figure 1). The fluorescent-labeled M13 primers and PCR amplification system and program were also described by Guo et al. [39]. After that, a set of 16 primer pairs showing high polymorphism and good stability were obtained. Finally, these excellent SSR markers were selected for the amplification and analysis of all 322 tree peony cultivars.


**Figure 1.** Flower photos showing the thirty-six tree peony (*Paeonia* section *Moutan* DC.) cultivars used in this study. Note: No. 1–22: Zhongyuan group; No. 22–32: Northwest group; No. 33–36: Jiangnan group.

### 2.4. Data Analysis

To ensure the accuracy of the results, high-clarity and -stability SSRs obtained through two rounds of screening were amplified. Subsequently, the amplified products were separated with the ABI 3730XL DNA capillary electrophoresis analyzer (Applied Biosystems, Foster City, CA, USA) alongside the GeneScan-500LIZ size standard. Alleles of the SSR markers were validated using GeneMarker ver. 2.2.0 (SoftGenetics LLC, State College, PA, USA). The parameters of genetic diversity were estimated using GenAlEx ver. 6.501 (Australian National University, Canberra ACT 0200, Australia), including the number of different alleles (*Na*), the effective number of alleles (*Ne*), Shannon's information index (*I*), the diversity (*H*), the inbreeding coefficient ($F_{IS}$), Wright's fixation index ($F_{IT}$), the fixation index ($F_{ST}$), and the gene flow (*Nm*) [41,42]. Polymorphic information content (*PIC*) values were calculated for each SSR primer pair using PowerMarker ver. 3.25 software (North Carolina State University, NC, USA [43]. Furthermore, a principal coordinate analysis (PCoA) was conducted using genotype data obtained from SSR markers to examine the dissimilarities within and between populations.

An algorithm called Bayesian model-based clustering was used with the software package STRUCTURE ver. 2.2.2 to analyze the population genetic structure based on sixteen SSR markers [44]. For this study, ten separate runs were performed for each value of *K* (*K* = 1–20), using a burn-in period of 10,000 iterations and 10,000 Markov chain Monte Carlo (MCMC) replications, and the default values were used for the remaining parameters. The online Structure Harvester tool (https://taylor0.biology.ucla.edu/structureHarvester/, accessed on 12 April 2023) was employed to determine the most probable value of K using the delta *K* (Δ*K*) method [45]. Following, the bar plot of membership probability, based on the Q-matrix results, was generated using the CLUMPAK ver. 1.1.2 [46] and Distruct ver. 1.1 programs [47].

## 3. Results

### 3.1. Screening of Polymorphic Microsatellites

After two rounds of screening, a total of sixteen primer pairs with high polymorphism and good universality were selected from the initial pool of 115 SSR markers. The selected sixteen SSR primers consistently amplified all five populations of tree peony under standard conditions and were polymorphic in all the populations investigated. Detailed information regarding these sixteen primers can be found in Table 1.

**Table 1.** Sixteen SSR markers' information of 322 tree peony (*Paeonia* section *Moutan* DC.) cultivars in this study.

