forests

Study on the Genetic Variation of *Triadica sebifera* (Linnaeus) Small Populations Based on SSR Markers

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Abstract: *Triadica sebifera* (Linnaeus) Small is a tree species native to China. The seeds of *T. sebifera* are rich in oil and are widely used in industrial fields. To explore the genetic diversity and genetic differentiation of *T. sebifera* germplasm resources, 10 pairs of microsatellite markers were applied to 203 samples collected from eight populations. Forty-three alleles were detected. The average expected heterozygosity (He = 0.491) revealed a low level of genetic diversity. The genetic differentiation among *T. sebifera* populations was low (Fst = 0.026), which might be related to high gene flow (average Nm = 11.151). Genetic distance and structure results further confirmed that the genetic compositions of the eight populations were quite similar. One of the possible reasons for this phenomenon is that the early introduction and cultivation of *T. sebifera* were common, so gene exchange was frequent among populations. However, UPGMA clustering results indicated that the eight *T. sebifera* populations could still be divided into three categories. The classification was related to their geographical location: the southwestern group (ZY), central group (HG and XY) and eastern group (LS, HS, LX, XZ and LY). The reason for this differentiation might be severe deforestation following the decline in *T. sebifera* economic status. In addition, the central XY population had the largest number of rare alleles (4). In conclusion, although *T. sebifera* germplasm resources had a low level of genetic diversity, several rare alleles were detected in the central populations, which are valuable for breeding. These resources should be conserved to maintain genetic diversity in the *T. sebifera* populations. Moreover, geographical distances were important reasons for the limited genetic variations among the populations.

Keywords: *Triadica sebifera* (Linnaeus) Small; genetic diversity; rare allele; germplasm resource conservation

1. Introduction

Triadica sebifera (Linnaeus) Small is a tree species native to China that belongs to the genus *Sapium* (*Triadica*) in the family Euphorbiaceae. It is mainly distributed in Jiangsu, Zhejiang, Fujian, Henan and other provinces (regions) [1]. The seeds of *T. sebifera* are rich in oil and have been widely used in soap, paint and other industrial products, making it an ideal tree species for oil production [2]. However, the demand for *T. sebifera* gradually decreased due to the impact of imported oil. Therefore, many *T. sebifera* trees have been cut down, which has resulted in a sharp decrease in the germplasm resources of this species [3]. Recently, *T. sebifera* has been planted widely in gardens because of its high ornamental value. The success of *T. sebifera* breeding depends on the available germplasm resources. It is necessary to extensively collect *T. sebifera* germplasm resources and analyse their distribution patterns and genetic differentiation levels to protect and utilize them more scientifically and rationally.

At present, research on *T. sebifera* is mainly focused on phylogenetic analysis, chemical composition, biological activity and other economic traits [4–7]. However, there have been

few studies on its germplasm resources and genetic diversity, with the exception of studies on the screening of elite clones, introduction tests, etc. [8–10]. Although there have been studies on the genetic diversity of *T. sebifera*, most of them used intersimple sequence repeat (ISSR) markers [11,12], which are dominant markers and cannot distinguish heterozygotes. This approach is not conducive to the genetic evaluation of *T. sebifera* germplasm resources because of the limited information provided. In contrast, simple sequence repeat (SSR) markers have the advantages of large numbers, a wide distribution, and codominance. They have been widely used to study the genetic aspects of many woody plants, such as poplar, bamboo, and *Eucalyptus robusta* Smith [13–15]. When molecular markers are used for genetic analysis, more widely collected samples can reveal genetic differentiation and genetic diversity more comprehensively and accurately. Dewalt et al. [16] performed research on *T. sebifera* populations using SSR markers, but the number of samples was small, making it difficult to obtain comprehensive genetic characteristics of the *T. sebifera* populations.

Because of these research limitations, many questions remain, such as the following: What are the levels of genetic diversity and genetic structure in existing *T. sebifera* forests? How can the existing *T. sebifera* forest resources be protected? With these perspectives in mind, 10 pairs of SSR markers were used to explore the genetic variation of eight *T. sebifera* populations in the distribution area. The aim was not only to comprehensively explore the existing genetic resources of *T. sebifera* populations from a population genetics perspective, including aspects of genetic diversity and genetic structure, but also to explore the characteristics and origin of the differentiation of *T. sebifera* populations. This study is expected to provide a more reliable basis for formulating protection and utilization measures for *T. sebifera*.

