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Genetic Diversity and Population Genetic Structure of Ancient *Platycladus orientalis* L. (Cupressaceae) in the Middle Reaches of the Yellow River by Chloroplast Microsatellite Markers

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Abstract: Ancient trees are famous for their life spans of hundreds or even thousands of years. These trees are rare, a testament to history and are important for scientific research. *Platycladus orientalis*, with the longest life span and a beautiful trunk, has become the most widely planted tree species and is believed to be sacred in China. Extensive declines in habitat area and quality pose the greatest threats to the loss of genetic diversity of ancient *P. orientalis* trees in the middle reaches of the Yellow River. Strengthening the protection of *P. orientalis* genetic resources is of great significance for the long-term development of reasonable conservation and breeding strategies. To better understand the genetic diversity and population structure of *P. orientalis*, we successfully analyzed four polymorphic chloroplast simple sequence repeat (cpSSR) loci and applied them to diversity and population structure analyses of 202 individuals from 13 populations in the middle reaches of the Yellow River. Based on the cpSSR data, 16 alleles were detected across 202 individuals, and a moderate level of genetic diversity was inferred from the genetic diversity parameters (H = 0.367 and A_R = 1.964). The mean pairwise genetic differentiation coefficient (Fst) between populations was 0.153, indicating relatively high genetic population differentiations. Analysis of molecular variance (AMOVA) showed that only 8% of the variation occurred among populations. Structure analysis divided the 13 *P. orientalis* populations into two groups with no significant geographic population structure, which was consistent with the unweighted pair group method with arithmetic mean (UPGMA) and Mantel test results. These results may indicate that transplanting and cultivation by ancient human activities are the main factors responsible for the revealed pattern of genetic differentiation of ancient *P. orientalis* populations. Our research is of great significance for the future establishment of protection schemes and scientific breeding of *P. orientalis*.

Keywords: *Platycladus orientalis*; ancient trees; cpSSR; genetic diversity; population structure

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1. Introduction

Ancient trees are those that have lived for hundreds or even thousands of years [1]. They are precious specimens of natural resources and have important scientific research value [2]. As resources of genetic diversity and reproductive ability, ancient tree populations have many valuable genes in their gene pool [3].

Platycladus orientalis (L.) Franco, belonging to the genus *Platycladus* (Cupressaceae) of evergreen coniferous tree species, is the main afforestation tree species in the arid regions of the Loess Plateau of Northwest China [4–6]. Due to its long lifespan, beautiful trunk, sacred status and important economic value, *P. orientalis* has been planted in gardens, temples and the vicinity of mausoleums since ancient times [7]. It is the most widely planted cemetery tree species in China [8]. The age of *P. orientalis* can reach hundreds or even thousands of years [9]. An ancient *P. orientalis* tree at the Mausoleum of the Yellow Emperor in Shaanxi

Province has witnessed the history and culture of China for more than 4000 years, and is known as the oldest ancient *P. orientalis* tree in the world [1].

The middle reaches of the Yellow River are considered to be one of the important birthplaces of Chinese civilization [10], mainly including Henan Province, Shaanxi Province, Shanxi Province and parts of Gansu Province [11]. This region is not only an area of the dense distribution of ancient *P. orientalis* trees in China [12,13], but also a region with a long history of human settlement [10]. Temples, cemeteries, and ancient academies in the middle reaches of the Yellow River have the largest number of ancient trees of *P. orientalis*, and have become tourist destinations with unique cultural value in China [1]. Recently, due to rapid changes in climate and the environment, intense human activities and overexploitation of forest resources, many populations of ancient *P. orientalis* have decreased their size or even disappeared, and their excellent genetic resources are facing great challenges and threats [9,14].

Accurate assessment the genetic diversity and population structure of ancient trees is an important prerequisite for the protection of their genetic resources [14,15]. Molecular marker technology has greatly facilitated assessments of the genetic resources of plants and the level of genetic diversity in tree species [16], and has been recognized as an effective means of genetic analysis of coniferous species [17]. Chloroplast simple sequence repeats (cpSSRs) are a new and efficient molecular marker technology developed in recent years [18]. Due to their high polymorphism, uniparental inheritance through chloroplast DNA (cpDNA) and lack of sexual recombination [19], cpSSRs allowing to trace haplotype diversity have become ideal markers in population genetic diversity evaluation, population structure analysis and phylogenetic studies [20,21]. Previous research has established that the inheritance pattern of cpDNA in *P. orientalis* is patrilineal [22], which also makes it an ideal marker for monitoring gene exchange and genetic differentiation among populations [23].