| Primer ID | Primer Sequence (5′-3′) | Primer (3′-5′) | Annealing Temperature (°C) | Expected Size (bp) | Repeat Motif |
|---|---|---|---|---|---|
| PS371 | CATTGAGCCACCCATAGA | GCAACAATCCTGGTAGTGA | 58 | 219 | $(CAC)_5$ |
| PS119 | GCAAAGACAACAGCCTCG | CTCACCATCCAATCCCAC | 57 | 289 | $(CAG)_6$ |
| 49A | TCTGGGTGATAGGTGGAGCTGGTGC | GGAAGACGCCCACAATGAAATCACA | 55 | 314 | $(TGC)_5$ |
| PS308 | ACTACTCTATTGCGAAACC | GTCTTATGGCGGCTATGT | 53 | 189 | $(TC)_7$ |
| PS074 | TGCCTTGCTCCTCCTTGT | CGGTTAGCCATGAATCCC | 57 | 236 | $(CT)_7$ |
| PS052 | CAAATCTGCTAATTAAAGAC | GATAGAAGGGAAAGGAAG | 49 | 235 | $(CT)_7$ |
| PS118 | CGTAGCCGTGCTTCTTTC | CCCATCAACCCATAATCC | 54.5 | 199 | $(TGG)_5$ |
| PS068 | CTTTGGCATTCTCATTCA | GGTGGTATTGGGCTTCTT | 52.5 | 174 | $(TC)_7$ |
| PS311 | AACGCCACCATCACCTTT | CCTCCTCCCTGTTCTTCT | 60 | 277 | $(TTC)_6$ |
| PS144 | CAACCTACAATCCGACAATG | TGTGGGTAGTGGTTTGTTAG | 54.5 | 317 | $(TGC)_5$ |
| PS24 | TTGAGCAATCAGGTTCATTAGG | TAGCCTCCGGTTCTGAATTG | 56.4 | 155 | $(CAA)_5$ |
| PS36 | TCCAAGCTACTCCATGCCTTA | GAATACTCACTCGCGGCTTC | 58.8 | 277 | $(TCT)_5$ |
| PS47 | TCTCAGCTTCTAATCTTCTCCTCA | ATGTCATGCCTCCAATCTCC | 57.5 | 246 | $(AG)_6$ |
| PS50 | TTACAGCAGGCCACGACTG | CATGACATCATGTGGTCCAA | 55.9 | 262 | $(AGC)_6$ |
| PS57 | GCGACAGTACATTCCATCAA | GTCAACCACACGTCTGCAAG | 57.7 | 128 | $(TC)_7$ |
| PS64 | GATTCTGTCTGGCATTGACG | CCATCTGTCTGGATCGACCT | 58.1 | 293 | $(GA)_6$ |

### 3.2. Genetic Diversity Analysis

A total of 391 alleles were detected at 16 SSR loci in 322 samples, with the number of different alleles (*Na*) ranging from 1.400 (PS144) up to 12.600 (PS308) with an average of 5.113 alleles across all loci. The range of the number of effective alleles (*Ne*) extended from 1.073 (PS144) to 4.294 (PS308), with an average value of 2.155. Shannon's information index (*I*) had an average value of 0.842 with the lowest value (0.101) for marker PS144 and the highest value (1.727) for marker PS308. The gene diversity (*H*) and the polymorphism information content (*PIC*) was consistent, with the lowest values at PS144 (0.012), the highest at PS308 (0.834 and 0.820, respectively), and averages of 0.532 and 0.503 over all loci, respectively. The calculated gene flow *Nm* was 1.951, indicating moderate levels of gene flow (Table 2).