2. Materials and Methods

2.1. Sample Information

Germplasm resources were collected in the main distribution areas of *T. sebifera*, covering seven provinces, namely, Guizhou, Zhejiang, Anhui, Hubei, Henan, Jiangsu and Shandong (Figure 1). Two-year-old branches were collected from different geographical regions, and the sample information for each population was recorded. A total of 203 samples from eight populations were ultimately collected, with 19 to 34 samples from each population. The samples were preserved by grafting in the *T. sebifera* Germplasm Resource Garden, which is located at the Zhejiang Academy of Forestry Sciences (30°13′09″ N, 120°01′44″ E). The information for each population is provided in Figure 1 and Table 1. Young leaves were collected and stored at −80 °C for later use.

Table 1. The origins of the eight *T. sebifera* (Linnaeus) populations.

| Population Code	Location	Longitude (E)	Latitude (N)	Altitude (m a.s.l.)	Annual Rainfall (mm)	N
ZY	Zunyi, Guizhou	27°41′57″	106°54′41″	209	1200	19
HG	Huanggang, Hubei	31°26′38″	114°24′06″	124	1400	24
XY	Xinyang, Henan	31°39′35″	115°23′35″	229	1200	28
HS	Huangshan, Anhui	30°01′16″	118°0′7″	224	2395	27
LY	Linyi, Shandong	35°15′40″	117°58′19″	174	840	20
XZ	Xuzhou, Jiangsu	34°16′37″	118°26′41″	198	876	34
LX	Lanxi, Zhejiang	29°19′19″	119°41′56″	98	1158	28
LS	Lishui, Zhejiang	28°41′18″	119°17′08″	326	1350	23
Total						203
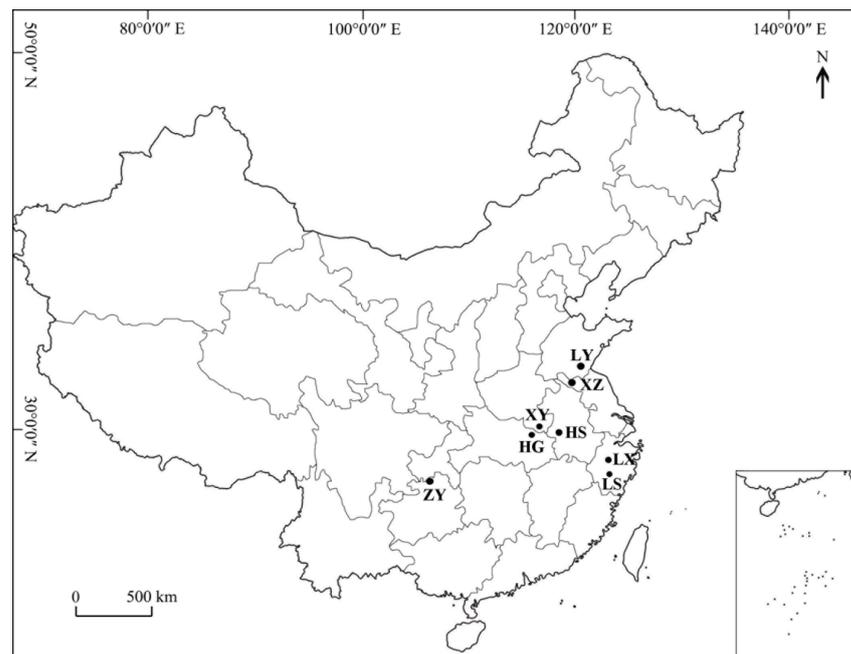
Figure 1. Geographical distribution map of the eight *T. sebifera* (Linnaeus) populations.

2.2. Experimental Method

The cetyl trimethyl ammonium bromide (CTAB) method was used to extract total genomic DNA [17]. A total of 120 pairs of SSR primers were designed by our team based on transcriptome sequences, and 10 of them were selected for this study. The primer information is shown in Table 2.

