forests

MDPI

Article

Genetic Evaluation of Ancient *Platycladus orientalis* L. (Cupressaceae) in the Middle Reaches of the Yellow River Using Nuclear Microsatellite Markers

Bei Cui [1,2], Ping Deng [1,3], Linghong Tian [1,4], Qingqi Wang [1,2], Sheng Zhang [1,2,*] and Zhong Zhao [1,2,*]

1 Key Comprehensive Laboratory of Forestry, College of Forestry, Northwest A & F University, Xianyang 712100, China; cuibei@nwsuaf.edu.cn (B.C.); d_p@nwsuaf.edu.cn (P.D.); tianlinghong@nwsuaf.edu.cn (L.T.); wangqinqi@nwsuaf.edu.cn (Q.W.)
2 Research Center for the Conservation and Breeding Engineering of Ancient Trees, Xianyang 712100, China
3 College of Biology and Pharmacy, Yulin Normal University, Yulin 537000, China
4 Forestry Experimental Bureau of Xiaolongshan, Tianshui 741022, China
* Correspondence: zhangsh@xju.edu.cn (S.Z.); zhaozhlunwen2010@126.com (Z.Z.); Tel.: +86-029-8708-2072 (Z.Z.)

Abstract: As a precious and rare genetic resource, ancient *Platycladus orientalis* L. (Cupressaceae) has important scientific, cultural and historical value. The ancient temples and royal cemeteries in the middle reaches of the Yellow River contain the most concentrated and abundant distributions of ancient *P. orientalis*. Due to unfavorable conditions, the genetic resources of ancient trees are facing great threats and challenges; thus, it is urgent to strengthen the evaluation of the genetic resources of ancient *P. orientalis*. In this study, we used nine polymorphic nuclear simple sequence repeats (nSSRs) to evaluate the genetic resources of 221 individuals in 19 ancient *P. orientalis* populations in the middle reaches of the Yellow River. These selected polymorphic nSSR loci can be used reliably and rapidly in *P. orientalis* genetic studies. Our study showed that the 19 ancient *P. orientalis* populations have high genetic diversity (mean H = 0.562, He = 0.377). High historical gene flow (mean Nm = 1.179) and high genetic differentiation (mean Fst = 0.184) were observed in the ancient *P. orientalis* population. The analysis of molecular variance (AMOVA) showed that higher genetic variation existed within populations (93%) rather than among populations (7%). The genetic structures showed that the 19 populations were divided into two groups. The Mantel test and neighbor-joining (NJ) tree analysis showed no geographical distribution characteristics among populations, which may indicate a history of transplanting by ancient humans. Our research provides a theoretical basis for the protection and utilization of ancient *P. orientalis* germplasm resources and exploration of the historical origin and genetic relationships among the populations of *P. orientalis* on a large scale in the future.

Keywords: *Platycladus orientalis*; ancient trees; nSSR marker; genetic diversity; population structure; conservation

Citation: Cui, B.; Deng, P.; Tian, L.; Wang, Q.; Zhang, S.; Zhao, Z. Genetic Evaluation of Ancient *Platycladus orientalis* L. (Cupressaceae) in the Middle Reaches of the Yellow River Using Nuclear Microsatellite Markers. *Forests* **2021**, *12*, 1616. https://doi.org/10.3390/f12121616

Academic Editor: Claudia Mattioni

Received: 22 October 2021
Accepted: 19 November 2021
Published: 23 November 2021

1. Introduction

Platycladus orientalis L. (Cupressaceae), which belongs to the family Cupressaceae, is a monoecious, evergreen conifer that originates in China and is distributed as far away as Japan, North Korea and Europe [1]. The presence of *P. orientalis* has been historically demonstrated by records of 'The Book of Songs', which was the first anthology of Chinese poetry written approximately 3000 years ago [2]. Moreover, ancient coffins excavated in various parts of China have provided archaeological evidence proving that the extensive cultivation of *P. orientalis* has a history spanning thousands of years [3].

According to the national ancient tree resource survey conducted by the National Greening Committee and the National Forestry Administration in 2005, ancient *P. orientalis* are mainly located at temples, historical sites and cemeteries in the vicinity of the middle reaches of the Yellow River. The middle reaches of the Yellow River are considered one of

the most important birthplaces of Chinese civilization. The area is bounded by Yinshen Mountain to the north and the Qinling Mountains to the south. It extends to the west of the Sun Moon Mountain and east of Taihang Mountain [4]. Temples and royal cemeteries were important places for major ceremonies held by successive dynasties in China [5]. The most representative population is the ancient *P. orientalis* population located at the Yellow Emperor's Mausoleum, Yan'an city, Shaanxi Province [6], which is regarded as an important symbol of the sacrificial culture of various periods in ancient China. Tree ages in this population range from 2000 to 5000 years. As a special gene bank, ancient *P. orientalis* populations are not only rare materials for the study of population genetics but also an important symbol of changes over the history of China [7], with important scientific, cultural and historical value [8].

Due to the existence of various unfavorable factors, such as global climate change, natural disasters, human-made damage, diseases and insect pests, ancient *P. orientalis* populations have weakened and even died gradually [8,9]. The number of *P. orientalis* populations has shown a downward trend, and the species is endangered throughout its distribution range. Conservation efforts are required to maintain the genetic integrity and survival of remnant populations [10,11]. Within this context, it is essential to carry out genetic evaluations of endangered populations [12]. The evaluation of the genetic diversity and genetic structure of plant populations is the core of species genetic evaluation [13,14]. Genetic diversity provides templates for the survival, adaptation and evolution of species [15]. In addition, to unlock the full potential of *P. orientalis* germplasm collections, it is also necessary to understand the genetic relationships among various populations and the amount and distribution of genetic variation within them [16].

For plants, molecular marker technology has become a popular method for studying the population genetics of species [17–20]. Selecting suitable molecular markers is the key to accurately assessing the genetic diversity and genetic structure of endangered species populations [21,22]. Nuclear microsatellite markers (nuclear simple sequence repeats, nSSRs) with biparental inheritance have the advantages of high polymorphism, codominant inheritance and widespread presence throughout the entire genome [23,24]. Thus, nSSRs stand out among the many known molecular markers [25]. Microsatellite markers have been successfully applied in various studies on conifer species, such as core collection sorting, genetic resource assessment and relationship establishment [17].

Previous studies on ancient *P. orientalis* have mainly focused on revealing its longevity mechanism [26,27] and mapping the climatically suitable habitat of *P. orientalis* for introduction and cultivation on a global scale [28,29]. Zhu et al. [9,30] used nine SSR loci to analyze the genetic diversity of 94 300-year-old *P. orientalis* individuals growing near ancient temples or cemeteries in Beijing and confirmed that ancient *P. orientalis* played an important role in maintaining the genetic diversity and reproductive ability of the tree species. Chang et al. [26] used the specific-locus amplified fragment (SLAF)-specific locus sequencing method to detect SNPs in the genomes of 100 individuals that were obtained from 13 ancient *P. orientalis* populations in China. The results suggested that the relationships among many individuals were inconsistent with their geographical locations. The authors speculated that it may have a history of transplantation and cultivation. Although there are several earlier studies on the genetic diversity of ancient *P. orientalis*, large-scale data on the evaluation of the patterns of variation in the precious germplasm resources of ancient *P. orientalis* populations on a large scale remain limited. Despite their high concentration and relative abundance in the middle reaches of the Yellow River basin, no research has been reported on these populations. In this context, our previous study analyzed the genetic diversity and structure of 202 ancient *P. orientalis* individuals from 13 populations using four polymorphic chloroplast simple sequence repeat (cpSSR) loci [8]. Valuable genetic information that is needed to protect the germplasm resources of ancient *P. orientalis* populations is still lacking. In most gymnosperms (such as *P. orientalis*), the chloroplast genome can be used to reveal the paternal lineage, as pollen is inherited from the paternal line [31]. The nuclear genome is inherited from both parents and reflects the

gene flow in seeds and pollen [32]. Therefore, in this study, the patterns of variation that can be detected using parental markers (nSSRs) could provide supplementary information for the protection of ancient *P. orientalis* populations.