**Table 2.** Analysis of the genetic diversity of 322 tree peony cultivars with 16 simple sequence repeat (SSR) loci.

| Locus | *N* | *Na* | *Ne* | *I* | *Ho* | *He* | *H* | *PIC* | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ | *Nm* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PS57 | 28 | 5.800 | 2.699 | 1.002 | 0.948 | 0.577 | 0.757 | 0.722 | −0.642 | −0.577 | 0.040 | 6.040 |
| PS074 | 27 | 5.400 | 2.138 | 0.930 | 0.620 | 0.512 | 0.644 | 0.599 | −0.211 | −0.160 | 0.042 | 5.702 |
| PS36 | 20 | 4.000 | 1.721 | 0.632 | 0.330 | 0.340 | 0.595 | 0.536 | 0.030 | 0.182 | 0.156 | 1.349 |
| PS24 | 22 | 4.800 | 2.156 | 0.953 | 0.659 | 0.525 | 0.623 | 0.601 | −0.254 | −0.181 | 0.058 | 4.032 |
| PS47 | 19 | 3.800 | 1.703 | 0.754 | 0.372 | 0.396 | 0.597 | 0.542 | 0.061 | 0.356 | 0.314 | 0.546 |
| PS119 | 17 | 3.600 | 1.740 | 0.643 | 0.393 | 0.354 | 0.587 | 0.544 | −0.110 | 0.450 | 0.504 | 0.246 |
| PS068 | 29 | 6.000 | 2.254 | 1.033 | 0.680 | 0.531 | 0.432 | 0.421 | −0.280 | −0.197 | 0.064 | 3.627 |
| PS118 | 21 | 4.400 | 2.820 | 1.136 | 0.351 | 0.637 | 0.647 | 0.595 | 0.449 | 0.540 | 0.165 | 1.263 |
| PS308 | 54 | 12.600 | 4.294 | 1.727 | 0.540 | 0.732 | 0.834 | 0.820 | 0.262 | 0.301 | 0.053 | 4.496 |
| PS50 | 25 | 5.000 | 1.406 | 0.444 | 0.183 | 0.198 | 0.502 | 0.485 | 0.071 | 0.187 | 0.124 | 1.762 |
| PS64 | 36 | 7.600 | 3.445 | 1.444 | 0.712 | 0.696 | 0.775 | 0.741 | −0.023 | 0.123 | 0.143 | 1.495 |
| PS052 | 14 | 2.800 | 1.381 | 0.442 | 0.028 | 0.242 | 0.387 | 0.359 | 0.883 | 0.894 | 0.093 | 2.448 |
| PS311 | 24 | 4.800 | 1.973 | 0.767 | 0.169 | 0.388 | 0.284 | 0.278 | 0.565 | 0.738 | 0.397 | 0.380 |
| PS144 | 7 | 1.400 | 1.073 | 0.101 | 0.000 | 0.056 | 0.012 | 0.012 | 1.000 | 1.000 | 0.102 | 2.193 |
| PS371 | 30 | 6.000 | 1.820 | 0.686 | 0.266 | 0.302 | 0.331 | 0.323 | 0.122 | 0.266 | 0.164 | 1.272 |
| 49A | 18 | 3.800 | 1.855 | 0.772 | 0.388 | 0.421 | 0.501 | 0.469 | 0.079 | 0.518 | 0.477 | 0.274 |
| Mean | | 5.113 | 2.155 | 0.842 | 0.415 | 0.432 | 0.532 | 0.503 | 0.125 | 0.277 | 0.181 | 2.320 |

*N*: number of alleles; *Na*: number of different alleles; *Ne*: number of effective alleles; *I*: Shannon's information index; *Ho*: observed heterozygosity; *He*: expected heterozygosity; *H*: gene diversity; *PIC*: polymorphism information content; $F_{IS}$: inbreeding coefficient; $F_{IT}$: Wright's fixation index; $F_{ST}$: fixation index; *Nm*: gene flow.

At the population level, the Zhongyuan population had the largest numbers of different and private alleles among populations, probably due to the larger sample size. The highest *H* (0.508) and *PIC* (0.479) values were observed in the Japan population, indicating a rich diversity. The second most diverse population was that of Southwest, with *H* and *PIC* values of 0.498 and 0.456, respectively. The *He* of the total population was larger than the *Ho*, showing the presence of a certain degree of heterozygous deletion phenomenon. The average $F_{ST}$ value was 0.154, indicating that the peony population was at a moderate level of genetic differentiation (Supplementary Tables S3 and S4).

### 3.3. Population Structure Analysis

The population structure analysis of the tree peony population showed that $\Delta K$ was the highest when $K = 2$ (Figure 2A,B). These results and many K values ($K = 2$–4) suggested that the current collection of germplasm could be classified into two primary subpopulations, labeled as subpopulations I and II (Figure 2C). Consequently, the *Q* values of the five populations were analyzed and categorized into two subgroups (Table 3). The individuals of the Zhongyuan population were mainly distributed in subpopulation II (92.83%), the other four population individuals were mainly distributed in subpopulation I, the Northwest population with 98.15% and the Southwest population with 87.50%, respectively. All individuals in the Japan ($n = 12$) and Jiangnan ($n = 11$) populations were placed in cluster I, indicating the presence of a strong population structure (Table 3). Following, a principal

coordinates analysis (PCoA) was performed by the unweighted genetic distances method showing that the level of genetic differentiation of the Zhongyuan population was higher than that of the other four populations (Figure 3).