Table 2. Information on the 10 SSR loci.

Locus Code	Repeat Motif	Primer Sequence (5′~3′)	Fragment Size (bp)	TM (°C)
E-SSR25	(AAG)10	AGGTTGACGACTTCTGTGTT AGTTAGCCTGACCATTTCTC	351	53 °C
E-SSR29	(CT)11	ACCTTGCGAATGTTTATCC GGGGAAAAACAGATGGAAT	339	50 °C
E-SSR52	(AG)12	CTTTTACCTTTGATGTCGG GTTTCGGCAATTTCTCTGT	491	53 °C
E-SSR53	(AG)10	AAACAAGTGAAGTGCCCAT TTAGCCCAGCCCATTATTA	380	51 °C
E-SSR55	(TCT)10	GCGTACCTTCTTCAATGCTC TTCAACTTCTCTTTCCGTCA	428	53 °C
E-SSR58	(AGA)11	TCCACCTAGCGAAGTTTTG TTGATTCCTCCCCTTGTTT	295	52 °C
E-SSR61	(AAG)12	GGTTTCTTTTGCTCTCTTC CCGGTTACTGCATTTCATA	279	50 °C
E-SSR85	(CT)10	TTGCTCTTGGGACCTATTA TTCTTCCCTTGTGAGTTGT	290	50 °C
E-SSR103	(TC)10	CTACCCAATCACCTCTTTC TTCTTCTCTGTTCTGGCTC	287	50 °C
E-SSR106	(AGG)10	TCCCAGTTGACTGACGAACA CGAGGGTGAGGTCAGAGAAG	197	55 °C

Polymerase chain reactions (PCRs) were performed using the GeneAmp PCR System 9700 (Applied Biosystems Inc., Carlsbad, CA, USA) after the upstream sequence of the SSR primer was fluorescently labelled with FAM. The total PCR volume was 20 µL, which included 60 ng of genomic DNA, 10 µL of 2× TSINGKE Master Mix, and 1.5 µL (10 µmol/L) of EST-SSR forward primer and reverse primer. Finally, ddH2O was added to reach a total volume of 20 µL. PCR products were separated on an ABI 3730xl instrument for short tandem repeat (STR) typing. The typing results were read by Peak Scanner version 1.0 software (Applied Biosystems, Foster City, CA, USA) [18].

2.3. Data Analysis

POPGENE version 1.32 software (University of Alberta, Edmonton, Canada) was used to calculate population genetic parameters, such as Shanno's information index (I), the average number of observed alleles (Na), the effective number of alleles (Ne), expected heterozygosity (He), and the genetic differentiation coefficient (Fst) [19].

PowerMarker version 3.25 software (North Carolina State University, Raleigh, NC, USA) [20] was used to calculate the polymorphism information content (PIC) and linkage disequilibrium. Allelic richness (AR) was analysed by FSTAT version 2.9.3 software (University of Lausanne, Lausanne, Switzerland) [21]. GenAlEx version 6 software (Rutgers University, New Brunswick, NJ, USA) was used for analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA) [22].

Population genetic structure was analysed by Structure version 2.3.4 software (Stanford University, San Francisco, USA) [23]. For clustering from K = 1 to K = 9 (number of populations + 1), the Markov chain Monte Carlo simulation (MCMC) algorithm [24] was applied under admixed ancestry and an allele frequency model. The Evanno method was used to determine the optimal K value as implemented in STRUCTURE HARVESTER [25]. CLUMPP version 1.1.2 (Stanford University, Stanford, CA, USA) and DISTRUCT version 1.1 (Stanford University, San Francisco, USA) [26,27] were used to plot the results. The unweighted pair group method with arithmetic mean (UPGMA) cluster diagram and Mantel test were completed using NTSYS version 2.10 software (State University of New York, NY, USA) [28].

