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Population Genetic Diversity and Structure of Ancient Tree Populations of *Cryptomeria japonica* var. *sinensis* Based on RAD-seq Data

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Abstract: Research highlights: Our study is the first to explore the genetic composition of ancient *Cryptomeria* trees across a distribution range in China. Background and objectives: *Cryptomeria japonica* var. *sinensis* is a native forest species of China; it is widely planted in the south of the country to create forests and for wood production. Unlike *Cryptomeria* in Japan, genetic Chinese *Cryptomeria* has seldom been studied, although there is ample evidence of its great ecological and economic value. Materials and methods: Because of overcutting, natural populations are rare in the wild. In this study, we investigated seven ancient tree populations to explore the genetic composition of Chinese *Cryptomeria* through ddRAD-seq technology. Results: The results reveal a lower genetic variation but higher genetic differentiation (Ho = 0.143, F_{ST} = 0.1204) than Japanese *Cryptomeria* (Ho = 0.245, F_{ST} = 0.0455). The 86% within-population variation is based on an analysis of molecular variance (AMOVA). Significant excess heterozygosity was detected in three populations and some outlier loci were found; these were considered to be the consequence of selection or chance. Structure analysis and dendrogram construction divided the seven ancient tree populations into four groups corresponding to the geographical provinces in which the populations are located, but there was no obvious correlation between genetic distance and geographic distance. A demographic history analysis conducted by a Stairway Plot showed that the effective population size of Chinese *Cryptomeria* had experienced a continuing decline from the mid-Pleistocene to the present. Our findings suggest that the strong genetic drift caused by climate fluctuation and intense anthropogenic disturbance together contributed to the current low diversity and structure. Considering the species' unfavorable conservation status, strategies are urgently required to preserve the remaining genetic resources.

Keywords: *Cryptomeria japonica* var. *sinensis*; genetic diversity; population structure; demographic history; SNP; RAD-seq; ancient tree; conservation

1. Introduction

Cryptomeria (Cupressaceae) is a relic genus that was widely distributed throughout Eurasia during the Cenozoic era [1]. Today, there is only one extant species, *Cryptomeria japonica* (Linn. f.) D. Don, which has three recognized varieties. Two Japanese varieties, var. *japonica* and var. *radicans*, are found in the moist temperate region from Aomori Prefecture to Yakushima Island on the Japanese

archipelago [2]; var. *japonica* mainly occurs on the Pacific Ocean side and var. *radicans* mainly on the Sea of Japan side. These two varieties are present in 44% of all Japanese planted forests [3], and the species is known as "the national tree" of Japan. The third variety, var. *sinensis*, is limited to southern China, with a few natural occurrences in Fujian (Nanping), Jiangxi (Lushan mountain), and Zhejiang (Tianmu mountain) provinces. However, these wild forests are hard to distinguish from an enormous number of artificial stands [4]. Planted forests in China are not as common as in Japan, but still play an important role in forestry in the south of the country. This species has been long cultivated in China, and the earliest historical document can be traced back to 1279, referring to the Tianmu mountains area. Some ancient trees are still well preserved in villages such as Fengshui forest, and they are supposed to bring fortune and happiness. Since the founding of modern China, the government has launched a series of greening campaigns. Because var. *sinensis* is a common and popular species, it has been widely planted throughout southern China for afforestation and to exploit as timber in the future. Recently, studies have revealed that Chinese *Cryptomeria* (*Cryptomeria japonica* var. *sinensis* Miquel) forests have great ecological benefits with respect to soil properties, water infiltration, and biodiversity, and also have substantial economic benefits in terms of wood production. However, we know little about its population genetics.

Knowledge of population genetic diversity and structure is of fundamental importance for conifer conservation and breeding programs [5]. Chen et al. [6] investigated the demographic structure of Chinese *Cryptomeria* on Tianmu mountain using microsatellites markers. This area contains the most famous and largest ancient tree population. Luo et al. [7] examined the genetic diversity of 96 clones from 12 provenances in seed orchards, using 26 polymorphic microsatellite loci. However, a large-scale study on natural or artificial resources of Chinese *Cryptomeria* has yet to be conducted.

Single-nucleotide polymorphisms (SNP) have proved to be the most abundant form of variation within a species at the genome level and can provide detailed insight into the genetic basis of a population [8]. Combined with Next-Generation Sequencing (NGS) technology, SNP markers are having substantial impacts on population genetics as well as plant breeding [9,10]. Among large-scale sequencing-based approaches, Restriction-site Associated DNA sequencing (RAD-seq) technology has been shown to be cost-effective for generating genome-wide markers for a large number of samples simultaneously [11–14]. This approach has great advantages, including generating a large quantity of data across the genome, having reasonable costs, needing simpler procedures for library construction, needing a short duration of experiment, having no requirements for a reference genome, and having a well-developed pipeline for data treatment and analysis [15,16].

In the present study, we employed ddRAD-seq (double digest RAD-seq) technology, based on 122 samples from seven ancient tree populations in China, to (1) evaluate the level of genetic diversity, (2) explore the genetic structure among current ancient tree populations, and (3) estimate the demographic history. We also used six natural Japanese populations to (4) compare the genetic diversity between Japanese and Chinese *Cryptomeria*. From this analysis, we hope to gain insight into the status of genetic resources of *C. japonica* var. *sinensis*, and put forward some suggestions for conservation strategies.