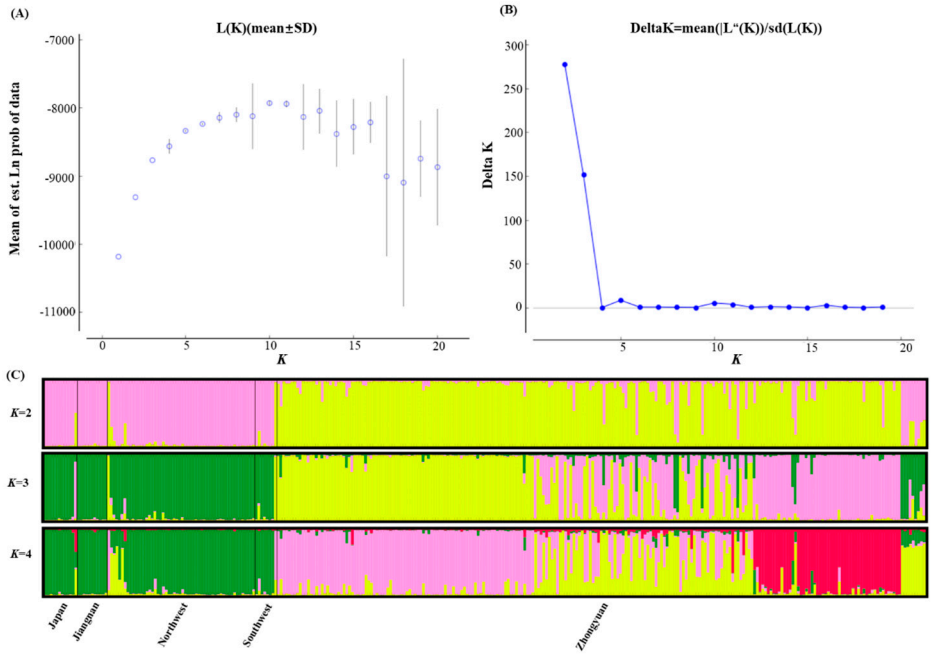


**Figure 2.** Population genetic structure analysis of 322 test samples of tree peony using 16 SSR markers. (**A**) *LnP*(D) for each K value; (**B**) The estimation of the optimal number of subpopulations based on the appropriate K value revealed that the mean Δ*K* values across the 10 runs reached a maximum of *K* = 2. (**C**) The population structure and clustering of the 322 tree peony individuals were estimated for K values ranging from 2 to 4. The population from left to right are as follows: Japan population, Jiangnan population, Northwest population, Southwest population, Zhongyuan population.

**Table 3.** The distribution of samples within each subpopulation (*K* = 2).

| Subpopulation | Japan | Jiangnan | Northwest | Southwest | Zhongyuan | Total | Mean Q-Value |
|---|---|---|---|---|---|---|---|
| I | 12 | 11 | 53 | 7 | 17 | 100 | 0.939 |
| II | 0 | 0 | 1 | 1 | 220 | 222 | 0.938 |
| Total | 12 | 11 | 54 | 8 | 237 | 322 | - |