3. Results
3.1. Genetic Diversity

Ten pairs of SSR primers were applied to PCR amplification of 203 samples from eight populations. The STR typing results are shown in Figure 2. A total of 43 alleles were detected, with an average of 4.3 alleles (and range of 3 to 5) per SSR locus (Table 3). Overall, the 203 *T. sebifera* samples showed a moderate level of genetic diversity. The average Ne, He and I of each locus were 2.034, 0.491, and 0.854, respectively. Among the 10 SSR loci, E-SSR58 had the lowest polymorphism level. Its Ne, He, I and PIC were the smallest, at 1.410, 0.292, 0.598, and 0.269, respectively. In contrast, E-SSR85 had the highest polymorphism level. Its Ne, He, I and PIC were the highest, at 2.899, 0.657, 1.214, and 0.614, respectively. The level of polymorphism shown by E-SSR85 was the highest, followed by that of E-SSR25. In addition, except for the E-SSR58 locus, all the loci significantly deviated from Hardy–Weinberg equilibrium ($p < 0.05$).

The genetic diversity levels of the eight *T. sebifera* populations were not significantly different (average He = 0.486) (Table 4). The XZ population had the most genetic diversity, with the largest effective allele number (Ne = 2.079) and the highest degree of genetic diversity (AR = 3.185) and expected heterozygosity (He = 0.503), followed by the XY population. In contrast, the LS population had the lowest level of genetic diversity, with the lowest Na (1.908) and He (0.466). The average inbreeding coefficient (F) was −0.203 among the eight *T. sebifera* populations. All populations showed F < 0, indicating excess heterozygotes and insufficient homozygotes.
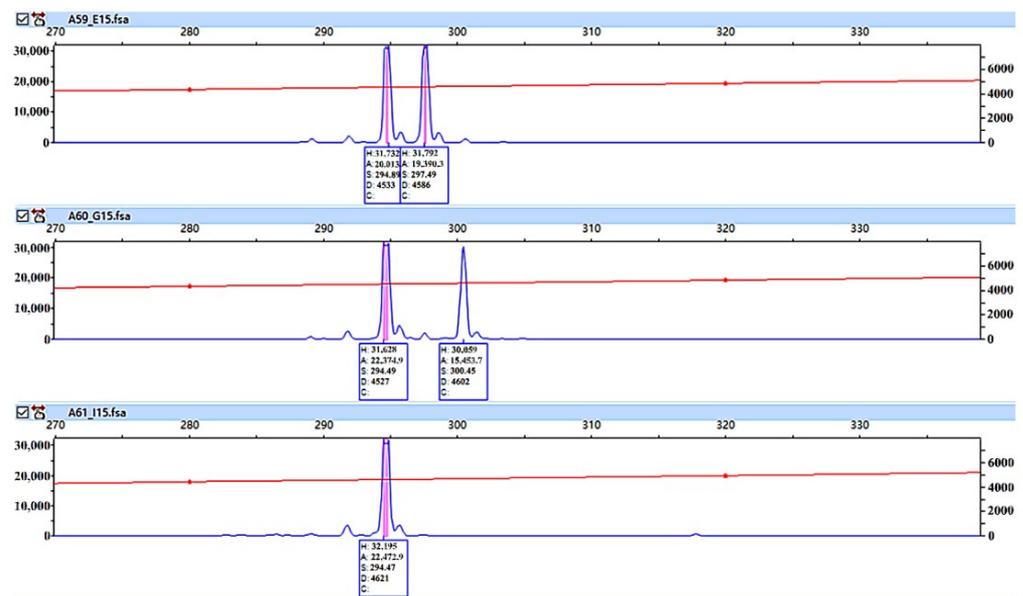
Figure 2. Amplification map of the SSR marker E-SSR58.

Table 3. Polymorphism information of the 10 SSR loci.

Locus	Na	Ne	He	I	PIC	Fst	Nm	HWE
E-SSR25	5	2.411	0.587	1.043	0.520	0.042	5.706	**
E-SSR29	5	1.662	0.399	0.730	0.329	0.037	6.484	*
E-SSR52	3	2.084	0.521	0.797	0.408	0.017	14.091	**
E-SSR53	5	1.929	0.483	0.861	0.427	0.014	18.192	**
E-SSR55	4	2.208	0.5 48	0.891	0.446	0.024	10.312	**
E-SSR58	4	1.410	0.292	0.598	0.269	0.038	6.389	
E-SSR61	4	1.685	0.408	0.762	0.370	0.022	10.910	**
E-SSR85	5	2.899	0.657	1.214	0.614	0.039	6.239	**
E-SSR103	3	1.930	0.483	0.798	0.443	0.019	13.279	**
E-SSR106	5	2.124	0.531	0.846	0.417	0.012	19.911	**
Mean	4.3	2.034	0.491	0.854	0.424	0.026	11.151	