2. Materials and Methods

2.1. Population Sampling

We investigated the sites where natural forests supposedly occurred in China, according to previous studies [17]. Unfortunately, most forests have been exposed to severe disturbance as a result of human activities, and the species is now found in patches in villages and national forest parks. To avoid materials from unknown sources, only ancient trees with a DBH (diameter at breast height) greater than 100 cm were selected for this study. A total of 122 individuals from seven populations were collected, covering all the recorded natural forest sites. For each population, needles were collected from 10 to 22 mature trees from each population (Figure 1). The name, geographic location, altitude,

and sample size for each population are listed in Table 1. We also used six natural populations of *C. japonica* from Japan, covering the whole natural distribution range and the most important forests, in order to compare the genetic diversity with Chinese populations [18] (Table S1).

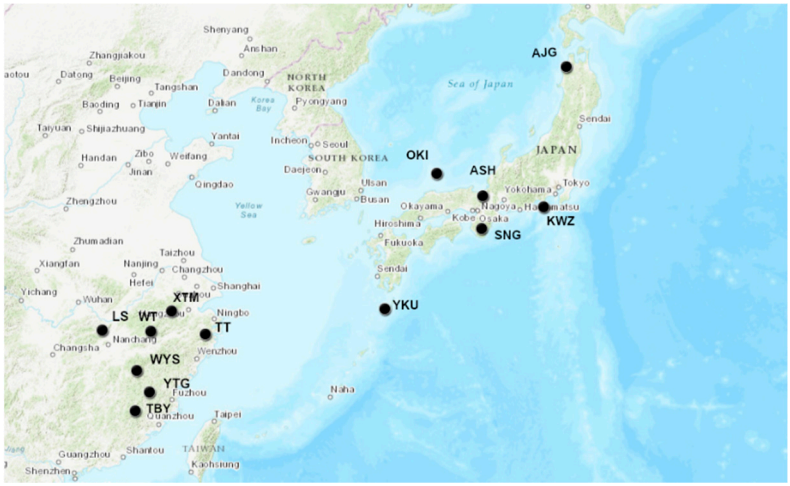

Figure 1. Sampling locations of seven populations of *C. japonica* var. *chinensis* in China and six populations of *C. japonica* in Japan.

Table 1. Geographic locations and sample size (*n*) of the seven Chinese *Cryptomeria* populations.

Populations		Longitude (E)	Latitude (N)	Altitude (m)	*n*
Province	**location**				
Jiangxi	Lushan (LS)	115.5801	29.3259	895 m	22
Anhui	Huangshan (WT)	118.1600	29.2743	436 m	17
Fujian	Tianbaoyan (TBY)	117.3044	25.5756	1132 m	17
	Nanping (YTG)	118.0848	26.4418	846 m	10
	Wuyishan (WYS)	117.4136	27.4535	892 m	18
Zhejiang	Tiantaishan (TT)	121.0541	29.1458	897 m	17
	Tianmushan (XTM)	119.2608	30.2017	1089 m	21
Total					122

2.2. DNA Extraction and RAD Sequencing

The total genomic DNA was extracted from fresh needles using a modified CTAB (cetyltrimethylammonium bromide) method [19]. Purified DNA was digested with *Pst*I and *Sph*I, ligated with Y-shaped adaptors, and amplified by PCR with KAPA HiFi polymerase (Kapa Biosystems, Wilmington, USA). After PCR amplification with adapter-specific primer pairs (Access Array Barcode Library for Illumina, Fluidigm, South San Francisco, USA), an equal amount of DNA from each sample was mixed and size-selected with the BluePippin agarose gel (Sage Science, Beverly, MA, USA). Approximately 450 bp library fragments were retrieved. Further details of the library preparation method are given by Ueno et al. [20]. The quality of the library was checked using a 2100 Bioanalyzer with a high-sensitivity DNA chip (Agilent Technologies, Waldbronn, Germany) and finally sequenced using an Illumina Hi-Seq X to generate paired-end reads 150 bp long.

2.3. SNP Calling and Filtering

SNPs were called by dDocent (version 2.17) [21], which is a pipeline containing a series of statistical tools. Because no reference genome was available for *Cryptomeria japonica*, a reference was constructed using the dDocent de novo assembly and optimized utilizing the reference optimization steps provided on the dDocent assembly tutorial. We followed the default settings of dDocent for mapping and SNP calling, and the resulting vcf-file was used for filtering by VCFtools [22] in the dDocent environment.

Specifically, for the first filtering sites with >50% missing data across all individuals and sites with a minor allele count <3 and quality value <30 were excluded. Secondly, we removed individuals with >10% missing data, and further filtered SNPs with the following criteria: mean depth ≥20, the proportion of missing data >95%, a Minor Allele Frequency (MAF) ≥0.05. In addition, we removed sites that deviated greatly from the Hardy–Weinberg equilibrium within populations and thinned sites that were tightly linked at <1 kb intervals using VCFtools. For the stairway plot analysis, we included all SNPs located less than 1 kb apart with no MAF filtering.