To date, the roles of genetic diversity and population structure in *P.orientalis* remain largely unexamined. A few published studies have attempted to detect the genetic diversity of *P. orientalis* with molecular markers such as isozymes [13], amplified fragment length polymorphisms (AFLPs) [24], simple sequence repeat markers (SSRs) [25,26], and single nucleotide polymorphisms (SNPs) [9]. However, Huang, et al. [27] established a set of cpSSR markers that can be transferred between different genera of Cupressaceae, and used eight nuclear SSRs (nSSRs) and four cpSSRs to reconstruct the family of *P. orientalis* seed gardens in Henan Province, China [27,28]. Surprisingly, no research has surveyed the genetic diversity and population structure of ancient *P. orientalis* using cpSSR markers. This includes the middle reaches of the Yellow River, an area with rich resources of ancient *P. orientalis* trees. This lack of research has hindered the protection and utilization of the precious genetic resources hidden in the ancient trees of *P. orientalis*.

Knowledge of the degree and distribution of genetic diversity can guide efficient gene conservation sampling designs [29]. This study set out to investigate the usefulness of four polymorphic cpSSR loci for evaluating the haplotype diversity level of 13 *P. orientalis* populations in the middle reaches of the Yellow River and to analyze the genetic diversity and genetic structure of the populations. These results provide a theoretical basis for the protection and utilization of ancient *P. orientalis* germplasm resources and reveal the population history of *P. orientalis* in the middle reaches of the Yellow River.

2. Materials and Methods

2.1. Plant Material

We collected a total of 202 individuals from 13 populations of ancient *P. orientalis* in four provinces (Gansu, Shanxi, Henan and Shaanxi) of China (the oldest and best-preserved ancient *P. orientalis* populations in the middle reaches of the Yellow River) (Figure 1, Table 1). The decision for sample collection was approved by the Yellow Emperor Mausoleum Administration (Letter No. 036-043). All of the samples were carefully identified by Professor Yongxiang Kang of Northwest A&F University based on the descriptions in Flora

of China. All ancient trees of *P. orientalis* were over 500 years old. The age was based on the diameter at breast height (DBH) [9] of the selected trees and the historical records provided by the local government department responsible for managing ancient trees. The fresh young leaves were collected in the field preserved with silica gel, transferred to the laboratory of the Ancient and Famous Tree Protection and Breeding Engineering Technology Research Center of the National Forestry and Grassland Administration, and stored at −80 °C until the DNA was extracted.

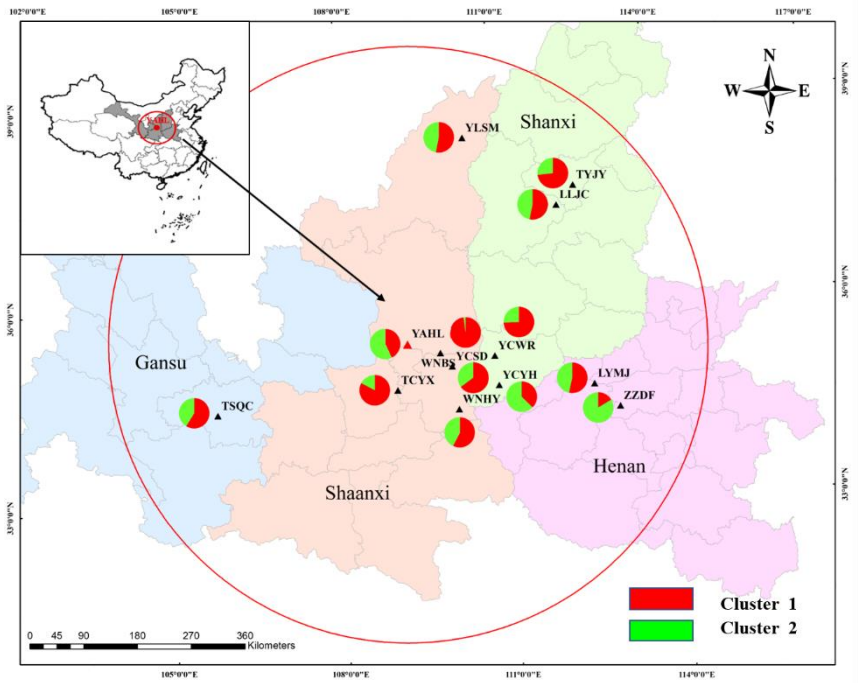

Figure 1. Geographical distribution of plant materials. Triangle points indicate the populations that were collected in the middle reaches of the Yellow River. The sector map shows the mean cluster membership proportions of the analyzed individuals in each of the 13 *P. orientalis* populations based on the structure at K = 2.

Table 1. Geographical location and genetic variability statistics for 13 ancient *P. orientalis* populations in the middle reaches of the Yellow River.