Na—Observed number of alleles, Ne—Effective number of alleles, He—Expected heterozygosity, PIC—Polymorphism information content, Fst—Genetic differentiation index, Nm—Gene flow, I—Shannon's information index, HWE—Hardy–Weinberg Equilibrium, *: Significant ($p < 0.05$), **: Extremely significant ($p < 0.01$).

Table 4. Information on genetic diversity in the eight populations based on 10 SSR loci.

Population Code	Na	Ne	AR	Ho	He	F
ZY	2.9	2.047	2.890	0.563	0.492	−0.146
HG	3.3	1.974	3.293	0.613	0.497	−0.233
XY	3.5	2.021	3.245	0.554	0.498	−0.113
HS	3.3	1.990	3.192	0.556	0.480	−0.157
LY	3.2	1.989	3.415	0.595	0.485	−0.228
XZ	3.4	2.079	3.185	0.641	0.503	−0.276
LX	3.3	1.969	3.125	0.596	0.471	−0.265
LS	3.0	1.908	3.295	0.565	0.466	−0.212
Mean	3.24	1.997	3.205	0.585	0.486	−0.203

AR—Allelic richness, Ho—Observed heterozygosity, F—Inbreeding coefficient.

3.2. Genetic Differentiation and Genetic Structure

The genetic differentiation coefficient (Fst) of the eight *T. sebifera* populations was 0.026, indicating low genetic differentiation among them (Table 3). The results of AMOVA further revealed different levels of genetic variation between regions and populations. However, the variation was mainly concentrated within the populations, reaching an extremely significant level ($p < 0.01$, Table 5). Four percent of the genetic variation occurred among regions (southwest and mideast, $p = 0.001$). One percent of the total variation occurred among populations within regions, and 95% of the genetic variation occurred within populations ($p = 0.001$).

Table 5. AMOVA of 203 samples from eight *T. sebifera* populations.

Source of Variance	Variance Component	Percentage of Total	*p* Value
Among regions	0.155	4%	**
Among populations within regions	0.038	1%	
Within populations	3.823	95%	**
Total	4.016	100%	

**: Extremely significant ($p < 0.01$).

The common ancestor relationship of *T. sebifera* populations was analysed for further research on population genetic structure. According to the maximum ΔK value, the optimal K was 3 (Figure 3A). When K = 3, the genetic information divided the *T. sebifera* populations into three different ancestral populations (Figure 3B). However, the genetic information sources of the eight populations were quite similar when K = 2, 3 or 8. In addition, a cluster diagram (Figure 3C) was constructed based on the genetic distances between the populations (Table 6). There was a certain degree of genetic differentiation among the eight populations. They could be roughly divided into three categories: the ZY population in the southwest was divided into a separate class, the HG and XY populations in the central region were divided into one class, and the LS, HS, LX, XZ and LY populations were grouped into one category. The classification results of the eight populations and their distribution positions were associated. The results of the Mantel test performed on the eight *T. sebifera* populations further proved this result, implying a significant association between the genetic and geographical distances between populations (r = 0.6697, $p = 0.9860$, Figure 4).

Table 6. Pairwise genetic distances between *T. sebifera* populations.