2.4. Genetic Diversity and Genetic Differentiation

Neutral loci tests of the genotype data for all populations and markers were conducted using BayeScan [23] and the "Fsthet" package [24]. Genetic indices, such as the number of alleles (*N*a), number of effective alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and Fixation index (F_{IS}) were estimated within each population using GenALEx 6.502 [25]. HP-Rare v.1.1 [26] was employed to calculate the allelic richness (*Ar*) and private allelic richness (*pAr*) with a minimum sample size of eight.

To examine differences between populations, genetic differentiation coefficients were calculated following Meirmans's method [27] in GenALEx 6.502 and Weir and Cockerham's method [28] in the R "hierfstat" package. Hierarchical analysis of molecular Variance (AMOVA) [29] was performed using GenALEx 6.502. Gene flows (*Nm*) based on F_{ST} and private alleles were calculated using GenALEx 6.502 and GENEPOP v4.3 [30]. Genetic distance matrices of pairwise population F_{ST} and pairwise population gene flow were also calculated in GenALEx 6.502.

2.5. Population Structure

We inferred the most likely number of genetic clusters using STRUCTURE v.2.3.4 [31]: 10 independent runs were performed at K = 1–10 with a burn-in period of 50,000 iterations and 100,000 MCMC repetitions, using no prior information, under the admixture and correlated allele frequencies models. The outputs of STRUCTURE were analyzed in Structure Harvester [32] to determine the most likely number of clusters according to ΔK [33] and mean LnP(K) [31]. CLUMPP v.1.1 [34] was then used to calculate the average pairwise similarity of runs based on the Greedy method, and finally the outputs of CLUMPP were visualized in Distruct v.1.1 [35].

The pairwise F_{ST} distance matrix was used to generate a dendrogram in MEGA v.7.0.26 [36] with the neighbor-joining method [37] and a network in SplitsTree v.4.14.8 [38] with the neighbor-net method [39]. In addition, we tested the correlations between genetic distance and geographic distance by correlating $F_{ST}/(1 - F_{ST})$ with geographic distance (km) in a Mantel test with 9999 permutations, as implemented in GenAIEx.

In order to assess the relationship structure within each population, we employed the COANCESTRY software to calculate the pairwise relatedness for all individuals with Wang estimator [40]. These relatedness coefficients(r) vary from 0 to 1, and a value of 0.5 indicates that individuals are first-order relatives, such as parents–offspring or full siblings. A value of 0.25 indicates second-order relatedness, such as half sibling, grandparents–grandchildren, avuncular, or double first cousins.

2.6. Demographic History

The variation in effective population size (*Ne*) over time was inferred using the composite likelihood approach with a multi-epoch model implemented in the Stairway plot software [41]. This method evaluates the difference between the observed site frequency spectrum (SFS) and its expectation under a specific demographic history [42]. The software was run using the two-epoch method, following the recommended 67% of sites for training and 200 bootstraps on the folded SFS. We excluded singletons from the estimation to minimize errors due to genotype calling. We assumed a mutation rate per generation of 1.50×10^{-9} based on a previous study by Moriguchi et al. [43]. *Cryptomeria japonica* is a

long-lived species, and there are many ancient trees older than 1000 years in the wild. Suzuki and Susukida [44] estimated that 100 to 300 years were necessary for the regeneration of the natural forest on Yakushima Island, thus we set the generation time to 150, 200, or 300 years in different runs.

3. Results

3.1. Genetic Diversity and Differentiation

A total of 922 SNPs were obtained and used to assess the genetic diversity of seven populations of Chinese *Cryptomeria*. The SNP data were deposited in Dryad (DOI: https://doi.org/10.5061/dryad.nk98sf7rf); the loci did not depart from neutrality according to Bayescan. The number of alleles in each population ranged from 1.550 to 1.939, with an average of 1.789. The observed heterozygosity and expected heterozygosity were in the ranges *Ho* = 0.187 to 0.307 and *He* = 0.174 to 0.316, with an average of *Ho* = 0.269 and *He* = 0.253, respectively. The fixation index (F_{IS}) for populations LS and WT indicated significant inbreeding, while populations YTG, WYS, and XTM exhibited significant excess heterozygosity. The allelic richness varied from 1.42 in population WT to 1.77 in population LS. Notably, population LS had a relatively higher private allelic richness of 0.03 and the other populations were lower (*pAr* = 0.01 or 0). Overall, the highest diversity within a population was in LS and the lowest was in WT (Table 2).

Table 2. Genetic diversity indices of the seven populations of *C. japonica* var. *sinensis* based on 922 loci.