In this study, nine polymorphic nSSR loci were used to evaluate the genetic resources of 19 ancient *P. orientalis* populations from different geographic areas in the middle reaches of the Yellow River. The aims of the present study were to (1) provide polymorphic nSSR markers that can be used for genetic research of *P. orientalis* populations; (2) assess the genetic diversity and population structure of the 19 ancient *P. orientalis* populations; and (3) reveal the variability among different populations. The genetic evaluation data obtained from this study, provide an important reference basis for formulating conservation strategies for *P. orientalis* germplasm resources.

2. Materials and Methods

2.1. Sample Collection

A field survey was conducted on extant ancient *P. orientalis* populations from April 2019 to August 2020. The administrative division includes Shaanxi Province, Shanxi Province, Henan Province and Gansu Province. A set of young leaves of 221 individuals from 19 ancient *P. orientalis* populations (covering almost all ancient *P. orientalis* populations in the region) were collected. The healthy leaves collected were immediately transferred to the molecular laboratory and stored at −80 °C. The abbreviations of place names for the populations are shown in Figure 1 and Table 1. The age of all *P. orientalis* individuals was 500–4000 years. Tree age determination was based on historical records provided by the local ancient trees management department [7]. The sample collection was approved by the local ancient tree administration (Letter No. 036-045). These plant samples were identified by Professor Zhong Zhao of Northwest A&F University concerning the Flora of China (percent identity: 100%). The voucher specimens of the sample were kept in the Engineering Research Center of Conservation and Breeding of Ancient and Famous Trees of Northwest A&F University.

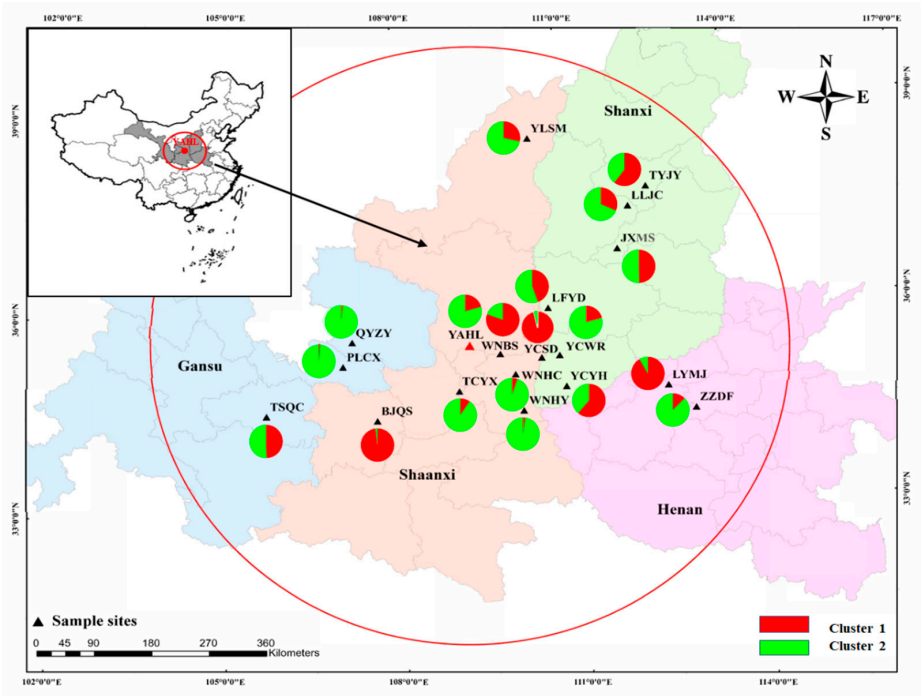

Figure 1. Distribution of the ancient *P. orientalis* population in the middle reaches of the Yellow River. The triangular points represent the locations of the sample collection sites. The four colors in the image represent four different provinces in China. The sector map represents the distribution of dependency coefficients within 19 ancient *P. orientalis* populations (k = 2).

Table 1. Sampling Locations for the 19 ancient *P. orientalis* populations.

Population	Location	Number of Samples	Latitude (N)	Longitude (E)	Elevation (m)	Average Age (Years)
WNBS	Cangjie Temple, Weinan, Shaanxi	29	35°22′	109°41′	816	2314
WNHY	Xiyue Temple, Weinan, Shaanxi	13	34°34′	110°06′	315	1518
WNHC	Sima Temple, Weinan, Shaanxi	4	35°22′	110°24′	396	1075
TCYX	Medicine God Temple, Tongchuan, Shaanxi	12	34°54′	108°59′	717	1238
BJQS	Zhougong Temple, Baoji, Shaanxi	3	34°29′	107°35′	824	967
YAHL	Xuanyuan Temple, Yanan, Shaanxi	19	35°35′	109°16′	865	2711
YLSM	Shenmu County, Yulin, Shaanxi	19	38°40′	110°25′	1172	1668
LLJC	Mt. Gua shan Scenic, Lvliang, Shanxi	6	37°34′	112°07′	884	1200
TYJY	Jinci Temple, Taiyuan, Shanxi	7	37°42′	112°26′	816	1871
JXMS	Mt. Mianshan Scenic, Jiexiu, Shanxi	2	36°56′	111°53′	985	1975
LFYD	Guangsheng Temple, Linfen, Shanxi	4	36°03′	110°29′	460	1600
YCSD	Shun emperor Mausoleum, Yuncheng, Shanxi	6	35°07′	110°54′	398	2417
YCYH	Guan emperor Temple, Yuncheng, Shanxi	12	34°54′	110°50′	325	1308
YCWR	Wanrong County, Yuncheng, Shanxi	8	35°21′	110°48′	770	2250
LYMJ	Guangwu emperor Mausoleum, Luoyang, Henan	30	34°50′	112°35′	124	1500
ZZDF	Zhongyue Temple, Zhengzhou, Henan	17	34°27′	113°04′	350	1435
TSQC	Fuxi Temple, Tianshui, Gansu	24	34°34′	105°42′	1173	929
PLCX	Chongxin County, Pingliang, Gansu	2	35°19′	107°01′	1168	2250
QYZY	Zhenyuan County, Qingyang, Gansu	4	35°40′	107°11′	1235	1900

WNBS: Baishui, Weinan city; WNHY: Huayin, Weinan city; WNHC: Hancheng, Weinan city; TCYX: Yao county, Tongchuan city; BJQS: Qishan, Baoji city; YAHL: Huangling, Yan'an city; YLSM: Shenmu, Yulin city; LLJC: Jiaocheng, Lvliang city; TYJY: Jinyuan, Taiyuan city; JXMS: Mianshan, Jiexiu city; LFYD: Yaodu, Linfen city; YCSD: Shun emperor Mausoleum, Yuncheng city; YCYH: Yanhu, Yuncheng city; YCWR: Wanrong county, Yuncheng city; LYMJ: Mengjin, Luoyang city; ZZDF: Dengfeng, Zhengzhou city; TSQC: Qinchuan, Tianshui city; PLCX: Chongxin, Pingliang city; QYZY: Zhenyuan, Qingyang city.

2.2. Extraction and PCR Amplification

Fresh leaves weighing 0.1 g were ground in liquid nitrogen to a fine powder, and the total genomic DNA was extracted and purified using a New Rapid Plant Genome DNA extraction Kit (BioTeke, Beijing, China). The quality of DNAs was verified by 1% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (BioTek Instruments, Winooski, VT, USA). The DNA samples were diluted to a final concentration of 20 ng/μL for polymerase chain reaction (PCR) [33–35]. According to the maximum number and stability of polymorphic bands, nine polymorphic nSSR loci were selected for ancient *P. orientalis*. Detailed information from this study is provided in Table 2 which includes four polymorphic nSSR loci (M11, M22, M23 and M25) that were selected from the study of Ding et al. [36] (Supplementary Table S1) and five polymorphic nSSR loci (M01, M04, M07, M17 and M19) that were developed based on the obtained root transcriptomic sequencing data of *P. orientalis* [37]. All primers were synthesized by Sangon Biotech (Shanghai), and synthetic primer pairs were verified by PCR using M13-tail technology, in which 5′ M13-tail forward primers were combined with fluorescent labels (FAM, TAMRA, HEX and ROX).

Table 2. Information on nine nSSR loci used for PCR amplification and diversity estimates in the analysis of genetic diversity.