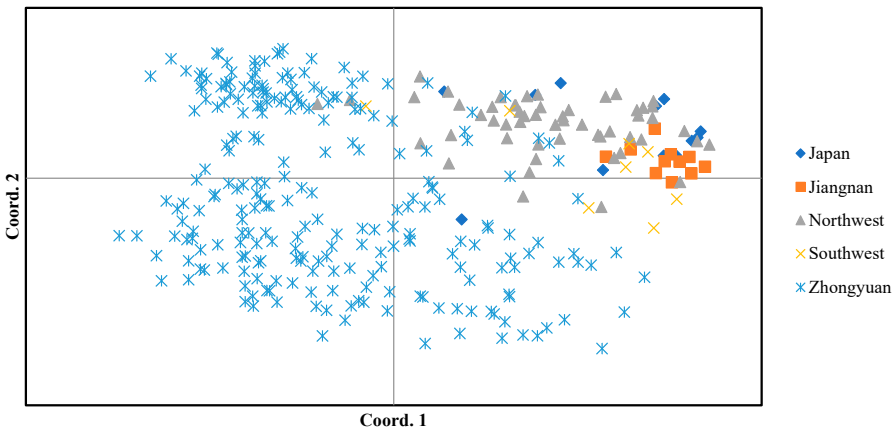


**Figure 3.** The distribution of 322 tree peony accessions, utilizing genotyping data from sixteen SSR markers, is visually represented through a PCoA using the first two coordinates. Axis 1 accounts for 14.29% of the variation, while axis 2 explains 9.83% of the variation.

### 3.4. Population Differentiation Analysis

Nei's genetic identity, calculated through pairwise comparisons, ranged from 0.663 (between Zhongyuan and Jiangnan) to 0.902 (between Southwest and Northwest), with an average of 0.804. The majority of Nei's genetic distance ranged from 0.103 (between Southwest and Northwest) to 0.231 (between Zhongyuan and Southwest), with an average of 0.183 (Table 4). A Mantel test was performed to examine the correlation between genetic distance and geographical distance among tree peony populations, which indicated no significant correlation ($r = -0.243$, $p = 0.340$) (Figure 4).

**Table 4.** Nei's genetic identity (below diagonal) and genetic distance (above diagonal).

| Population | Japan | Jiangnan | Northwest | Southwest | Zhongyuan |
|---|---|---|---|---|---|
| Japan | | 0.175 | 0.178 | 0.180 | 0.355 |
| Jiangnan | 0.839 | | 0.164 | 0.209 | 0.411 |
| Northwest | 0.837 | 0.849 | | 0.103 | 0.231 |
| Southwest | 0.835 | 0.812 | 0.902 | | 0.215 |
| Zhongyuan | 0.701 | 0.663 | 0.794 | 0.806 | |

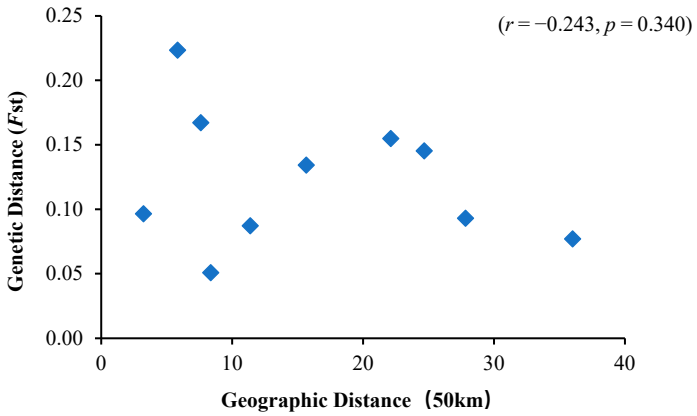


**Figure 4.** Mantel test between genetic distance ($F_{ST}$) and geographic distance for all tree peony populations in Luoyang.

The degree of genetic differentiation ($F_{ST}$) between any two populations was calculated for all five populations (Table 5). Among all populations, $F_{ST}$ values for the global and pairwise multilocus analysis ranged from 0.051 (Southwest vs. Northwest) to 0.223 (Jiangnan vs. Zhongyuan) with an overall $F_{ST}$ value of 0.123. Seven pairs of $F_{ST}$ combinations were significant differences and the others were not significant. An AMOVA was performed based on 999 permutations and revealed the genetic variation among and within populations for tree peony samples (Table 6). The AMOVA showed that 16% of the total genetic variation occurred among populations and a significant amount (84%, $p < 0.05$) of the total variation occurred within populations.