Pop		*N*	*Na*	*Ne*	*Ho*	*He*	*F*	*Ar*	*pAr*
LS	Mean	16	1.939	1.532	0.307	0.316	0.023 *	1.77	0.03
	SE		0.008	0.011	0.006	0.005	0.009		
WT	Mean	11	1.550	1.309	0.187	0.174	0.023 *	1.42	0.00
	SE		0.016	0.013	0.009	0.007	0.015		
TBY	Mean	15	1.900	1.458	0.286	0.277	−0.023	1.70	0.01
	SE		0.010	0.011	0.006	0.005	0.009		
YTG	Mean	8	1.614	1.351	0.239	0.209	−0.123 *	1.53	0.00
	SE		0.016	0.012	0.008	0.006	0.011		
WYS	Mean	11	1.871	1.466	0.306	0.279	−0.087 *	1.71	0.00
	SE		0.011	0.011	0.007	0.006	0.009		
TT	Mean	17	1.838	1.429	0.266	0.257	−0.033	1.64	0.01
	SE		0.012	0.011	0.007	0.006	0.08		
XTM	Mean	15	1.807	1.427	0.279	0.257	−0.082 *	1.64	0.01
	SE		0.013	0.011	0.007	0.006	0.007		
Total	Mean	13.3	1.789	1.424	0.267	0.253	−0.042	1.63	0.0086
	SE		0.005	0.004	0.003	0.002	0.004		

N: sample size; *Na*: No. alleles; *Ne*: No. effective alleles; *Ho*: observed Heterozygosity; *He*: expected Heterozygosity; F_{IS}: Fixation index; *Ar*: Allelic richness; *pAr*: private Allelic richness. * Significance (>confidence interval 99%).

The overall population differentiation coefficient (F_{ST}) among all Chinese populations for the 922 loci was 0.119 (Meirmans's method) and 0.134 (Weir and Cockerham's method). Correspondingly, the gene flows (*Nm*) based on F_{ST} were 1.858 and 1.618 respectively, while the *Nm* based on private allele frequency was only 0.0811. The AMOVA results (Table 3) showed that the proportion of variation among populations was 12%; among individuals, it was 12%' and within individual, it was 76%. The majority of variation occurred within individuals. The pairwise F_{ST} and pairwise *Nm* for each population in this study suggested significant differentiation in every pair of populations, and the gene flow varied widely between different pairs. The greatest gene flow occurred between populations TBY and WYS (7.846), and the lowest occurred between populations YTG and WT (0.696) (Table 4).

Table 3. Analysis of Molecular Variance (AMOVA) for the seven populations of *C. japonica* var. *sinensis.*

Source	df	SS	MS	Est. Var.	%
Among Pops	6	4033.190	672.198	18.885	12%
Among Indiv	89	14,142.633	158.906	18.552	12%
Within Indiv	96	11,693.000	121.802	121.802	76%
Total	191	29,868.823		159.239	100%
F_{ST}	0.119				
Nm	1.858				

Table 4. Pairwise *Nm* (above the diagonal) and pairwise genetic differentiation (F_{ST}) (below the diagonal) of the seven populations of *C. japonica* var. *sinensis.*

	LS	WT	TBY	YTG	WYS	TT	XTM
LS	-	1.014	2.460	1.251	2.630	1.533	1.963
WT	0.198 ***	-	1.296	0.696	1.293	0.995	1.047
TBY	0.092 ***	0.162 ***	-	1.927	7.846	2.902	3.259
YTG	0.167 ***	0.264 ***	0.115 ***	-	2.287	1.282	1.490
WYS	0.087 ***	0.162 ***	0.031 **	0.099 ***	-	2.780	4.953
TT	0.140 ***	0.201 ***	0.079 ***	0.163 ***	0.083 ***	-	4.788
XTM	0.113 ***	0.193 ***	0.071 ***	0.144 ***	0.048 **	0.050 ***	-

Significance levels: ** $p < 0.01$, *** $p < 0.001$.

Six Japanese populations were also sequenced and merged with the Chinese populations into a CHN-JPN dataset. We obtained 183 SNPs from this dataset to compare the genetic diversity and structure between the Chinese and Japanese groups. Japanese populations (*Na* = 1.842, *Ne* = 1.393, *He* = 0.267, *Ho* = 0.245) showed higher genetic diversity than the Chinese populations (*Na* = 1.511, *Ne* = 1.232, *He* = 0.150, *Ho* = 0.143). Interestingly, the highest diversity population in China, LS, harbors a very similar level of diversity to the Japanese populations (Table S2). In addition, a higher genetic differentiation among Chinese populations (F_{ST} = 0.1204) was detected than among Japanese populations (F_{ST} = 0.0455).

3.2. Genetic Structure

We explored the genetic structure of the Chinese and Japanese populations based on 183 SNPs. Two groups were clearly identified, but the population LS was placed with the Japanese group in the network (Figure S1).

We subsequently analyzed the genetic structure within Chinese populations using the 922 SNPs. The Bayesian cluster analysis assigned the seven populations into four distinct clusters (Figure 2). The results based on Δ*K* and mean LnP(*K*) indicated optimal values of 4 and 5, respectively (Figure S2). The presence of four clusters is consistent with division according to the four geographical provinces in which the populations are located, but note that population WYS from cluster 3 (Fujian prov.) shows a certain amount of mixing with cluster 4 (Zhejiang prov.). When *K* = 5, population YTG from Fujian province is allocated to a separate cluster. The other values of *K* also provided some additional information. Population LS (Jiangxi prov.) was the first to split from the other populations when *K* = 2, followed by population WT (Anhui prov.) when *K* = 3. Separation occurred within cluster 3 (Fujian prov.) when *K* = 5–7. Cluster 4, two populations from Zhejiang province, were always closely related. Similar results were obtained from the dendrogram based on the pairwise F_{ST} matrix (Figure 3) (Figure S3).