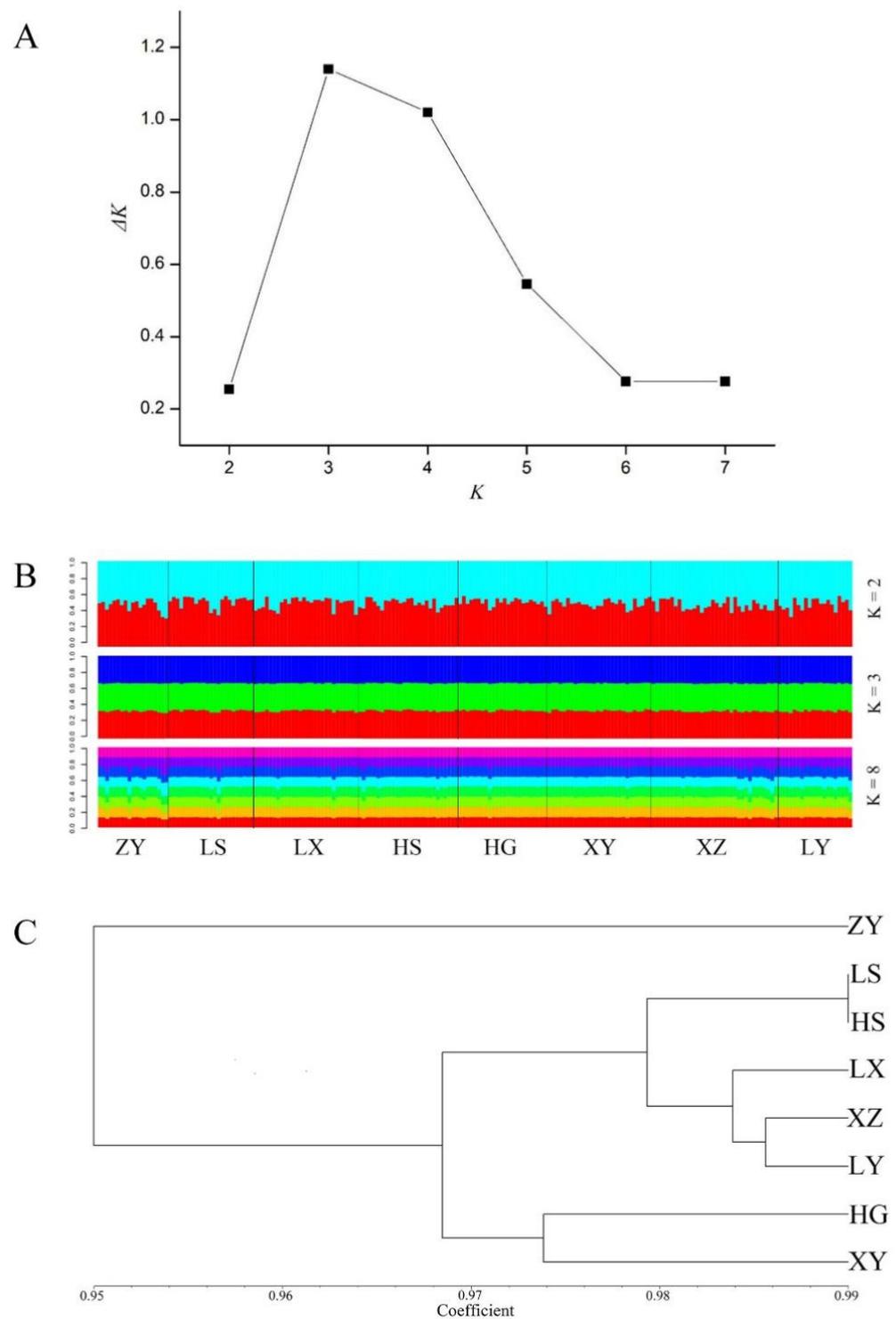|	ZY	LS	LX	HS	HG	XY	XZ	LY
ZY	0							
LS	0.043	0						
LX	0.051	0.025	0					
HS	0.038	0.009	0.019	0				
HG	0.059	0.026	0.026	0.035	0			
XY	0.047	0.031	0.032	0.028	0.024	0		
XZ	0.036	0.021	0.013	0.015	0.030	0.024	0	
LY	0.051	0.017	0.016	0.014	0.027	0.031	0.013	0