Locus	Primer Sequence (5′–3′)	Repeat Motif	Tm (°C)	Allele Size (*bp*)	*Na*	*Ne*	*I*	*Ho*	*He*	*H*	PIC	*Fst*	*Nm*	FNA
M01	F: ATCCCACCATGAAGCTGTTC R: TTTACCCCCTACAGCCACTG	(TGATA)5	55	155	10	5	1.781	0.484	0.516	0.818	0.792	0.189	1.070	0.176
M04	F: TGAGGGTCACTGGGAGAATC R: CAGCGTAAAATTGCCTGGTT	(AT)6	55	243	11	3	1.385	0.851	0.149	0.663	0.605	0.222	0.875	0.037
M07	F: AGTTTTGGCGGGTGTTACAG R: AGGAGCAAGCCACGAGAATA	(CTC)5	55	280	4	2	1.049	0.742	0.258	0.548	0.520	0.135	1.607	0.020
M11	F: CTTCGTCCCCGATACAAGAG R: CATCATGCCCGATATCATCA	(CAG)6	55	252	6	2	0.872	0.629	0.371	0.431	0.403	0.254	0.734	0.006
M17	F: TCGCAGCTATGAACTCCAAT R: TCGCAGCTATGAACTCCAAT	(TC)6	55	204	5	2	0.707	0.624	0.376	0.460	0.365	0.130	1.671	0.000
M19	F: GCCATCATCCCATTCATCAT R: GAGCTCAGGGGAGAGTTGTG	(CT)6	55	261	8	2	0.816	0.620	0.380	0.431	0.378	0.154	1.373	0.132
M22	F: TGCATTCTATGCGCTTGTTC R: GAATGGCTTGCATGCATCTT	(AC)6	55	276	3	2	0.709	0.453	0.548	0.495	0.377	0.169	1.234	0.087
M23	F: CCTACCTTTTGCTACCACGG R: CTAGGGTGAATCGCCATGTT	(AT)6	55	319	7	2	1.203	0.425	0.575	0.579	0.546	0.227	0.851	0.000
M25	F: AGTGCATGCGTTCATCTCAG R: GCCATCAAACAATCAAGCCT	(TG)6	55	223	11	3	1.410	0.778	0.222	0.635	0.600	0.173	1.196	0.000
Mean	-	-	-	-	7.222	2.556	1.104	0.623	0.377	0.562	0.510	0.184	1.179	-

Na: observed number of alleles per locus; *Ne*: mean number of effective alleles; *I*: Shannon–Wiener index; *Ho*: mean observed heterozygosity; *He*: expected heterozygosity; *H*: Nei's gene diversity index; PIC: polymorphism information content; *F*st: genetic differentiation coefficient; *N*m: gene flow; FNA: frequency of null alleles.

PCR was performed in a 25 μL reaction mixture containing 2.5 μL of 10X Taq Buffer, 20 ng of genomic DNA, 10 pmol of each primer, 2.5 U Taq Plus DNA (Sangon Biotech, Shanghai, China) and 10 μM dNTPs. The cycling parameters were programmed to 1 cycle at 95 °C for 3 min, 10 cycles at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, 35 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The fluorescence-labeled PCR products were determined by capillary electrophoretic separation using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the data were analyzed using GeneMapper v4.0 software (Applied Biosystems) (Supplementary File S1).

2.3. Data Analysis

Microchecker software 2.2.3 was used to estimate the frequency of null alleles (FNA) and scoring errors [38]. POPGENE ver.1.3.2 [39] was used to estimate the number of alleles per locus (*N*a), the number of effective alleles (*N*e), the Shannon–Wiener index (*I*), the observed heterozygosity (*H*o), the expected heterozygosity (*H*e), Nei's gene diversity index (*H*) and gene flow (*N*m), as well as perform the Ewens–Watterson test for neutrality at each locus. PIC CALC v.0.6 [40] was used to calculate the polymorphism information content (PIC) of genes. ARLEQUIN ver. 3.0 [41] was used to calculate the genetic differentiation coefficients (*F*st) of each microsatellite locus in 19 ancient *P. orientalis* populations. Genetic parameters in the population genetic analysis were estimated by GenALEx version 6.5 [42], including the proportion of polymorphic loci (PPB), the fixation index (*F*), the mean number of private alleles (*A*priv) per population, the pairwise population matrix of Nei's genetic distance (GD) and the pairwise population matrix of Nei's genetic identity. In addition, to test for correlations between GDs and geographical distances (in kilometers) among populations, the Mantel matrix correspondence test was performed. The allelic richness (*A*r, based on a randomization of a minimum of two individuals at one locus) was determined via FSTAT 2.9.3 software [43]. The Hardy–Weinberg equilibrium at each population and the linkage disequilibrium of each locus pairwise combination in each population was determined by GENEPOP ver.1.2 [44]. Recent bottleneck events in each population were tested through a stepwise mutation model (SMM) and two-phase models (TPM) in BOTTLENECK ver.1.2 [45]. The significance of these tests was assessed by the one-tailed Wilcoxon signed rank test. The percentage of SMM was set to 70% under the default settings.

The ARLEQUIN ver.3.11 [41] was used to test the significance of the variance components in the analysis of molecular variance (AMOVA) on the basis of 1000 permutations.

The STRUCTURE ver. 2.3.4 was determined to analyze the population structure with a Bayesian clustering approach [46]. Upon setting the correlated allele frequency admixture model, 10 separate runs of the number of clusters in dataset *K* (the number of putative clusters) were implemented for *K* from 1 to 10 at 100,000 Markov Chain Monte Carlo (MCMC) repetitions with a burn-in period of 500,000. To determine the optimal value of *K*, STRUCTURE HARVESTER [47] was used to detect the highest lnP(D) that best fit the dataset based on Δ*K*, as proposed by Evanno et al. [48]. The obtained optimal value of *K* was used to display the summary of ancestry coefficients generated by the STRUCTURE analysis and to generate a pie chart for 19 populations.

The tree topologies of all individuals were constructed based on the neighbor-joining (NJ) method [49] using NTSYS ver. 2.11 (Applied Biostatistics, Port Jefferson, New York, NY, USA) and MEGA 7.0 software [50].

3. Results

3.1. Microsatellite Analysis

In total, 65 different alleles were detected at nine loci across all samples, and the largest number of alleles was detected at M04 and M25 locus with a mean value of 7.222 alleles per locus. The mean *N*e value was 2.556 (2–5). The *I* value ranged from 0.707 (M17) to 1.781 (M01). The PIC is an important indicator measuring the degree of microsatellite DNA polymorphism. Regarding PIC values, five loci (M01, M04, M07, M23 and M25) had highly informative alleles with values greater than 0.5, while four loci (M11, M17, M19 and M22) had less informative alleles, with values less than 0.5. The mean *H*o and *H*e values were 0.623 (0.425–0.851) and 0.377 (0.149–0.575), respectively. The *H* value ranged from 0.431 (M11 and M19) to 0.818 (M01), with a mean value of 0.562. The mean *F*st for each locus was 0.184 (0.130–0.254), revealing a high degree of genetic differentiation among the populations. The *N*m range of each microsatellite locus was 0.734–1.671, with a mean value of 1.179, revealing a high level of historical gene flow among the ancient *P. orientalis* populations. According to the Ewens–Watterson test, all the nSSR loci were selectively neutral (Supplementary Table S2). In summary, the nine loci selected in this study had a high degree of polymorphism in the ancient *P. orientalis* population.

3.2. Population Genetic Diversity and Mantel Test

At the population level, the data showed that the *N*a in each population was 3.105 (1.556–4.778), with an *N*e of 2.107 (1.555–2.727). The mean *I* was 0.787 (0.385–1.081). Except for WNHC and JXMS, the *H*o of the other populations was slightly lower than the *H*e (mean *H*o = 0.374 and *H*e = 0.454). The *F* value ranged from −0.600 (JXMS) to 0.345 (TSQC), indicating that there were different degrees of genetic differentiation among the populations. The mean PPB was 94.210% (56–100%). These parameters suggested a high level of genetic diversity in ancient *P. orientalis*. The values of GDs among the populations ranged from 0.031 (YAHL/YLSM) to 0.721 (JXMS/PLCX). Similarly, the genetic identity varied from 0.486 (JXMS/PLCX) to 0.969 (YAHL/YLSM) (Supplementary Table S3). During the bottleneck analysis, two targeted populations (JXMS and PLCX) were significantly deficient in heterozygotes. This result suggests that there were signs of bottlenecks in these 2 populations, and 17 populations in the study did not exhibit bottlenecks (Table 3).

Table 3. Genetic variability statistics of 9 nSSR loci in 19 ancient *P. orientalis* populations.