In summary, we partitioned the seven populations of *C. japonica* var. *sinensis* into four clusters (LS from Jiangxi prov.; WT from Anhui prov.; WYS, TBY, and YTG from Fujian prov.; and TTS and XTM from Zhejiang prov.) which seems to be a reasonable classification. However, population LS should be regarded as Japanese *Cryptomeria* (Figure S1).

We did not detect a significant correlation between geographic distance and genetic distance based on a Mantel test (R^2 = 0.007, p = 0.382) (Figure 4). No isolation by distance (IBD) was found.

Here, we considered six populations, excluding LS because this population appears to be an old plantation derived from Japanese stock.

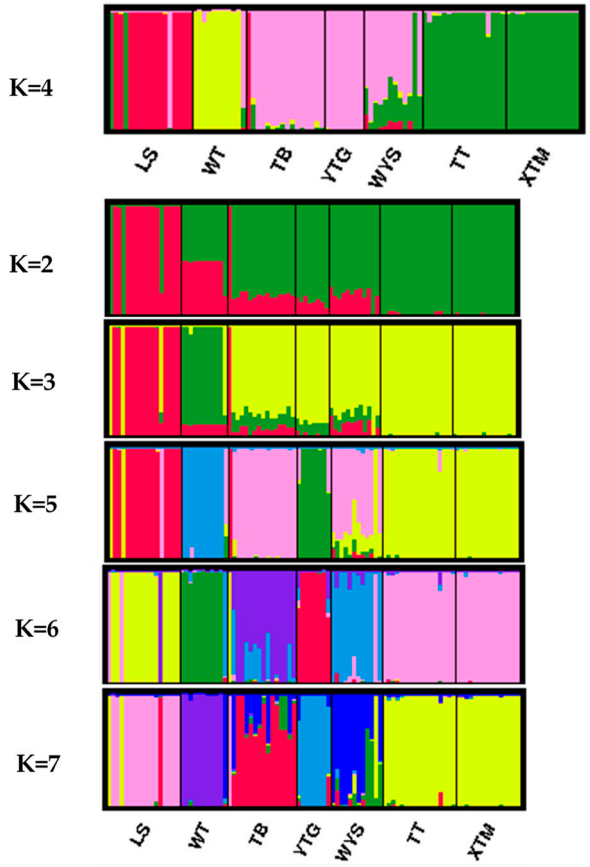

Figure 2. Population genetic structure of seven populations of *C. japonica* var. *sinensis* by STRUCTURE.

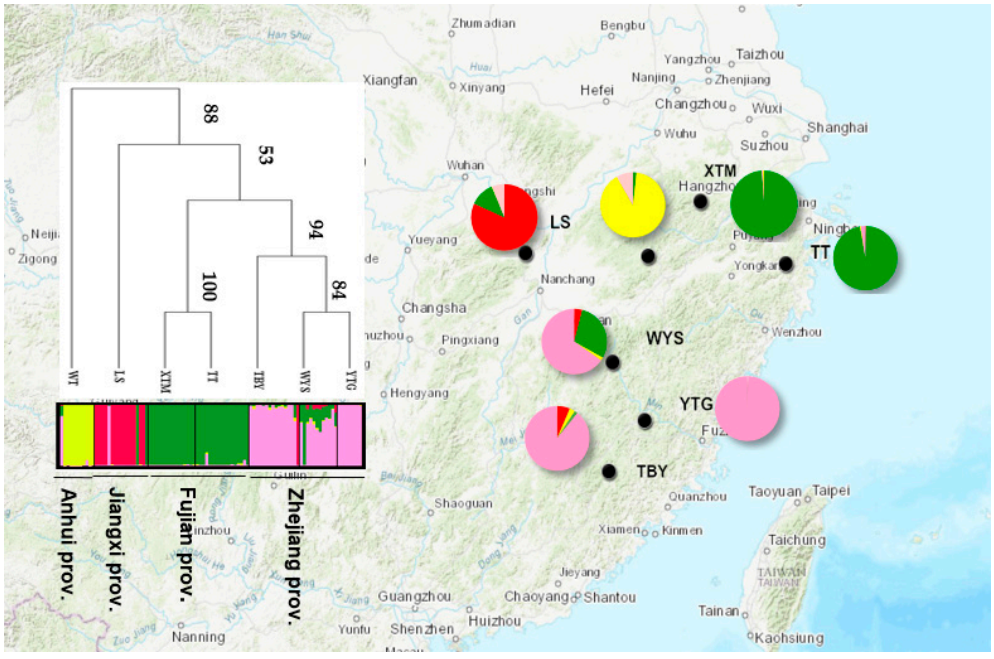

Figure 3. Genetic structure of *C. japonica* var. *sinensis* based on 922 SNPs. The pie diagrams on the map represents the membership coefficients to the four clusters inferred in Structure software. The neighbor-joining dendrogram based on pairwise F_{ST} values with 1000 bootstrap replicates.