Figure 3. Genetic structure of eight *T. sebifera* populations. (**A**) Relationship between the number of clusters (K) and the corresponding ΔK statistic; (**B**) Results of the structure analysis of *T. sebifera* populations when K = 2, 3, and 8. Different colors (light-blue, red, blue, green, dark purple, purple, light-green, and orange) represent different genetic compositions; (**C**) Cluster diagram of the eight populations.
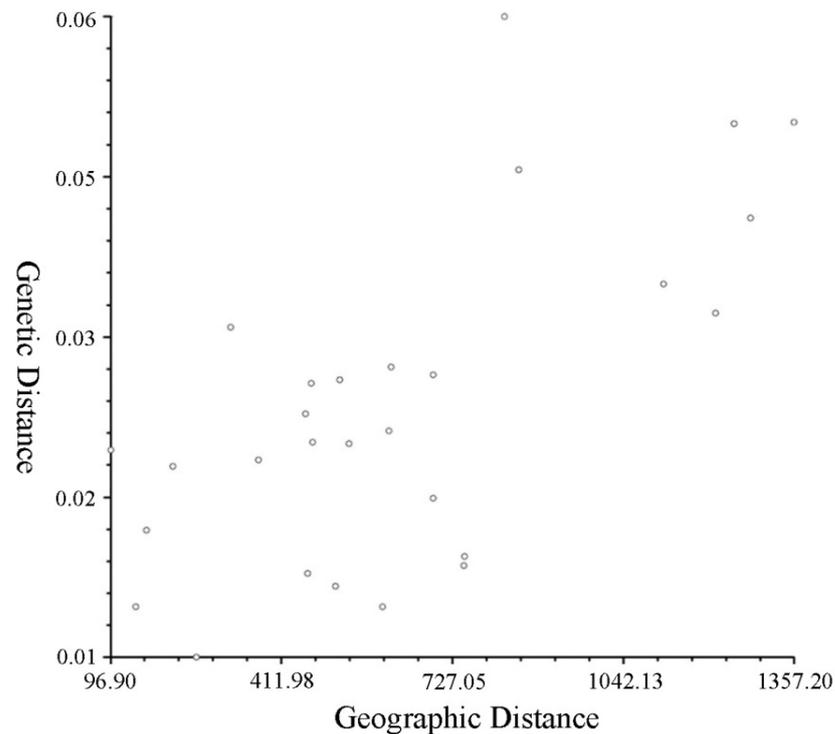
Figure 4. The result of the Mantel test.

3.3. Rare Alleles

The frequencies of the 43 alleles ranged from 0.0049 to 0.8350, with an average of 0.2326. The minimum allele frequencies (MAFs) of the 10 SSR loci were 0.0049 to 0.0591, and the average was 0.0195. Rare alleles with a frequency less than 0.1 were selected, and the frequency of each rare allele in the populations was calculated (Table 7). Six rare alleles were found among the 10 SSR loci, which were distributed at 5 SSR loci. Two rare alleles were detected at the G-SSR106 locus, and one rare allele each was detected at the other four loci. The central XY population and HG population had the largest numbers of rare alleles, with four and three, respectively. In contrast, the western and eastern populations had 0~2 rare alleles.

Table 7. Rare allele frequencies within the *T. sebifera* populations.

	E-SSR29-E	E-SSR55-E	E-SSR61-E	E-SSR85-C	E-SSR106-D	E-SSR106-E
ZY	0.079					
HG			0.021	0.042		0.021
XY	0.018	0.018		0.018	0.018	
HS						
LY					0.075	
XZ						0.044
LX		0.018				
LS		0.022	0.022			

4. Discussion

4.1. Genetic Diversity

Genetic diversity is an important indicator used to measure the ability of a species to resist changes in the external environment. It is affected by selection, genetic drift, and breeding system. Research on the genetic diversity of *T. sebifera* populations could provide a theoretical reference for the protection and utilization of *T. sebifera*. Li [11] used ISSR markers to study 32 *T. sebifera* leaf samples from six populations, and the H and I

values were 0.2822 and 0.4321, respectively. Zhang [12] also used ISSR markers to analyse 72 samples in four main production areas and obtained similar results (He = 0.281). In this study, the average He of 203 *T. sebifera* samples from eight populations based on SSR markers was 0.486, which was higher than that in previous studies. There are two possible explanations for this discrepancy. First, the marker types used were different. Compared with ISSR markers, SSR markers can reveal more genetic variation. Second, there were differences in the number of samples. The number of samples selected in this study was relatively large. In addition, the distribution range covered the main distribution areas of *T. sebifera*. DeWalt et al. [16] used six pairs of SSR markers to analyse 129 samples from 12 *T. sebifera* populations in China, and the values were higher than those of this study (average He = 0.70). The reason was inferred to be sample differences. In recent years, many older trees might have been transplanted or traded with the gradual popularization of *T. sebifera* application. This contributed to the loss of *T. sebifera* resources and a decrease in the genetic diversity of the *T. sebifera* populations.