Populations	Sample Size	Locations	Latitude (N)	Longitude (E)	Elevation (m)	Average Age (years)	*Na*	*Ne*	A_R	*Apriv*	*I*	*TPM*	*SMM*
WNBS	29	Weinan, Shaanxi	35°22′	109°41′	816	2314	2.750	1.436	2.123	0.250	0.510	0.290	0.253
WNHY	13	Weinan, Shaanxi	34°34′	110°06′	315	1518	2.250	1.611	2.180	0.000	0.546	0.562	0.384
TCYX	12	Tongchuan, Shaanxi	34°54′	108°59′	717	1238	1.750	1.148	1.571	0.250	0.213	0.241	0.217
YAHL	19	Yan'an, Shaanxi	35°35′	109°16′	865	2711	2.250	1.689	2.063	0.250	0.588	0.686	0.680
YLSM	19	Yulin, Shaanxi	38°40′	110°25′	1172	1668	2.750	1.583	2.294	0.250	0.610	0.229	0.200
LLJC	6	Lvliang, Shanxi	37°34′	112°07′	884	1200	1.500	1.346	1.500	0.000	0.286	0.739	0.715
TYJY	7	Taiyuan, Shanxi	37°42′	112°26′	816	1871	2.000	1.366	1.989	0.000	0.404	0.081	0.063
YCSD	6	Yuncheng, Shanxi	35°07′	110°54′	398	2417	1.250	1.096	1.250	0.000	0.113	0.516	0.477
YCYH	12	Yuncheng, Shanxi	34°54′	110°50′	325	1308	2.000	1.414	1.869	0.000	0.411	0.444	0.481
YCWR	8	Yuncheng, Shanxi	35°21′	110°48′	770	2250	1.750	1.321	1.725	0.000	0.324	0.661	0.713

Table 1. *Cont.*

Populations	Sample Size	Locations	Latitude (N)	Longitude (E)	Elevation (m)	Average Age (years)	*Na*	*Ne*	A_R	*Apriv*	*I*	*TPM*	*SMM*
LYMJ	30	Luoyang, Henan	34°50′	112°35′	124	1500	2.750	1.693	2.330	0.000	0.668	0.634	0.562
ZZDF	17	Zhengzhou, Henan	34°27′	113°04′	350	1435	2.500	1.858	2.275	0.000	0.659	0.658	0.629
TSQC	24	Tianshui, Gansu	34°34′	105°42′	1173	929	2.750	1.709	2.371	0.250	0.678	0.634	0.577
Mean	15.538	-	-	-	-	1720	2.173	1.482	1.964	0.096	0.462	0.490	0.457

Note: Observed number of alleles per locus (*Na*); Mean number of effective alleles (*Ne*); Allelic richness (A_R); Mean number of private alleles (A_{priv}); Shannon-Weiner index (*I*); *P* value of bottleneck under two phase model (*TPM*); *P* value of bottleneck under stepwise mutation model (*SMM*). Baishui, Weinan city (WNBS); Huayin, Weinan city (WNHY); Yaoxian, Tongchuan city (TCYX); Huangling, Yan'an city (YAHL); Shenmu, Yulin city (YLSM); Jiaocheng, Lvliang city (LLJC); Jinyuan, Taiyuan city (TYJY); Shundi, Yuncheng city (YCSD); Yanhu, Yuncheng city (YCYH); Wanrong, Yuncheng city (YCWR); Mengjin, Luoyang city (LYMJ); Dengfeng, Zhengzhou city (ZZDF); Qinchuan, Tianshui city (TSQC).

2.2. DNA Extraction and cpSSR Analysis

Total genomic DNA was extracted with a Plant Genome DNA Extraction Kit (BioTeke, Beijing, China). The quality and concentration of DNA were verified by 1.5% agarose gel electrophoresis and a NanoDrop2000 (BioTeke Instruments, Winooski, VT, USA). For cpSSR analysis, DNA was diluted to 50 ng/μL and stored at −20 °C. The screening of cpSSR polymorphic primers was based on a set of cpSSR marker systems established by Huang et al. [27], which was transferable among different genera of Cupressaceae. We used all DNA samples from 13 ancient populations of *P. orientalis* for polymerase chain reaction (PCR) amplification. A total of 22 of the 26 cpSSR loci (N1, N2, N4, N6, N8, N9, N10, N13, N14, N15, N18, N19, N20, N22, N23, N25, N27, N28, N31, N32, N33, and N35) in the ancient *P. orientalis* trees were amplified successfully (Table S1), and used an ABI 3730 sequencer to perform fluorescence capillary electrophoresis to detect their polymorphism levels. However, only four cpSSR loci were polymorphic (N1, N2, N6, and N33) (Table 2). All the primers used in this study were synthesized by Sangon Biotech (Shanghai, China).

Table 2. Characteristics of four cpSSR loci for PCR amplification in *P. orientalis*.

ID	Primer Sequence (5′–3′)	Repeat Motif	Expected PCR Product Size (bp)	Location	Tm (°C)	Species
N1	TTCTAGCTCGCACCCAAACT TTGTTTCGCCGATATGTTCA	(AC)6	260	rbcL/accD IR	56	*Cupressus gigantean*
N2	TGGTCATACCATTGCTGTTCA TGGGCTACTCTACGTGCTTT	(AT)5	395	rps19/rp122 IR	56	*Cupressus gigantean*
N6	GGGAACAACCAGAATTGGAA GCCACTTTTATGGCACGACT	(TA)5	360	ycf1	56	*Cupressus gigantean*
N33	CTGTTCCCCTGTGCATCATA AGGAGGAAAATCCGTTGGTT	(TCT)5	400	trnF-GAA/trnL-UAA IR	56	*Juniperus scopulorum*

PCR amplifications were performed using 25 μL reactions containing 50 ng of genomic DNA, 2 × Taq Master Mix, and 10 pmol of forward and reverse primers. The PCR conditions were as follows: an initial denaturation at 95 °C for 3 min; followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C; and a final extension 5 min at 72 °C. An ABI3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) was used to separate fluorescently labeled PCR products (FAM, HEX, TAMRA, ROX), and the data were analyzed with Gene Mapperv4.0 software (Applied Biosystems, Foster City, CA, USA).