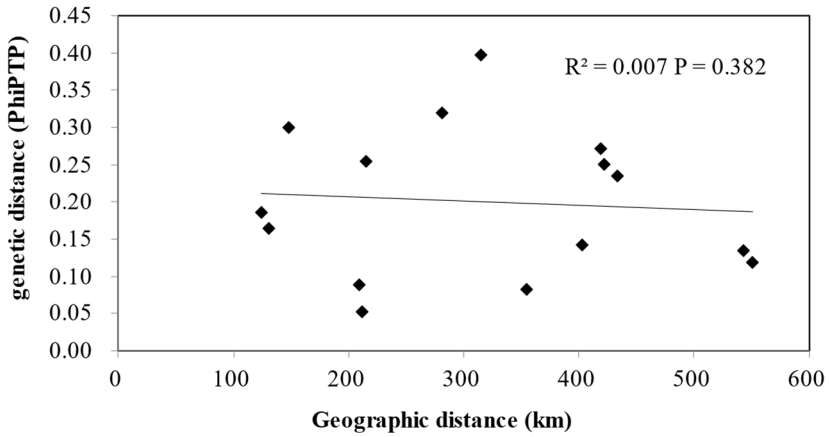

Figure 4. Mental test between geographical distance (km) and genetic distance (PhiPTP) for only Chinese populations where suspected Japanese individuals in LS were removed.

3.3. Demographic History

We obtained very similar trends in the three different scenarios (generation time = 150, 200, and 300 years)—namely, that the effective population size of Chinese *Cryptomeria* has experienced a continuous decline from the mid-Pleistocene to the present. The first decline occurred from 1 Mya to 0.4 Mya BP, coinciding with the onset of the Naynayxungla Glaciation (0.8–0.5 Mya) in China. The second decline began ca. 0.1 Mya to 0.06 Mya BP, when the Last Glacial Period (LGP) commenced. However, the range of *Cryptomeria* in China did not increase but continued to decline throughout the Holocene (Figure 5).

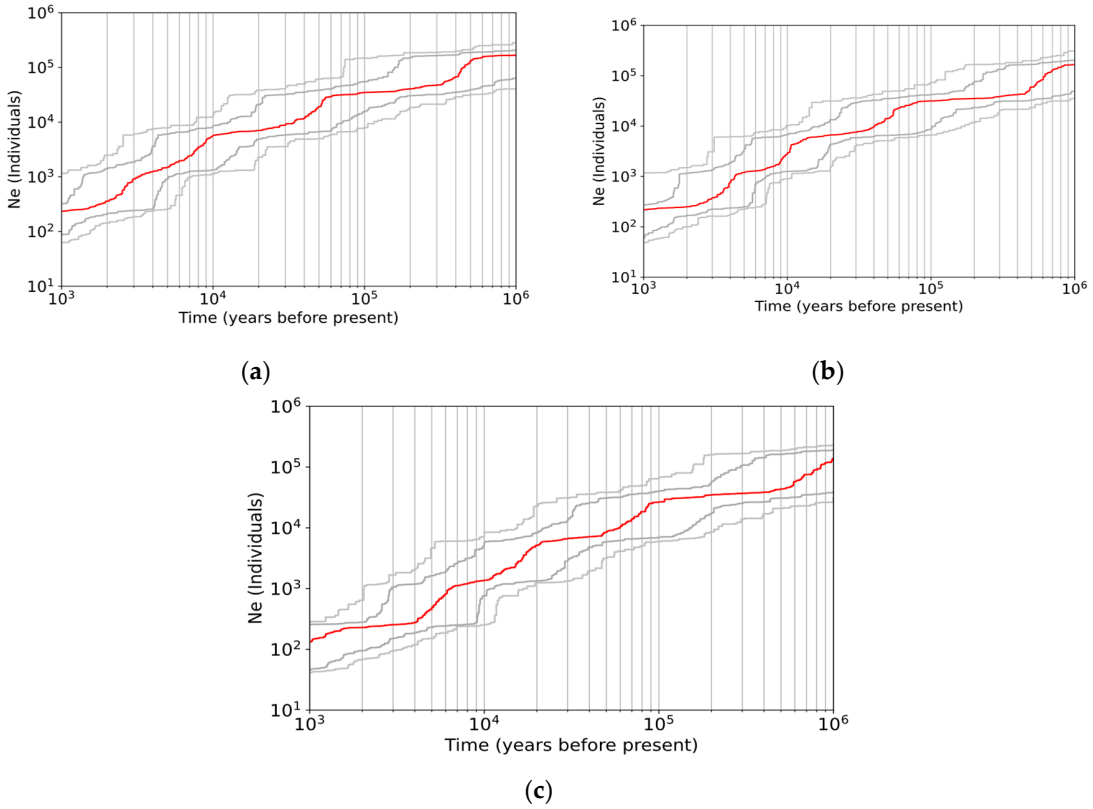

Figure 5. Effective population size (Ne) estimated based on folded SFS (Site Frequency Spectrum) with a generation time of (**a**) 150 years, (**b**) 200 years, and (**c**) 300 years, according to the Stairway Plot software. Red and grey lines represent the medians and the 2.5 and 97.5 percentiles, respectively.