Compared with the results from SSR markers in other angiosperms, the genetic diversity of *T. sebifera* (He = 0.491) was significantly lower than that of *Ginkgo biloba* Linn (He = 0.808) [29], *Quercus variabilis* BI. (He = 0.707) [30], *Cunninghamia lanceolata* (Lamb.) Hook (He = 0.557) [31] and other species. *T. sebifera* has been widely promoted and planted as an important oil tree species. However, with industrial development and changes in the national industrial structure, the economic status of *T. sebifera* has declined, and the natural populations have been reduced excessively. Therefore, its genetic resources are difficult to preserve, resulting in a significant reduction in genetic diversity. Therefore, protecting the genetic diversity of *T. sebifera* is an important goal for preserving its natural resources.

4.2. Genetic Differentiation and Genetic Structure

Natural selection contributes to population differentiation and is the most important evolutionary force. Gene flow plays an important role in selection. For perennial outcrossing woody plants, genetic differentiation among populations is generally low. When Fst is between 0 and 0.05, there is basically no differentiation among populations. There is a moderate degree of differentiation when Fst is 0.05~0.15. When Fst is 0.15~0.25, the degree of differentiation is relatively high. There is strong differentiation when Fst is greater than 0.25 [32]. The differentiation of *T. sebifera* populations detected in this study (Fst = 0.026) was much weaker than that of *Liriodendron chinense* (Hemsl.) Sarg. (Fst = 0.302) [33], *Q. variabilis* (Fst = 0.067) [30], *Pinus massoniana* Lamb (Fst = 0.072) [34], etc. Our results were quite different from those of Li [11] (Gst = 0.439), which might be due to the use of different markers and numbers of samples.

The results of the AMOVA further indicated that the variation among *T. sebifera* populations was quite low, and most of the genetic variation existed within the populations. Cluster analysis also showed that the eight *T. sebifera* populations had similar ancestral origins. The small genetic distances (the highest was only 0.051) also indicated close kinship among the populations. *T. sebifera* was widely distributed in the early stage because of its high economic value. The mutual introduction and cultivation in different regions led to frequent genetic exchange among geographic groups. This also resulted in similar origins among different geographic populations.

However, there was still a certain degree of genetic differentiation among the eight *T. sebifera* populations. The UPGMA clustering results showed that the eight populations could be divided into three categories, which were related to their geographical location, namely, the southwestern group (ZY), the central group (HG and XY) and the eastern group (LS, HS, LX, XZ and LY). The results of the Mantel test further indicated that there was a significant positive correlation between genetic distance and geographic distance. Many *T. sebifera* resources have been removed, contributing to the differentiation among geographical populations, while introduction and cultivation have been greatly reduced due to the decline of the *T. sebifera* industry.

4.3. Genetic Resource Conservation Strategy of T. sebifera

Abundant germplasm resources are the key to successful forest tree breeding, and they guarantee the continuation of tree species [35]. The overall genetic diversity of *T. sebifera* was low, and there has been a downward trend in recent years. Even though quite similar amounts of genetic information were observed between populations, six rare alleles were detected in this study. The populations in the central regions had more rare alleles. These resources with rare alleles are not only important for maintaining species diversity but also valuable breeding materials. Therefore, the protection and utilization of various *T. sebifera* populations should be strengthened in the future, especially for the central populations with more rare alleles.

5. Conclusions

In general, our study included a genetic evaluation of eight populations of *T. sebifera*. The genetic compositions of the eight populations were similar, and the genetic diversity was low. One possible reason was that introduction was common a long time ago, so gene exchange was frequent among populations. However, the UPGMA results still classified these populations into three categories, indicating that they began to diverge geographically. Finally, rare alleles were detected in some populations, which will be of great help in maintaining the level of genetic diversity in *T. sebifera* populations.

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