2.3. Statistical Analysis

POPGENE ver.1.3.2 [30] and GenALEx ver.6.5 [31] were used to characterize and calculate the polymorphism level of four cpSSR loci (Table 2). The metrics calculated included the number of alleles per locus (*Na*), the number of effective alleles per locus (*Ne*), the mean number of private alleles (*Apriv*) per locus, Shannon–Weiner index (*I*), Nei's gene diversity (*H*), the genetic differentiation coefficient (*F*st) and gene flow (*N*m), *N*m = [(1/*F*st)

− 1]/4. The allelic richness (A_R, based on a randomization of a minimum of six individuals at one locus) was determined via FSTAT 2.9.3 software [32]. PIC_CALC v.0.6 [33] was used to calculate the polymorphism information content (PIC) of genets. Tests of deviation from Hardy–Weinberg equilibrium per locus and the linkage disequilibrium between the pairs of loci in each population were performed at a significance level of 0.05, using GENEPOP v1.2 [34].

To test for recent bottleneck events in the targeted populations, Bottleneck v.1.2 [35] was applied (with the stepwise mutation model (SMM) and two-phase model (TPM), and their significance was tested. The proportion of the SMM was set to 70% under default settings. Genetic analysis in the Excel (GenAlEX ver.6.5) package was used to determine Nei's genetic distance (D) and Nei's genetic identity among populations. The significance of *F*st values for the targeted populations across all loci was calculated by Holm's sequential Bonferroni correction.

Analysis of molecular variance (AMOVA) was performed using Arlequin version 3.1 and significance was tested based on 1000 permutations [36]. In addition, to test for a correlation between genetic distances and geographical distances (in kilometers) among populations, the Mantel matrix correspondence test was performed [37].

Nei's genetic identity was applied to evaluate the genetic relationships among populations. The unweighted pair group method with arithmetic mean (UPGMA) was used to perform cluster analysis on the genetic similarity data, and NTSYS ver. 2.1 (Applied Biostatistics, Port Jefferson, New York, NY, USA) software was used to obtain tree diagrams [38].

Population structure was explored by using STRUCTURE ver.2.3.4 with a Bayesian clustering approach [39]. Testing ten independent runs with K from 1 to 13, each run had a burn-in period of 100,000 iterations and 500,000 Monte Carlo Markov iterations, assuming an admixture model. The targeted populations were separated into groups by the Structure Harvester program [40] based on the ΔK values [41].

3. Results

3.1. Microsatellite Analysis

Sixteen different alleles were detected for the four cpSSR loci across all 202 individuals in the thirteen populations of *P. orientalis* (Table 3, File S1). The N2 locus exhibited the largest *N*a (six), while the N1 locus exhibited the largest *N*e (1.833), *I* (0.793), PIC (0.397), *H* (0.455) and *F*st (0.210). However, locus N33 showed lower than mean values of mentioned above statistics. The *I* ranged from 0.548 (N33) to 0.793 (N1) with a mean of 0.673. In terms of PIC values, three loci (N1, N2 and N6) had highly informative alleles with values higher than 0.3, while only N33 had less informative alleles, with a value less than 0.25. The mean *H* and *N*m values were 0.367 (0.258–0.455) and 1.503 (0.221–1.935), respectively. This shows that there was a high level of historical gene exchange among populations. The mean *F*st for the loci was 0.153 (0.114–0.210). The results showed that the four cpSSR loci selected in this study had high polymorphism in the thirteen populations of *P. orientalis* in the middle reaches of the Yellow River of China.

Table 3. Diversity statistics of the four cpSSR loci across 202 *P. orientalis* individuals.

ID	*Na*	*Ne*	*I*	PIC	*H*	*Fst*	*Nm*
N1	4	1.833	0.793	0.397	0.455	0.210	0.938
N2	6	1.5339	0.761	0.332	0.350	0.115	1.919
N6	2	1.669	0.590	0.320	0.400	0.170	0.221
N33	4	1.348	0.548	0.245	0.258	0.114	1.935
Mean	4	1.597	0.673	0.324	0.367	0.153	1.503

Note: Observed number of alleles per locus (*Na*); Mean number of effective alleles (*Ne*); Shannon-Weiner index (*I*); Polymorphism information content (PIC); Nei's gene diversity index (*H*); Genetic differentiation coefficient (*F*st); Gene flow (*N*m).