4. Discussion

4.1. An Old Plantation Derived from Japanese Origin

The result of the NeighborNet based on F_{ST} revealed a clear separation between the Chinese and Japanese *Cryptomeria* (Figure S1), with population LS from Lushan mountain in Jiangxi province clustered with the Japanese group. Moreover, the genetic diversity of this population was apparently higher than that of the other Chinese populations and showed a very similar level of genetic diversity to the Japanese populations. There are historical records showing that, from ancient times, certainly as early as the 1st century BC, there was trade and the movement of people between Japan and China. After that, this kind of relationship continued until now. As a result, many items were exchanged between the two countries. Useful and important goods and ideas were shared, with rice cultivation being one of the best examples, having originated in China and then been exported to Japan. It is likely that a visitor to Japan in ancient times saw a huge *Cryptomeria* tree, brought back the seeds, and planted them in China. More recently, large-scale introductions of C. japonica from Japan occurred in the early 20th century. We, therefore, consider the population LS to be a Japanese *Cryptomeria* population based on our genetic data and historical evidence. However, some Chinese *Cryptomeria* individuals are present in this population, because individuals LS001 and LS017 belong to the Chinese population according to the NeighborNet result (Figure S4). Bayesian cluster analysis also showed a similar result to that generated by NeighborNet. With regard to individual LS001, this famous tree is recorded in an ancient book, "Travel Notes of Xu Xiake", written by a geographer in 1618, and it is estimated to be more than 600 years old (Figure S5). The *Cryptomeria* forest in Lushan mountain is, therefore, a mix of Chinese and Japanese *Cryptomeria*, and we found no significant phenotypic difference between them during our field investigation.

4.2. Low Genetic Diversity and High Genetic Differentiation in Chinese Cryptomeria

Ancient tree populations of *C. japonica* var. *sinensis* in China harbor a very low genetic diversity, but there is high genetic differentiation between populations compared to its congener, *C. japonica* in Japan. Here, we excluded LS population when discussing the genetic diversity and differentiation of Chinese *Cryptomeria.* Previous studies such as those by Chen et al. [6] and Tsumura et al. [18] also indicate a lower genetic variation in Chinese populations. Theoretically, inbreeding, genetic drift, restricted gene flow, and small population size all contribute to a reduction in genetic diversity [45]. In this study, we found evidence of a continuous decline in effective population size since the mid-Pleistocene. Genetic drift caused by climate fluctuation probably played an important role during the evolutionary process. Moreover, there was no expansion after the retreat of glaciers. We speculate that habitat loss or degradation and artificial selection caused by intense human activities have further accelerated the decline of the already low diversity, also leading to the great differentiation between different regions. Although gene flow in *Cryptomeria*, an allogamous, wind-pollinated conifer species, is expected to be high, we detected restricted gene flow (Nm = 1.618) between all the ancient tree populations, lower than the normal value of $Nm > 3$ in conifers [46]. Given that 76% of the variation occurred within populations, we consider that limited gene flow is also a factor accounting for the low genetic diversity. However, we did not find any sign of inbreeding in any population except WT. The result of relatedness analysis also presented a low proportion of kinship in most cases except the population of WT and YTG (Figure S6). The low diversity in WT may be caused by family relationship, while in YTG is even in the small sample size (only 8) there is no inbreeding (F_{IS} = −0.123) found in it. As far as other Chinese *Cryptomeia* populations go, we believe that the low level of genetic diversity can be attributed to climate change and strong human activity. Interesting, three populations (WYS, YTG, and XTM) showed signs of significant excess heterozygosity, which we consider probably to be the consequence of selection. However, the F_{IS} values of the three populations are not particularly high (WYS = −0.087, YTG = −0.123, and XTM = −0.082, Table 2) and thus this result may be related to the

relatively small number of individuals investigated or may just be a chance occurrence. Some outlier loci under selection were detected by "Fsthet", and these might also be related to this result (Figure S7).

Similar situations with low genetic variation and high genetic differentiation have been found in some isolated and threatened species, such as *Tsuga caroliniana* Englem. [47], *Podocarpus sellowii* Klotzsch ex Endl. [48], and *Lupinus alopecuroides* Desr. [49]. Chinese *Cryptomeria*, as an important timber species, has been widely planted throughout southern China, and the unexpectedly low genetic diversity is probably associated with population size reduction because of global climate change, while the high genetic differentiation is probably the result of long isolation and human disturbance.

4.3. Specific Genetic Structure with an Absence of IBD

We separated the seven ancient tree populations into four groups coinciding with the four different administrative provinces in which the trees are located. Population LS from Lushan province and population WT from Anhui province were clearly separated, while the divergence between the Zhejiang and Fujian groups had relatively lower support (Figure 3). A certain mixing of the two groups occurred in the contact population, WYS. Ln(K) is also provided an alternative optimal structure that showed differentiation within the Fujian province group when $K = 5$ (Figure S2) (Figure 2). We noticed that it was YTG, not the contact population of WYS, that was separated. As we mentioned before, this may related to the small sample size of that population.