Populations	*Na*	*Ne*	PPB%	*Ar*	*Apriv*	*I*	*Ho*	*He*	*F*	*p* Value of Bottleneck	
										TPM	*SMM*
WNBS	4.111	2.146	100	2.058	0.222	0.915	0.345	0.510	0.294	0.590	0.875
WNHY	3.333	2.162	100	2.038	0.111	0.848	0.462	0.473	−0.011	0.674	0.820
WNHC	2.222	1.555	89	1.738	0.000	0.532	0.333	0.319	−0.025	0.994	0.994
TCYX	3.333	2.399	100	2.189	0.111	0.949	0.509	0.555	0.064	0.015	0.125
BJQS	2.111	1.614	78	1.800	0.000	0.526	0.296	0.321	0.044	0.594	0.594
YAHL	3.444	2.095	100	1.996	0.111	0.835	0.345	0.471	0.261	0.545	0.633
YLSM	3.889	2.176	100	2.045	0.111	0.890	0.392	0.493	0.135	0.674	0.918
LLJC	2.889	2.118	100	2.027	0.111	0.778	0.370	0.455	0.264	0.500	0.545
TYJY	2.889	2.257	100	2.111	0.111	0.842	0.333	0.506	0.261	0.150	0.326
JXMS	1.556	1.556	56	1.556	0.000	0.385	0.444	0.278	−0.600	0.005	0.006
LFYD	2.556	2.095	89	2.059	0.000	0.735	0.250	0.441	0.415	0.230	0.230
YCSD	2.889	2.139	100	2.052	0.000	0.784	0.352	0.444	0.128	0.633	0.715
YCYH	3.667	2.145	100	2.081	0.000	0.894	0.343	0.493	0.235	0.545	0.898
YCWR	3.000	2.061	100	1.995	0.000	0.784	0.389	0.445	0.084	0.545	0.715
LYMJ	4.556	2.471	100	2.178	0.444	1.020	0.359	0.543	0.300	0.410	0.820
ZZDF	3.556	2.282	100	2.127	0.111	0.932	0.451	0.522	0.136	0.125	0.500
TSQC	4.778	2.727	100	2.265	0.444	1.081	0.352	0.561	0.345	0.544	0.633
PLCX	2.000	1.881	78	2.000	0.333	0.602	0.389	0.403	0.010	0.004	0.004
QYZY	2.222	2.146	100	1.873	0.000	0.620	0.389	0.396	−0.063	0.367	0.545
Mean	3.105	2.107	94.211	2.010	0.117	0.787	0.374	0.454	0.120	0.429	0.574

PPB: the percentage of polymorphic loci; *A*r: allelic richness; *A*priv: mean number of private alleles; F: fixation index; TPM: two-phase model; SMM: stepwise mutation model.

AMOVA revealed that the total variation was highly significant ($p < 0.001$) within populations (93%), and little genetic variance (7%) occurred among populations, indicating that a higher variation level existed within populations rather than among populations of *P. orientalis* (Table 4).

Table 4. Analysis of molecular variance (AMOVA) for 19 populations of ancient *P. orientalis*.

Source of Variation	df	Sum of Squares	Variance Components	Total Variation (%)	*p* Value
Among populations	18	128.821	0.183	7	<0.001
Among individuals within populations	202	614.351	0.672	26	
Within individuals	221	375.000	1.697	67	
Total	441	1118.172	2.552	100	

df: degrees of freedom.

Mantel test based on pairwise GDs and geographical distances (Supplementary File S2) among populations showed that there were no significant correlations ($R^2 = 0.0273$, $p = 0.130$) (Figure 2).

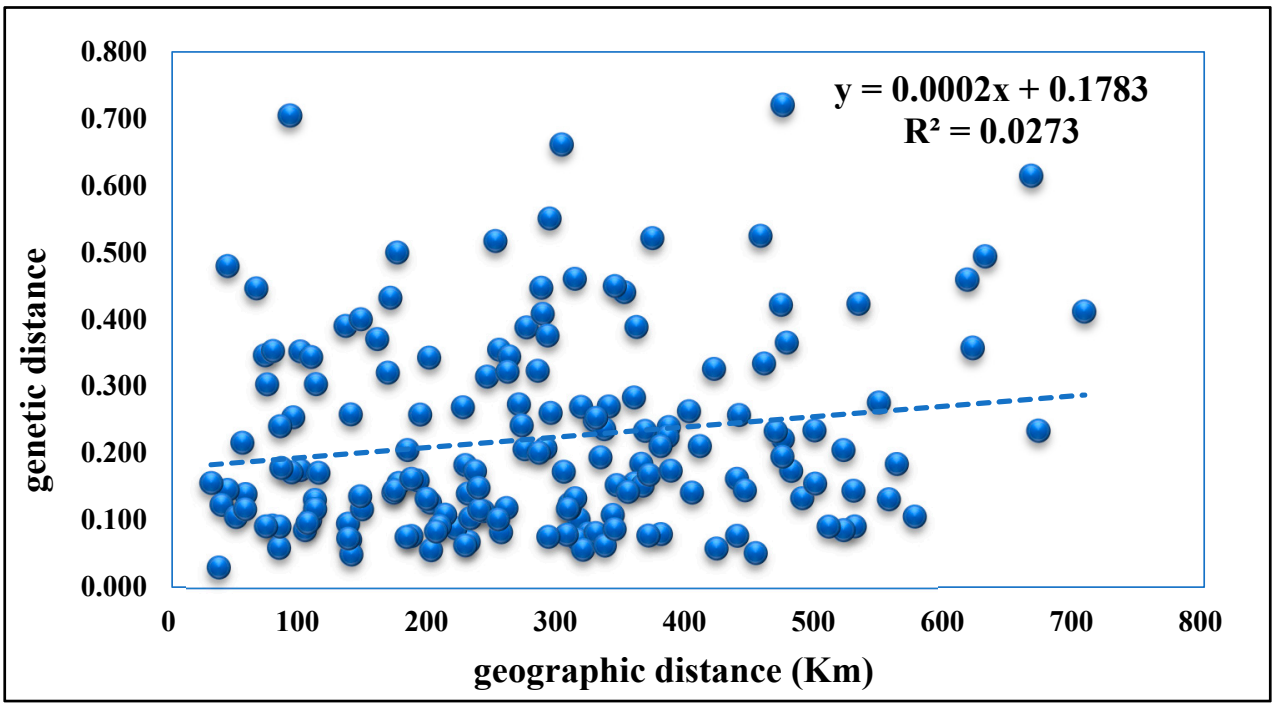

Figure 2. Mantel test between the genetic distances (GDs) and geographical distance of 19 ancient *P. orientalis* populations.

3.3. Population Structure and Genetic Relationships of Individuals

Bayesian analysis of the population structure yielded the highest ΔK value (526.492) with K = 2 (Figure 3B), and the largest lnP(D)-derived ΔK value (Figure 3A). The results showed that the 19 ancient *P. orientalis* populations can be divided into two clusters, which are denoted by red and green clusters (Figure 3C). According to the principle that individuals with a probability greater than 0.75 were considered pure; otherwise, they were considered admixed. The clusters labeled in red and green included 114 (108 pure and 6 admixtures) and 107 (106 pure and 1 admixture) individuals, respectively. The genetic structures of the WNBS, WNHY, WNHC, TCYX, BJQS, YAHL, YCSD, YCYH, LYMJ, ZZDF, PLCX and QYZY populations were relatively independent, but there was a small amount of genetic information mixing with other populations. Among them, the populations included in the red cluster were WNBS, BJQS, YCSD, YCYH and LYMJ,

and the populations included in the green cluster were WNHY, WNHC, TCYX, YAHL, ZZDF, PLCX and QYZY. However, the genetic information of the remaining seven ancient *P. orientalis* populations was relatively extensive and highly mixed. Among them, the genetic information of the YLSM, LLJC, JXMS, LFYD, YCWR and TSQC populations was similar (Figure 1).