3.2. Genetic Diversity and Mantel Test Results in P. orientalis

The four cpSSR loci were amplified consistently under standard conditions, produced clear products and were used to assess population genetic structure. Genetic diversity was recorded at the population level (Table 1). The data showed that the mean *N*a in the populations was 2.173 (1.250–2.750) and that the mean *N*e was 1.482 (1.096–1.858). The A_R of the ancient *P. orientalis* calculated for a minimum sample size of six individuals per population was compared with that of the other populations, which on the average was 1.964 (1.250–2.371). The mean of *A*priv was 0.096 (0.000–0.250). The YCSD population had the lowest values for allelic richness and private alleles (A_R = 1.250 and *A*priv = 0.000), and TSQC population (A_R = 2.371 and *A*priv = 0.250) had the highest values. The Shannon–Weiner index (*I*) ranged from 0.113 (YCSD) to 0.678 (TSQC), with an average of 0.462. In the bottleneck analysis, no significant heterozygote deficits were detected in the thirteen populations under the *TPM* and *SMM* models (Table 1). This suggests that none of the populations in this study exhibited signs of a recent bottleneck.

AMOVA was performed based on 295.614 permutations and revealed the genetic variation among and within populations for ancient *P. orientalis* (Table 4). The AMOVA showed that 8% of the total genetic variation occurred among populations and a significant amount (92%, $p < 0.001$) of the total variation occurred within populations.

Table 4. Analysis of molecular variance (AMOVA) of 202 individuals in 13 populations of *P. orientalis*.

Source of Variation	d.f.	Sum of Squares	Variance Components	Total Variation (%)	*p* Value
Among populations	9	38.979	0.062	8	<0.001
Among individuals within populations	189	256.635	0.679	92	
Total	403	295.614	0.741	100	

Note: degrees of freedom (d.f.).

We further analyzed the correlation between genetic distance and geographical distance (File S2) for the 13 populations using the Mantel test (Figure 2). Nei's genetic distances are listed in Table 5. There was no significant correlation between genetic distance and geographic distance (R^2 = 0.0002, p = 0.470).

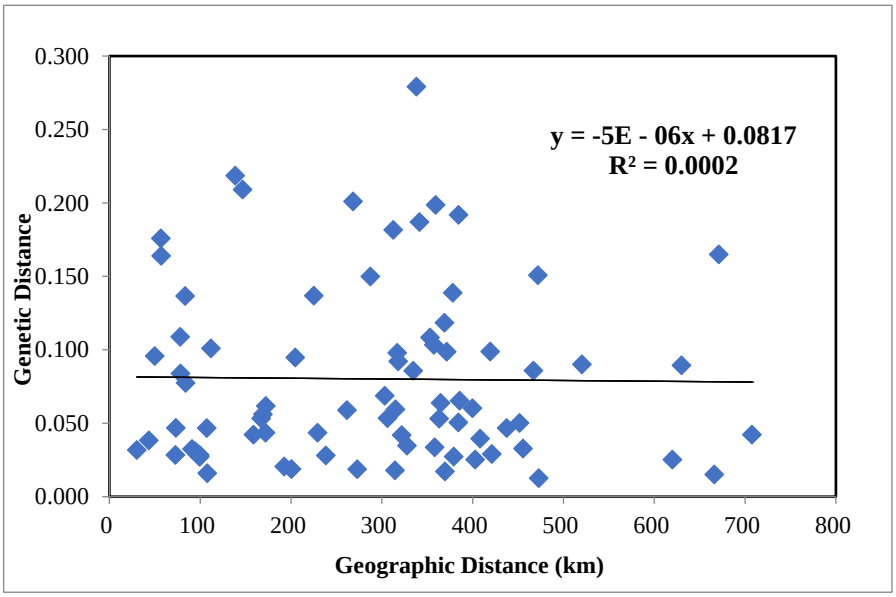

Figure 2. Mantel test between genetic distance and geographical distance of 13 *P. orientalis* populations.

3.3. Population Clustering and Genetic Structure of P. orientalis

The values of Nei's genetic distance among the studied populations ranged from 0.013 (YCWR/TSQC) to 0.279 (YAHL/TSQC). Similarly, the genetic identity varied from 0.756 (YCSD/ZZDF) to 0.987 (TYJY/YCSD) (Table 5). The clustering of populations according to the UPGMA dendrogram, which was based on pairwise genetic identity, showed that two groups were separated clearly at the population level (Figure 3). The resulting tree showed two groups among the 202 individuals from the 13 *P. orientalis* populations. The first group was composed of ten populations (WNBS, WNHY, YCWR, TCYX, TYJY, YCSD, YLSM, LLJC, LYMJ, and TSQC), and the second group was composed of three populations (YAHL, ZZDF, and YCYH).

Table 5. Nei's genetic identity (above diagonal) and genetic distance (D) (below diagonal) of 13 *P. orientalis* populations.