**Table 5.** Pairwise genetic distance based on fixation index $F_{ST}$ in tree peony accessions.

| Population | Japan | Jiangnan | Northwest | Southwest | Zhongyuan |
|---|---|---|---|---|---|
| Japan | | * | NS | NS | * |
| Jiangnan | 0.155 | | * | * | * |
| Northwest | 0.077 | 0.134 | | NS | * |
| Southwest | 0.093 | 0.167 | 0.051 | | * |
| Zhongyuan | 0.145 | 0.223 | 0.087 | 0.096 | |

* $p \leq 0.05$, NS: no significance.

**Table 6.** Analysis of molecular variance (AMOVA) partitioning variance into among populations and within populations for SSR markers.

| Source | d.f. | SS | MS | Est. Var. | % Variation | *p* |
|---|---|---|---|---|---|---|
| Among pops | 4 | 263.401 | 65.850 | 0.865 | 16 | <0.01 |
| Within pops | 317 | 2915.192 | 9.152 | 4.576 | 84 | <0.01 |
| Total | 322 | 3178.593 | | 5.441 | 100 | |

d.f.: degrees of freedom; SS: sum of squares; MS: mean square; Est. var.: estimated variance.

## 4. Discussion

Germplasm resources are not only the material basis for breeding excellent varieties but also an indispensable component of biodiversity, which plays an important supporting role in the construction of ecological civilization and sustainable economic development. Previous studies on the germplasm resources of tree peony have mainly focused on *Paeonia rockii* [15,48], *Paeonia ostii* [49], wild species [50,51], a few Zhongyuan cultivars [13,39,52], and some foreign varieties [10]. Most of these studies have developed markers based on sequencing results, but it is still unknown whether these markers are closely linked to the target traits. Because of the long breeding cycle of tree peony, using functional markers for research could better ensure the management and full utilization of germplasm resources. However, there are few studies on tree peony varieties using functional markers.

Genetic diversity, as measured by the variation in allelic frequencies and genetic composition within a population, is integral for the maintenance, adaptive capacity, and evolutionary potential of populations [21,53]. Information about genetic diversity and genetic structure is critical to revise a species management plan and conservation. A set of highly polymorphic and stable markers obtained was a prerequisite and foundation for conducting this study. In this study, a total of sixteen SSR primers with high polymorphism and good universality were selected from the initial pool of 115 SSR markers after two rounds of screening. Moreover, the average number of alleles per locus was 24.4, which was higher than that of 37 accessions from *Paeonia ostii* using EST-SSR [38]. The average of Shannon's information index value (*I*) was 0.842, which was lower than the genetic diversity analyzed by 34 SSR primers for 282 *Paeonia rockii* accessions ($I = 0.908$) [14] and 20 SSR primers for 274 specimens of wild species ($I = 1.1160$) [50]. These differences may be attributed to the different source of the samples (natural populations vs. cultivated varieties populations) and the number of SSR markers. Furthermore, the value of polymorphism information content (*PIC*) in this study was $PIC = 0.503$, that of the *Paeonia rockii* population was $PIC = 0.611$, and that of 274 specimens from 22 natural populations of nine wild species was $PIC = 0.53$. All these studies indicated that different types of tree peony populations exhibited high levels of genetic diversity. Furthermore, the genetic diversity levels of samples from natural populations were higher than those of cultivated varieties. This is consistent with the results of Zhou et al. [54], indicating that natural populations of tree peony have a stronger ability to adapt to the ecological environment.