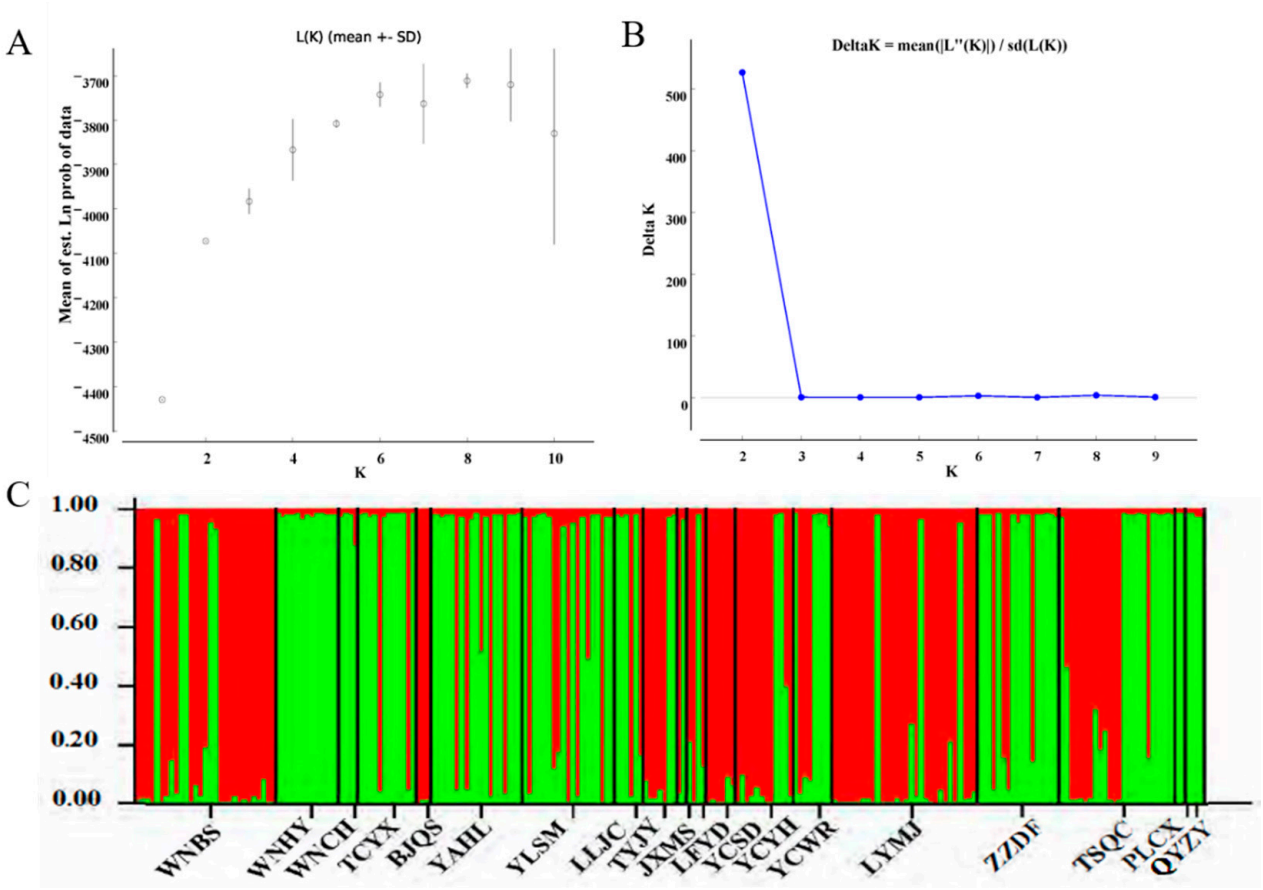

Figure 3. Population structure of 221 ancient *P. orientalis* genotypes based on 9 nSSR loci data: (**A**) estimation of population using mean of estimated lnP(D) with *K* values ranging from one to ten; (**B**) estimation of the number of clusters *K* and the plot of the ad hoc statistic Δ*K* calculated for different *K* values ranging from one to ten following the Δ*K* method proposed by Evanno et al. [48]; (**C**) assignment of 221 ancient *P. orientalis* genotypes to two clusters identified by the program STRUCTURE version 2.3.4.

The NJ tree constructed on the basis of a matrix of Nei's GDs among individuals using NTSYS 2.11 and MEGA 7.0 software showed that the relationship of 221 ancient *P. orientalis* individuals mainly had two branches. The first branch consisted of 69 individuals, and the second-largest branch consisted of 152 individuals. However, the admixed features of individuals of ancient *P. orientalis* among different populations were observed in each branch. The NJ tree was basically consistent with the STRUCTURE analysis, and the relationships among many individuals were inconsistent with their geographic location (Figure 4).

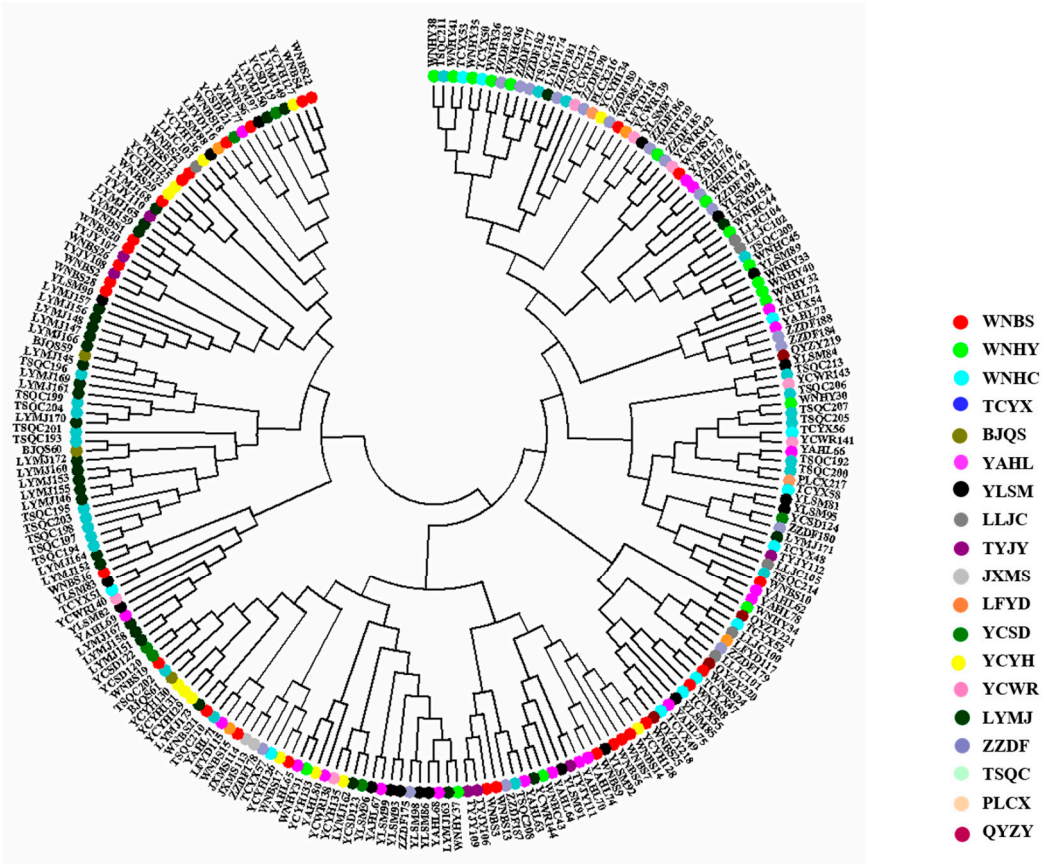

Figure 4. Neighbor-joining (NJ) tree constructed on the basis of Nei's GDs of 221 ancient *P. orientalis* in the middle reaches of the Yellow River based on nine polymorphic nSSR loci.

4. Discussion

Maintaining a high level of genetic diversity is important for the long-term sustainable development of a species [51–53]. As it contains genotypes related to longevity and resistance to harsh environmental conditions, ancient *P. orientalis* is an important species to include in breeding programs [7,27]. Therefore, assessing ancient *P. orientalis* germplasm on a large scale is essential for its efficient utilization.

In this study, nine highly polymorphic nSSR loci were used to assess the genetic diversity and population structure of ancient *P. orientalis*. The increase in the number of polymorphic markers provided more information for the effective protection of ancient *P. orientalis* germplasm resources. Factors such as the variation of the flanking microsatellite sequence or the deletion of large fragment alleles are the main causes of null alleles [54]. The existence of high FNA loci has a significant impact on the accuracy of population genetic analyses [55]. The locus selected in this study has a low FNA, indicating that there was a negligible effect on the accuracy of the genetic analysis [56]. The PIC value is an important indicator reflecting the level of polymorphism information provided by microsatellite markers [57]. Our research shows that the most suitable loci for genetic characterization of the analyzed ancient *P. orientalis* genotypes in terms of genetic resource evaluation and molecular breeding were M01, M04 and M25, which showed PIC values greater than or equal to 0.6.