PC	WNBS	WNHY	TCYX	YAHL	YLSM	LLJC	TYJY	YCSD	YCYH	YCWR	LYMJ	ZZDF	TSQC
WNBS	-	0.981	0.980	0.906	0.951	0.918	0.983	0.975	0.914	0.968	0.948	0.820	0.954
WNHY	0.027	-	0.962	0.902	0.938	0.918	0.982	0.959	0.870	0.971	0.967	0.825	0.951
TCYX	0.031	0.047	-	0.907	0.959	0.948	0.981	0.985	0.897	0.973	0.942	0.829	0.961
YAHL	0.176	0.219	0.084	-	0.925	0.959	0.909	0.860	0.910	0.897	0.904	0.948	0.945
YLSM	0.053	0.033	0.047	0.199	-	0.972	0.954	0.941	0.861	0.972	0.969	0.872	0.972
LLJC	0.060	0.027	0.040	0.187	0.021	-	0.940	0.914	0.849	0.957	0.943	0.912	0.966
TYJY	0.034	0.029	0.050	0.192	0.019	0.038	-	0.987	0.872	0.984	0.968	0.811	0.957
YCSD	0.032	0.028	0.028	0.137	0.051	0.042	0.064	-	0.848	0.975	0.937	0.756	0.934
YCYH	0.101	0.109	0.056	0.053	0.099	0.098	0.103	0.078	-	0.818	0.834	0.888	0.906
YCWR	0.033	0.016	0.044	0.209	0.017	0.019	0.018	0.047	0.096	-	0.973	0.804	0.954
LYMJ	0.059	0.044	0.035	0.092	0.086	0.054	0.086	0.028	0.042	0.062	-	0.839	0.970
ZZDF	0.182	0.201	0.099	0.118	0.090	0.108	0.139	0.150	0.095	0.137	0.164	-	0.920
TSQC	0.065	0.025	0.069	0.279	0.025	0.015	0.042	0.060	0.151	0.013	0.090	0.165	-

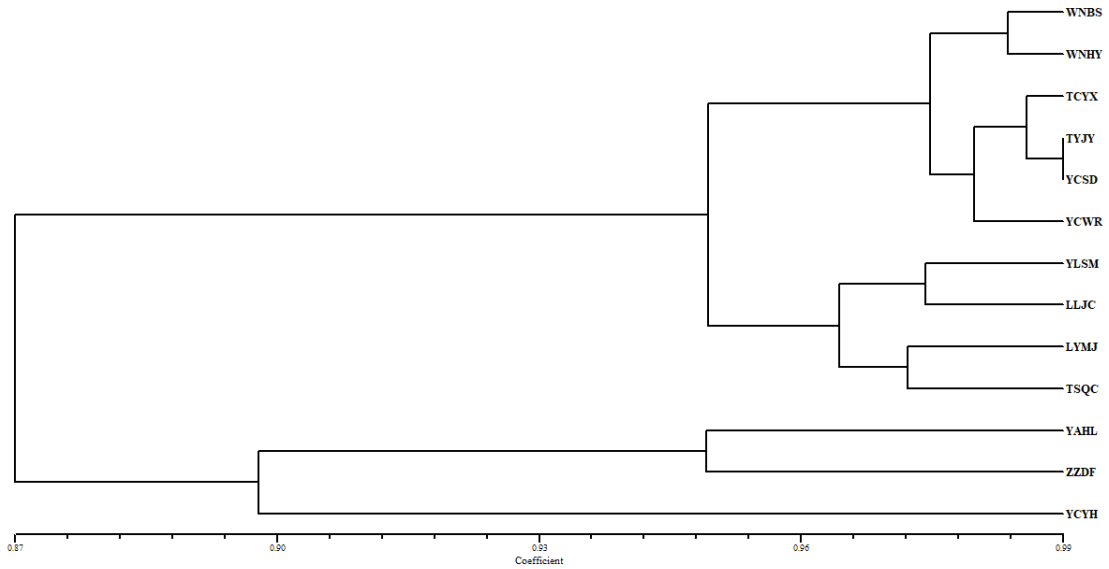

Figure 3. Genetic divergence among 13 populations of *P. orientalis* based on UPGMA clustering analysis.

In the population structure analysis, the highest ΔK value (307.899) (Figure 4A) had a clear peak for the 202 individuals from 13 populations when K = 2, indicating that 2 was the optimal number of genetic clusters and that all the studied plants exhibited admixture from two groups (Pop-red and Pop-green) (Figure 4B). The cluster membership probabilities of each individual from the 13 populations are shown in Figure 4B. Individuals with a proportion above 0.75 were considered pure; otherwise, they were considered admixed [42]. Pop-red contained 109 individuals, with 104 pure and five admixed individuals, while Pop-green contained 93 individuals, with 89 pure and four admixed individuals. Nearly all populations were composed of mixed individuals from two clusters (Pop-red and Pop-green), except the YCSD population, which was entirely composed of individuals from

the red cluster (Pop-red). On the basis of the Q values (the probability that the genomic variation of population No. I material originated from the K population), we also graphed the cluster membership probabilities for each population at K = 2 (Figure 1). The chart shows that there is no obvious geographical distribution among the populations, which is consistent with the results of the Mantel test and cluster analysis.