The expected heterozygosity is an important measure of gene diversity [55]. In our study, the average heterozygosity values *Ho* and *He* were 0.415 and 0.432, respectively. Similar results were obtained by Guo et al. [56] ($Ho = 0.5280$, $He = 0.5379$; *Paeonia suffruticosa* cultivar groups), Liu et al. [38] ($Ho = 0.57$, $He = 0.73$; *Paeonia rockii*), and Xue et al. [50] ($Ho = 0.1867$, $He = 0.5782$; wild species of tree peony) using SSR markers. Furthermore, *He* was lower than *Ho* at six loci (PS57, PS074, PS24, PS119, PS068, and PS64). Excess heterozygotes were also observed in the Jiangnan population ($Ho = 0.444$, $He = 0.373$) and Southwest population ($Ho = 0.427$, $He = 0.420$), respectively. These results suggested that the samples from the Jiangnan and Southwest populations in this study might have been derived from hybridization between multiple related species originating from different geographical locations [54,57]. We also found that most *He* values were higher than *Ho* values at the locus and population levels, this indicated that heterozygote deficiency was possible. Similar results were also reported for other species, such as, *Cunninghamia lanceolata* [19], *Eucalyptus cloeziana* [30], *Platycladus orientalis* (Cupressaceae) [58], *Lentinula*

*edodes* [59], and *Nelumbo nucifera* [60]. The presence of private alleles in these germplasms suggested the existence of valuable rare genetic variations, which could offer more possibility for selecting beneficial recombinants in future breeding efforts. Therefore, when developing a germplasm conservation and breeding program, it is necessary not only to preserve germplasm with high levels of genetic diversity but also to give special attention to individuals with private alleles.

The majority (70%) of pairwise comparisons of $F_{ST}$ showed significant differences ($p \leq 0.05$), indicating a significant genetic differentiation between populations. In addition, the $F_{ST}$ values between Jiangnan and Japan, Jiangnan and Southwest, and Jiangnan and Zhongyuan populations were 0.155, 0.167, and 0.223, respectively, indicating a high level of differentiation ($0.15 < F_{ST} < 0.25$). The $F_{ST}$ values between the other populations were at a moderate level of differentiation ($0.05 < F_{ST} < 0.15$). These results were consistent with previous studies of tree peony [50,61], indicating that the degree of genetic differentiation varies among populations from different sources. Therefore, it is important to consider their sources and diversity levels for the effective conservation of peony germplasm resources.

A population structure analysis of 322 tree peony samples was conducted, and two clusters were identified. The results derived from the genetic structure analysis utilizing multiple K-values and PCoA were consistent with the aforementioned findings. Interestingly, we found that the samples from the Zhongyuan population were predominantly assigned to cluster II, while the remaining four populations were primarily assigned to cluster I. These indicated that the materials from the Zhongyuan population exhibited distinct genetic backgrounds compared to the other four populations, corroborating the findings of previous studies conducted by Peng et al. [62]. Moreover, the complexity and diversity of the genetic background of Zhongyuan peony population might be attributed to its longstanding cultivation history in the Zhongyuan region, particularly in Luoyang, Henan Province, China [8,54]. However, further research is needed to fully understand the evolutionary and domestication history of Zhongyuan cultivars.

## 5. Conclusions

In this study, sixteen functional primer pairs with high polymorphism and good universality were selected from the initial pool of 115 SSR markers to analyze and infer the genetic diversity of tree peony germplasm. The tree peony germplasm resources from the nursery of Henan University of Science and Technology contained five populations and showed a high genetic diversity. These group samples could be categorized into two subpopulations, of which the Zhongyuan population was mainly distributed in subpopulation II, the other four population individuals were mainly distributed in subpopulation I. Furthermore, the observed variation was mainly attributable to within-population differences. The geographic distance is not the main driver of tree peony genetic structure. The findings provide comprehensive and important information for the breeding of tree peony and contribute to the sustainable management, conservation, and utilization of tree peony in the future.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14101945/s1; Figure S1: The original geographical sources of the tree peony population samples in this study; Table S1: Ninety-five EST-SSR markers information on the genetic linkage map in tree peony; Table S2: Twenty pairs of EST-SSR markers associated with phenotypic traits in tree peony populations; Table S3: Summary of private alleles in each population; Table S4. Analysis of the genetic diversity of tree peony populations in China.

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