A high level of genetic diversity of a species will increase resistance to the risk of extinction and worse environmental adaptation, which is an important step in the long-term sustainable deployment of a species [58,59]. Compared with the cpSSR data [8], the nSSR data in this study showed that the 19 ancient *P. orientalis* populations had high genetic diversity (*Na* = 3.105, *Ne* = 2.107, *Ar* = 2.010, *H* = 0.562 and *He* = 0.377). The

difference in the level of genetic diversity may mainly be due to the difference in sample size among populations.

The bottleneck analysis showed signs of bottlenecks in populations JXMS and PLCX. In our previous field survey, many individuals in the six studied populations withered, with no more than five surviving individuals. In particular, we found that only two surviving individuals of ancient *P. orientalis* were observed in the JXMS and PLCX populations. Habitat loss and man-made destruction are the main reasons for the substantial decreases in ancient trees [60,61]. The reduction in the size of these two populations will lead to the bottleneck effect of the ancient *P. orientalis* population and a heightened risk of extinction.

Differences in population genetic structures are an important aspect of the study of genetic diversity and are mainly manifested as genetic differentiation among populations [62]. A relatively continuous geographic scale and homogenous landscape distribution will promote gene flow among populations, resulting in few differences among populations [63]. As we expected, we found a high level of historical gene flow among populations ($Nm = 1.179$). This is closely related to the fact that the studied trees were collected in the center of the plains area where the landscape is homogenous. It is inferred that a large number of *P. orientalis* forests may have appeared in the middle reaches of the Yellow River over a long historical period. However, both the *F*st and AMOVA results in our study showed an overall high level of 19 ancient *P. orientalis* population differentiation with most of the total variation occurring within populations rather than among populations themselves. Factors that affect the level of genetic differentiation in long-lived plant species include the population history, gene flow, genetic drift and ancient human activities [64,65]. High levels of gene flow ($Nm > 1$) could offset genetic drift and prevent genetic differentiation [66]. Gene flow ($Nm = 1.179$) was obviously not the main reason for genetic differentiation among ancient *P. orientalis* populations. *Platycladus orientalis* is a wind-pollinated and cross-pollinated species [5]. We found that the distribution area of the ancient *P. orientalis* populations in the middle reaches of the Yellow River was fragmented in our field investigations and that the geographical distance among populations has far exceeded the distance over which pollen could spread effectively (100 km) [67]. Therefore, population history and habitat fragmentation by human activities may explain the genetic differentiation among ancient *P. orientalis* populations. Furthermore, compared with cpSSRs (*F*st = 0.153) [8], nSSRs showed a higher level of genetic differentiation (*F*st = 0.184). This is consistent with the low substitution rate and lack of cpDNA recombination of plant chloroplast DNA (cpDNA) sequences [68].

Population history plays an important role in the genetic structure of plant populations [61,69]. STRUCTURE analysis based on the Bayesian method revealed that none of the 19 ancient *P. orientalis* populations growing showed 100% dominant clusters, indicating that the ancient *P. orientalis* individuals could not be separated according to their populations. We observed similar results in our study using cpSSR markers [8]. The genetic structures of the WNBS, WNHY, WNHC, TCYX, BJQS, YAHL, YCSD, YCYH, LYMJ, ZZDF, PLCX and QYZY populations. The mixed genetic information was more obvious in the YLSM, LLJC, JXMS, TYJY, LFYD, YCWR and TSQC populations. It was inferred that the original sources of these ancient *P. orientalis* may have been two populations with great differences in genetic structure. This result was almost consistent with the results of the NJ tree analysis of 221 ancient *P. orientalis* individuals. Similarly, we also found no correlation between GDs and geographic distance in the Mantel test, possibly indicating a history of transplantation and cultivation by ancient humans. This result is similar to that of research exploring the phylogeography of ancient *P. orientalis* in China by SLAF sequencing [7]. Past human activities can have a significant impact on the present genetic diversity and population structure of ancient *P. orientalis* [70]. Thousands of years ago, temples and royal mausoleums, especially the Yellow Emperor Mausoleum in Yan'an city, Shaanxi Province, which is an important birthplace of Chinese civilization, attracted people from different regions to hold sacrificial activities and plant trees for blessings and longevity [71,72].

Currently, the populations of ancient *P. orientalis* are suffering severe and rapid declines and are on the verge of extinction. Accurate genetic evaluation of species and the development of suitable protection strategies are important prerequisites for ensuring the long-term evolution of an endangered species [73]. The analyzed ancient *P. orientalis* collection not only has high value for breeding but also provides a good sample for exploring the natural variation of *P. orientalis* in China. Therefore, genetic value together with habitat protection should be combined as a comprehensive method of protection, especially for populations with rich private alleles and allelic richness, such as the populations WNBS, LYMJ TSQC and PLCX. For populations with only a few remaining individuals of ancient *P. orientalis*, immediately carrying out in situ conservation and establishing a germplasm resource library are important measures to effectively protect the genetic diversity of the population.

5. Conclusions

In summary, this study provides the most representative analysis to date of ancient *P. orientalis* using biparentally inherited genomes, and reports the existing levels of genetic diversity, genetic differentiation, and structure within the middle reaches of the Yellow River. Our evaluation of the ancient *P. orientalis* populations revealed high genetic diversity and high genetic differentiation for materials in the entire collection. The 19 ancient *P. orientalis* populations were divided into two groups in the STRUCTURE analysis. The Mantel test and NJ tree analysis of ancient *P. orientalis* showed no geographical distribution characteristics among the populations, possibly indicating that past human activities have had an important impact on the genetic structure and genetic diversity of the ancient *P. orientalis* populations. In addition, the polymorphic nSSR markers selected in our study will be used reliably and rapidly in population genetic studies for *P. orientalis*. Our study provides useful information for the proper management of collections and genetic resource conservation in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12121616/s1, Table S1: Characteristics of 23 nuclear microsatellite loci for PCR amplification in ancient *P. orientalis*. Table S2: The results of overall Ewens–Watterson test of 19 ancient *P. orientalis* populations. Table S3: Genetic identities (above diagonal) and Nei's genetic distances (GDs) (below diagonal) among 19 populations of ancient *P. orientalis*. File S1: Statistical data of 19 ancient *P. orientalis* populations. File S2: Pairwise geographic distances among the 19 populations of ancient *P. orientalis*.

Author Contributions: Conceptualization, B.C. and P.D.; methodology, B.C.; software, B.C.; validation, B.C., L.T. and Q.W.; formal analysis, B.C. and P.D.; investigation, B.C., P.D., L.T. and Q.W.; resources, S.Z. and Z.Z.; data curation, B.C.; writing—original draft preparation, B.C.; writing—review and editing, B.C.; visualization, B.C.; supervision, S.Z. and Z.Z.; project administration, S.Z. and Z.Z.; funding acquisition, Z.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Forestry Industry Research Special Funds for Public Welfare Projects (China), grant number 201404302.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article or supplementary material.