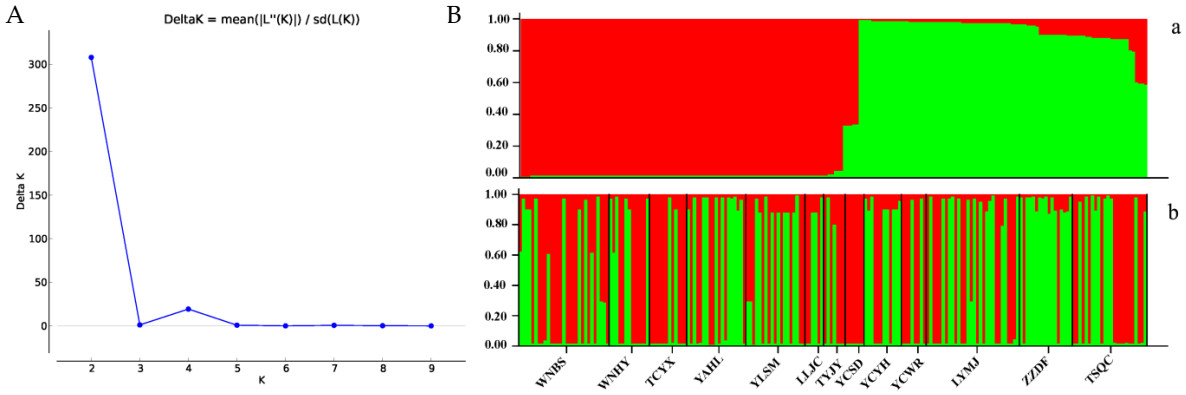

Figure 4. Population genetic structure. (**A**) Estimation of the best subpopulation numbers based on the appropriate K value: the mean Δ*K* values among the 10 runs reached a maximum at *K* = 2. (**B**) Genetic structural plot of 13 *P. orientalis* populations based on structure analysis. a *K* = 2, sorted by Q via STRUCTURE. b *K* = 2, samples displayed in order of collection. Red for Pop-A, and green for Pop-B in a and b.

4. Discussion

Ancient tree populations have specific alleles that are not found in other populations, constituting a set of unique genetic resources [14]. Nowakowska et al. [43] studied monumental Norway spruce trees in the Białowieża Primeval Forest in Europe on the basis of the frequencies of 11 nuclear microsatellite loci. Their results demonstrated that the loss of old spruce trees will cause a decrease in genetic variability in the Norway spruce population within the exceptionally valuable Białowieża Primeval Forest. The ancient trees of *P. orientalis* growing in the middle reaches of the Yellow River are precious plant genetic resources as well as important symbols and products of thousands of years of history and culture in China [8]. Prior studies have noted the importance of habitat loss and fragmentation as two of the greatest threats to the loss of diversity of *P. orientalis* [44,45]. Meanwhile, genetic diversity and genetic structure play an important role in the survival and adaptation of a species [29,46,47]. In our field investigation, we found that almost all of the ancient *P. orientalis* trees in the middle reaches of the Yellow River are scattered sporadically, growing slowly, or even withered. At some sample sites, there were fewer than ten individuals. Compared with other non-ancient tree species, the remaining samples are precious [9,14]. Therefore, a more exhaustive study on the genetic resources and genetic structure of ancient *P. orientalis* trees in the middle reaches of the Yellow River will provide a basis for establishing a germplasm resource bank of ancient *P. orientalis* and exploring the population history of this species in the future.

Xie et al. [13] used 19 isozymes to analyze the genetic structure of 14 natural *P. orientalis* populations and found that most variation occurred among individuals within populations. Wang et al. [24] used AFLP molecular markers to analyze the genetic diversity of 18 *P. orientalis* provenances from China and found that the genetic diversity of the *P. orientalis* provenances was relatively high. Zhu et al. [25] developed nine polymorphic nuclear microsatellite markers for *P. orientalis*, an evergreen tree. Jin et al. [26] used microsatellite loci developed based on *P. orientalis* transcriptome data to evaluate the genetic diversity of 192 elite individuals of *P. orientalis* in the seed orchards of Henan Province, and the obtained data will contribute to the upgrading of *P. orientalis* seed orchards in the future. Chang et al. [9] analyzed the distribution pattern of ancient *P. orientalis* in China based on 13 bioclimatic factors, and used the specific-locus amplified fragment (SLAF) sequencing

method to detect SNPs in 100 germplasm resources. Unfortunately, the study did not consider the genetic diversity and population structure of this collection of *P. orientalis*, which represents the most valuable germplasm resource bank of this species.