Acknowledgments: We thank the management region of Mausoleum of the Yellow Emperor Mausoleum Administration of Huangling County, which is the authority responsible for field studies. We thank Dinh Duy Vu (Institute of Tropical Ecology, Vietnam-Russia Tropical Centre) for his guidance on the data analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cheng, W.C.; Fu, L.K. Flora of China. Vol. 7. Gymnospermae. *Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita Tomus.* **1978**, *98*, 302–316.
2. Waley, A. The Book of Songs. *J. Am. Oriental. Soc.* **1960**, *80*, 188.
3. Zhang, C. *Research on Funerals in the Qin and Han Dynasties*; Shandong Normal University: Shandong, China, 2010.
4. Hua, S.Z.; Mou, J.Z. Harnessing and Development of the Loess Plateau in the Middle Reaches of the Yellow River. In *Taming the Yellow River: Silt and Floods*; Springer: Berlin/Heidelberg, Germany, 1989; pp. 517–528.
5. Jin, Y.Q.; Ma, Y.Q.; Wang, S.; Hu, X.G.; Huang, L.S.; Li, Y.; Wang, X.R.; Mao, J.F. Genetic evaluation of the breeding population of a valuable reforestation conifer *Platycladus orientalis* (Cupressaceae). *Sci. Rep.* **2016**, *6*, 34821. [CrossRef] [PubMed]
6. Wen, X. The Yellow Emperor's Mausoleum. *China Today* **1994**, *1*, 41–43.
7. Chang, E.M.; Tian, Y.X.; Wang, C.Y.; Deng, N.; Liu, C.X. Exploring the Phylogeography of Ancient *Platycladus Orientalis* in China by Specific-Locus Amplified Fragment Sequencing. *Int. J. Mol. Sci.* **2019**, *20*, 3871. [CrossRef]
8. Cui, B.; Deng, P.; Zhang, S.; Zhao, Z. Genetic Diversity and Population Genetic Structure of Ancient *Platycladus Orientalis* L. (Cupressaceae) in the Middle Reaches of the Yellow River by Chloroplast Microsatellite Markers. *Forests* **2021**, *12*, 592. [CrossRef]
9. Zhu, L.; Lou, A.R. Old-growth *Platycladus orientalis* as a resource for reproductive capacity and genetic diversity. *PLoS ONE* **2013**, *8*, e56489. [CrossRef]
10. Kirkpatrick, J.B.; Gilfedder, L. Maintaining integrity compared with maintaining rare and threatened taxa in remnant bushland in subhumid Tasmania. *Biol. Conserv.* **1995**, *74*, 1–8. [CrossRef]
11. Rodrigues, L.; Cássio, V.D.; Póvoa, O.; Monteiro, A. Low genetic diversity and significant structuring in the endangered Mentha cervina populations and its implications for conservation. *Biochem. Syst. Ecol.* **2013**, *50*, 51–61. [CrossRef]
12. Rajora, O.P.; Mosseler, A. Challenges and opportunities for conservation of forest genetic resources. *Euphytica* **2001**, *118*, 197–212. [CrossRef]
13. Wang, X.; Liu, X.J.; Xing, S.Y.; Kong, Q.Q.; Zhang, Y.H.; Sun, L.M.; Gao, Y. AFLP analysis of genetic diversity and a construction of the core collection of partial ancient *ginkgo* trees in China. *Acta Hortic. Sin.* **2016**, *43*, 249–260.
14. Lupini, A.; Aci, M.M.; Mauceri, A.; Luzzi, G.; Bagnato, S.; Menguzzato, G.; Mercati, F.; Sunseri, F. Genetic diversity in old populations of sessile oak from Calabria assessed by nuclear and chloroplast SSR. *J. Mt. Sci.* **2019**, *16*, 1111–1120. [CrossRef]
15. Booy, G.; Hendriks, R.; Smulders, M.; Groenendael, J.M.; Vosman, B. Genetic diversity and the survival of populations. *Plant Biol.* **2010**, *2*, 379–395. [CrossRef]
16. Forest, F.; Grenyer, R.; Rouget, M.; Davies, T.J.; Cowling, R.M.; Faith, D.P.; Balmford, A.; Manning, J.C.; Proche, E.; Bank, M. Preserving the evolutionary potential of floras in biodiversity hotspots. *Nature* **2007**, *445*, 757–760. [CrossRef]
17. Hou, L.; Cui, Y.H.; Li, X.; Chen, W.; Zhang, Z.Y.; Pang, X.M.; Li, Y.Y. Genetic evaluation of natural populations of the endangered conifer *Thuja koraiensis* using microsatellite markers by restriction-associated DNA sequencing. *Genes* **2018**, *9*, 218. [CrossRef]
18. Mandoulakani, B.A.; Sadigh, P.; Azizi, H.; Piri, Y.; Nasri, S. Comparative assessment of IRAP, REMAP, ISSR, and SSR markers for evaluation of genetic diversity of Alfalfa (*Medicago sativa* L.). *J. Agr. Sci. Technol.* **2015**, *17*, 999–1010.
19. Jiang, W.X.; Bai, T.D.; Dai, H.M.; Wei, Q.; Zhang, W.J.; Ding, Y.L. Microsatellite markers revealed moderate genetic diversity and population differentiation of moso bamboo (*Phyllostachys edulis*)—A primarily asexual reproduction species in China. *Tree Genet. Genomes* **2017**, *13*, 130. [CrossRef]
20. Le, R.J.; Wieczorek, A.M. Molecular systematics and population genetics of biological invasions: Towards a better understanding of invasive species management. *Annals Appl. Biol.* **2008**, *154*, 1–17.
21. Petit, R.J.; Mousadik, A.E.; Pons, O. Identifying populations for conservation on the basis of genetic markers. *Conserv. Biol.* **2010**, *12*, 844–855. [CrossRef]
22. Guizado, S.; Nadeem, M.A.; Ali, F.; Barut, M.; Baloch, F.S. Genetic Diversity and Population Structure of Endangered Rosewood from the Peruvian Amazon Using ISSR Markers. *Acta Amazon.* **2020**, *50*, 204–212. [CrossRef]
23. Varshney, R.K.; Graner, A.; Sorrells, M.E. Genic Microsatellite Markers in Plants: Features and Applications. *Trends Biotechnol.* **2005**, *23*, 48–55. [CrossRef] [PubMed]
24. Zhou, J.; Jie, Y.; Jiang, Y.; Zhong, Y.; Liu, Y.; Zhang, J. Development of simple sequence repeats (SSR) markers of ramie and comparison of SSR and inter-SSR marker systems. *Prog. Nat. Sci.* **2005**, *15*, 137–142.
25. Kalia, R.K.; Rai, M.K.; Kalia, S.; Singh, R.; Dhawan, A. Microsatellite markers: An overview of the recent progress in plants. *Euphytica* **2011**, *177*, 309–334. [CrossRef]
26. Chang, E.M.; Zhang, J.; Yao, X.M.; Tang, S.; Zhao, X.L.; Deng, N.; Shi, S.N.; Liu, J.F.; Jiang, Z.P. De novo characterization of the *Platycladus orientalis* transcriptome and analysis of photosynthesis-related genes during aging. *Forests* **2019**, *10*, 393. [CrossRef]
27. Zhou, Q.Y.; Jiang, Z.H.; Zhang, X.; Zhang, T.; Zhu, H.L.; Cui, B.; Li, Y.M.; Zhao, F.; Zhao, Z. Leaf anatomy and ultrastructure in senescing ancient tree, *Platycladus orientalis* L. (Cupressaceae). *PeerJ* **2019**, *7*, e6766. [CrossRef] [PubMed]
28. Hu, X.G.; Jin, Y.Q.; Wang, X.R.; Mao, J.F.; Li, Y. Predicting impacts of future climate change on the distribution of the widespread conifer *Platycladus orientalis*. *PLoS ONE* **2015**, *10*, e0132326. [CrossRef]
29. Jia, K.H.; Zhao, W.; Maier, P.A.; Hu, X.G.; Jin, Y.Q.; Zhou, S.S.; Jiao, S.Q.; El-Kassaby, Y.A.; Wang, T.; Wang, X.R. Landscape genomics predicts climate change-related genetic offset for the widespread *Platycladus orientalis* (Cupressaceae). *Evol. Appl.* **2020**, *13*, 665–676. [CrossRef]

30. Zhu, L.; Lou, A.R. Development and characterization of nine highly polymorphic microsatellite primers for *Platycladus orientalis* (Cupressaceae). *Am. J. Bot.* **2012**, *99*, e280–e282. [CrossRef]
31. Terauchi, R. A polymorphic microsatellite marker from the tropical tree *Dryobalanops lanceolata* (Dipterocarpaceae). *Genes. Genet. Syst.* **1994**, *69*, 567–576. [CrossRef]
32. Petit, R.; Duminil, J.; Fineschi, S.; Hampe, A.; Vendramin, G.G. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Mol. Ecol.* **2005**, *14*, 689–701. [CrossRef]
33. Vu, D.D.; Bui, T.T.X.; Nguyen, M.T.; Vu, D.G.; Nguyen, M.D.; Bui, V.T.; Huang, X.H.; Zhang, Y. Genetic diversity in two threatened species in Vietnam: *Taxus chinensis* and *Taxus wallichiana*. *J. For. Res.* **2017**, *28*, 265–272. [CrossRef]
34. Ujino, T.; Kawahara, T.; Tsumura, Y.; Nagamitsu, T.; Yoshimaru, H.; Ratnam, W. Development and polymorphism of simple sequence repeat DNA markers for *Shorea curtisii* and other Dipterocarpaceae species. *Heredity* **1998**, *81*, 422–428. [CrossRef] [PubMed]
35. Guo, W.Z.; Wang, W.; Zhou, B.L.; Zhang, T.Z. Cross-species transferability of G. arboreum-derived EST-SSRs in the diploid species of *Gossypium*. *Theor. Appl. Genet.* **2006**, *112*, 1573–1581. [CrossRef]
36. Ding, L.; Gao, D.D.; Han, J.; Liu, Z.H.; Pan, T.; Shi, L.Y.; Sun, X.J.; Sun, Z.L.; Wang, H.; Wu, B. Microsatellite records for volume 10, issue 3. *Conserv. Genet. Resour.* **2018**, *10*, 587–596. [CrossRef]
37. Zhang, S.; Zhang, L.L.; Zhao, Z.; Li, Y.M.; Zhou, K.K.; Su, L.; Zhou, Q.Y. Root transcriptome sequencing and differentially expressed drought-responsive genes in the *Platycladus orientalis* (L.). *Tree Genet. Genomes* **2016**, *12*, 79. [CrossRef]
38. Van Oosterhout, C.; Hutchinson, W.F.; Wills, D.P.M.; Shipley, P. Micro-checker: Software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **2004**, *4*, 535–538. [CrossRef]
39. Yeh, F.C.; Yang, R.C.; Boyle, T. *POPGENE Version 1.32 Microsoft Windows-Based Freeware for Populations Genetic Analysis*; University of Alberta: Edmonton, AB, Canada, 1999.
40. Nagy, S.; Poczai, P.; Cernák, I.; Gorji, A.M.; Hegedűs, G.; Taller, J. PICcalc: An online program to calculate polymorphic information content for molecular genetic studies. *Biochem. Gents.* **2012**, *50*, 670–672. [CrossRef] [PubMed]
41. Excoffier, L.; Laval, G.; Schneider, S. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinform.* **2005**, *1*, 47–50. [CrossRef]
42. Peakall, R.; Smouse, P.E. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **2006**, *6*, 288–295. [CrossRef]
43. Goudet, J. FSTAT (version 1.2): A computer program to calculate F-statistics. *J. Hered.* **1995**, *86*, 485–486. [CrossRef]
44. Raymond, M.; Rousset, F. GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. *J. Hered.* **1995**, *86*, 248–249. [CrossRef]
45. Cristescu, R.; Sherwin, W.B.; Handasyde, K.; Cahill, V.; Cooper, D.W. Detecting bottlenecks using BOTTLENECK 1.2.02 in wild populations: The importance of the microsatellite structure. *Conserv. Genet.* **2010**, *11*, 1043–1049. [CrossRef]
46. Pritchard, J.K.; Stephens, M.J.; Donnelly, P.J. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [CrossRef] [PubMed]
47. Earl, D.A.; Vonholdt, B.M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
48. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef] [PubMed]
49. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425.
50. Sudhir, K.; Glen, S.; Koichiro, T. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, 1870.
51. Ofori, D.A. Genetic diversity and its implications for the management and conservation of Milicia species. *Estud. Const.* **2001**, *12*, 29–54.
52. Schfer, D.; Vincent, H.; Fischer, M.; Kempel, A. The importance of genetic diversity for the translocation of eight threatened plant species into the wild. *Glob. Ecol. Conserv.* **2020**, *24*, e01240. [CrossRef]
53. Dervishi, A.; Jake, J.; Ismaili, H.; Javornik, B.; Tajner, N. Genetic Structure and Core Collection of Olive Germplasm from Albania Revealed by Microsatellite Markers. *Genes* **2021**, *12*, 256. [CrossRef]
54. Oddou-Muratorio, S.; Vendramin, G.G.; Buiteveld, J.; Fady, B. Population estimators or progeny tests: What is the best method to assess null allele frequencies at SSR loci? *Conserv. Genet.* **2009**, *10*, 1343–1347. [CrossRef]
55. Wang, Y.S.; Xing, S.Y.; Tang, H.X.; Feng, D.Q. Genetic diversity of *Platycladus orientalis* provenances. *Sci. Silva. Sin.* **2011**, *47*, 91–96.
56. Chapuis, M.P.; Estoup, A. Microsatellite null alleles and estimation of population differentiation. *Mol. Biol. Evol.* **2007**, *24*, 621–631. [CrossRef] [PubMed]
57. Liu, F.M.; Zhang, N.N.; Liu, X.J.; Yang, Z.J.; Jia, H.Y.; Xu, D.P. Genetic diversity and population structure analysis of *Dalbergia Odorifera* germplasm and development of a core collection using microsatellite markers. *Genes* **2019**, *10*, 281. [CrossRef]
58. Mandel, J.R.; Milton, E.F.; Donovan, L.A.; Knapp, S.J.; Burke, J.M. Genetic diversity and population structure in the rare Algodones sunflower (*Helianthus niveus* ssp. tephrodes). *Conserv. Genet.* **2013**, *14*, 31–40. [CrossRef]
59. Wu, Q.C.; Zang, F.Q.; Ma, Y.; Zheng, Y.Q.; Zang, D.K. Analysis of genetic diversity and population structure in endangered *Populus wulianensis* based on 18 newly developed EST-SSR markers. *Glob. Ecol. Conserv.* **2020**, *24*, e01329. [CrossRef]

60. Androsiuk, B.P.; Shimono, A.; Westin, J.; Lindgren, D.; Fries, A.; Wang, X.R. Genetic status of Norway spruce (*Picea abies*) breeding populations for northern Sweden. *Silvae Genet.* **2013**, *62*, 127–136. [CrossRef]
61. Reisch, C.; Mayer, F.; Rüther, C.; Nelle, O. Forest history affects genetic diversity—Molecular variation of *Dryopteris dilatata* (Dryopteridaceae) in ancient and recent forests. *Nord. J. Bot.* **2010**, *25*, 366–371. [CrossRef]
62. Ravishankar, H. Effect of planting density on growth parameters and fruit yield in guava (*Psidium guajava* L.) cv. Allahabad Safeda cultivated under mild humid conditions of Coorg. *J. Hortic. Sci.* **2008**, *3*, 123–126.
63. Slatkin, M. Gene flow and the geographic structure of natural populations: Slatkin, Montgomery. *Science* **1987**, *236*, 787–792. [CrossRef]
64. Hamrick, J.L.; Godt, M. Effects of life history traits on genetic diversity in plant species. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **1996**, *351*, 1291–1298.
65. Xiang, X.Y.; Zhang, Z.X.; Duan, R.Y.; Zhang, X.P.; Wu, G.L. Genetic diversity and structure of Pinus dabeshanensis revealed by expressed sequence tag-simple sequence repeat (EST-SSR) markers. *Biochem. Systemat. Ecol.* **2015**, *61*, 70–77. [CrossRef]
66. Wright, S. Evolution in mendelian populations. *Bull. Math. Biol.* **1990**, *52*, 241–295. [CrossRef] [PubMed]
67. Kremer, A.; Ronce, O.; Robledo-Arnuncio, J.J.; Guillaume, F.; Bohrer, G. Long-distance gene flow and adaptation of forest trees to rapid climate change. *Ecol. Lett.* **2012**, *15*, 378–392. [CrossRef] [PubMed]
68. Wolfe, K.H.; Li, W.H.; Sharp, P.M. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proc. Natl. Sci. Acad. USA* **1987**, *84*, 9054–9058. [CrossRef]
69. Lauterbach, D.; Ristow, M.; Gemeinholzer, B. Genetic population structure, fitness variation and the importance of population history in remnant populations of the endangered plant *Silene chlorantha* (Willd.) Ehrh. (Caryophyllaceae). *Plant Biol.* **2011**, *13*, 667–777. [CrossRef]
70. Bradshaw, R.H. Past anthropogenic influence on European forests and some possible genetic consequences. *Forest. Ecol. Manag.* **2004**, *197*, 203–212. [CrossRef]
71. Yu, H.X. Sacrificial Rites in the Ancesters' Temples and Their Cultural Features. *J. South* **2001**, *21*, 61–65.
72. Lee, Y.S. Structure and Change of Urban Sacrificial Centers in Late Imperial China: Confucian Temples and City-God Temples. *J. Ming-Qing Hist. Stud.* **2002**, *17*, 1–38. [CrossRef]
73. Margules, C.R.; Pressey, R.L. Systematic conservation planning. *Nature* **2000**, *405*, 243–253. [CrossRef]