Compared with nSSR markers, cpSSR are usually located within noncoding regions [48], with a lower evolutionary rate and higher transferability, which enables us to analyze the genetic diversity level and genetic structure of plant populations at the unique chloroplast genome level [27,49,50]. We used all 202 sample trees from different populations to amplify the polymorphisms of 26 cpSSR markers, and the results showed that only four cpSSR markers (N1, N2, N6 and N33) showed a certain degree of polymorphism. The other 22 cpSSR markers showed monomorphism with very low specificity or were even unable to be amplified. Two possible reasons for this result are the different degrees of genetic variation among the samples and the specificities among different species of Cupressaceae. In addition, in the study of Huang et al. [27], four species of Cupressaceae were selected for the development of polymorphic cpSSR markers, and the *P. orientalis* test materials were selected only from the *P. orientalis* seed orchard in Henan Province.

Numerous studies have shown that 4–6 high-level polymorphic loci are sufficient to assess genetic diversity and gene flow among populations [51–53]. In our study, a total number of 16 alleles were amplified with the use of four cpSSR loci was slightly lower than that reported by Huang et al. [28]. The number of alleles in the ancient tree populations can be driven by many factors, such as the initial genetic composition, the genetic selection, the genetic drift of the population, and direct and indirect human activity. In addition, our study showed that the *P. orientalis* populations in the middle reaches of the Yellow River have moderate genetic diversity, as measured by the *N*e, A_R, and *H*. The high diversity of germplasm resources is essential for the formulation of long-term effective breeding programs [26]. Thus, these trees should be protected as an important genetic resource bank. The results of this study are consistent with those of most current research revealing genetic diversity in ancient *P. orientalis* in Beijing [9,14]. However, compared with the genetic diversity metrics obtained with nSSRs, those obtained with cpSSRs for the ancient populations of *P. orientalis* were lower. The chloroplast genome of conifers has no inverted repeat (IR) region [54], which has a significant impact on the evolution rate of the plastid genome compared with that in angiosperms.

Population structure and genetic relationships are important for establishing the appropriate scale and subunits for conservation management [55]. Population structure is manifested mainly as genetic differentiation among populations [44]. In our study, the genetic differentiation of ancient *P. orientalis* populations across the four cpSSR loci was high (*F*st = 0.153). For long-lived tree species, population history seems to be the main factor responsible for this observation. We also observed similar results in previous studies [56,57]. Furthermore, due to the limited size of ancient *P. orientalis* populations in our study, genetic drift is also an important factor, which might shape the observed genetic differentiation [58,59]. The AMOVA results revealed that only 8% of the total variance were explained by differences among populations, whereas most of the genetic variation of the *P. orientalis* population from the middle reaches of the Yellow River was located among individuals. The most obvious finding that emerged from the UPGMA analysis was that the 13 *P. orientalis* populations could be divided into two groups (with the genetic similarity coefficient between them of 0.89) that did not correspond to their geographic distributions across China. Furthermore, both the Mantel test and population STRUCTURE analysis revealed that there were almost no obvious geographical boundaries for the 13 ancient *P. orientalis* populations. The influence of ancient human activities may be the main reason for this result. This supports the findings of previous research that past human activities had a significant impact on the population structure and genetic diversity of tree populations [60,61]. In ancient times, temples or royal cemeteries were important places for people to pray for blessings or hold large-scale sacrificial events. *P. orientalis* from different regions was separated from its original habitat and established spontaneous stands.

The ancient *P. orientalis* population with a history spanning several thousands of years has an abundance of genetic resources. Our results confirm those of previous studies that cemeteries and ancient temples played an important role in maintaining the genetic diversity and reproductive capacity of *P. orientalis* [14]. With the destruction of habitats and improper management, the populations of *P. orientalis* are gradually shrinking. The genetic consequences of reduced population sizes include increased genetic drift and inbreeding, leading to a loss of genetic variation [56,62]. Therefore, it is urgently important that in situ conservation of ancient populations of *P. orientalis* be carried out, especially populations with high genetic diversity and unique alleles, such as the YAHL population and the TCYX population in Shaanxi Province, the ZZDF population in Henan Province and the TSQC population in Gansu Province.

5. Conclusions

This is the first study to use cpSSR markers to investigate the genetic composition of *P. orientalis* populations in the middle reaches of the Yellow River. The results confirmed that ancient *P. orientalis* in the middle reaches of the Yellow River has a moderate level of genetic diversity and high genetic differentiations among populations. The 13 populations of *P. orientalis* in the middle reaches of the Yellow River were divided into two groups with no significant geographic population structure and extensive admixture. Our study sheds light on the population history of this species and genetic diversity among populations. Moreover, a reasonable plan to protect the precious genetic resources of *P. orientalis* should be established based on our research results to prevent the gradual loss of ancient *P. orientalis* populations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12050592/s1, Table S1: Characteristics of 26 cpSSR loci for PCR amplification in ancient *P. orientalis*; File S1: Raw and Statistical data-Excel. Zip, contains two files: File A: 1-Raw data of 13 ancient *P. orientalis* populations; File B: 2-Statistical data of 13 ancient *P. orientalis* populations; File S2: Pairwise geographic distance among the 13 populations of *P. orientalis*-Excel.

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