Even though a clear genetic structure was found, we did not detect a significant correlation between geographical distance and genetic distance, but Japanese *Cryptomeria* does exhibit such a correlation [50]. Generally, most tree species exhibit clear isolation by distance (IBD) if there is no strong human disturbance and selection [50,51]. In this case, geographical isolation is not the major factor responsible for the current structure. Since *Cryptomeria* is a wind-pollinated monoecious species, its mating system cannot explain the large differentiation either. As discussed above, the genetic drift associated with climate oscillations greatly reduced genetic diversity; one key factor may have been drought stress, which *Cryptomeria* is particularly sensitive to, as reported by Tsumura et al. [18] and Mori et al. [52]. On the other hand, the resulting habitat fragmentation also led to great genetic differentiation. We think that the reason for the unexpected absence of IBD was probably human disturbance. The topography of the range investigated, southern China, is characterized by numerous plains and basins between low hills [53]. In ancient times, the relatively flat terrain, with abundant grain cultivation, along with the development of handcraft industries resulted in the viability of commercial activities based on the timber and silk trade in this area, especially creating links between Fujian and Zhejiang province. A study of the genetic structure of horses suggested an important role for trade routes in facilitating exchange over topographically, ecologically, culturally, and politically diverse landscapes and large geographical distances [54]. Thus, we speculate that ancient trade routes in southern China may have provided opportunities for the transfer of material between different regions, resulting in ambiguities in the genetic origin of trees across the whole distribution range. However, given that we still found a pattern of genetic structure, the transfer of material must have been restricted.

Because of the very limited number of ancient trees currently in existence—we discovered only seven populations—the genetic structure presented in this study may deviate more or less from the original pattern without human interference. Despite this, we found a similar structure pattern—namely, an absence of IBD—to some species in southern China, including *Miscanthus lutarioriparius* L. Liou ex Renvoize & S.L. Chen [55], *Houpoea officinalis* (Rehder & E.H. Wilson) N.H. Xia & C.Y. Wu [56], and *Brasenia schreberi* J.F.Gmel. [57]. This may indicate a similar evolutionary history under anthropogenic pressure.

4.4. Continuous Decline of Population Size without Postglacial Recolonization

Climate oscillations throughout the Late Quaternary had a dramatic effect on the species ranges of both plants and animals in subtropical mainland Asia and the Japanese Archipelago [58]. The same

goes for Chinese *Cryptomeria*: a remarkable decline in effective population size has been detected since the mid-Pleistocene. However, unlike many widely spread temperate plant species in Japan and East China [59], there was no recolonization after contractions during the LGM, but the population kept declining throughout the Holocene. Tsumura et al. [18] also did not find an obvious range expansion in the mid-Holocene and during the present using Species Distribution Modelling (SDM). There are two possible explanations for the continuous decline in population size after the LGM. First, the Ne of Chinese *Cryptomeria* may have decreased to a threshold size that constrained recovery. The low genetic diversity may have undermined any adaptive potential of the population during migrations. Second, humans in the Holocene directly reduced population size by cutting trees and clearing land [60]. Similar cases can be seen in some plant species in eastern China, including the genus *Croomia* [61], *Davidia involucrate* Baill. [62], *Ostrya rehderiana* Chun [60], and *Kalopanax septemlobus* (Thunb.) Koidz. [63].

In Japan, the range of *Cryptomeria* contracted to several refugia, mainly concentrated in the southwestern part of Japan during the last glaciation [64], and some natural stands have been retained up to the present. Japan may have had a favorable environment, with sufficient precipitation and fertile soil [18], and less anthropogenic disturbance. Thus, *Cryptomeria* in Japan maintained a higher level of genetic diversity and also presumably a larger effective population size than in China.

4.5. Conversation Considerations

Our investigated sites included the most famous and well-conserved forest stand of Chinese *Cryptomeria*, population XTM, which is located in Tianmu national nature reserve, Zhejiang province. This area contains many relict species of the Paleogene glaciation and is known for various ancient trees, including *Ginkgo biloba* L., *Liquidambar formosana* Hance, and *Pseudolarix amabilis* (J. Nelson) Rehder; among them, *C. japonica* var. *sinensis* is the dominant species. Previous studies on *Ginkgo biloba* [65], *Liriodendron chinensis* (Hemsl.) Sarg. [66], and *Quercus acutissima* Carruth [67] presented some evidence of glacial refugia in this region. In our case, we found a moderate level of genetic diversity and some private alleles, but no evidence to show that this area is the origin of Chinese *Cryptomeria*, even though it may seem to be [18]. Chen et al. [6] also suggested that the ancient population of *C. japonica* var. *sinensis* on Tianmu mountain was introduced originally, with subsequent natural regeneration.

Before the 1950s, most of China's forests were naturally regenerated. Since then, demand for timber has resulted in the extensive cutting of forests, and timber harvests increased from 20 million m^3/year in the 1950s to 63 million m^3/year in the 1990s [68]. Large-diameter trees are the first targets of timber extraction [69]. Government policy did not require that native tree species be planted after logging, but promoted the planting of fast-growing tree species, such as larch (*Larix* sp.), poplar (*Populus* sp.), and Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook). As a consequence, forest coverage has increased substantially, while natural forest has declined to 30% of the total forest area in China [68]. Moreover, genetic diversity loss is irreversible. In 1998, the Chinese government established the National Forest Conservation program (NPCP) to protect existing natural forest from excessive cutting. However, natural stands of Chinese *Cryptomeria* are now very rare, and the ancient populations which probably contain the ancestral genetic signature exhibit very low diversity, highlighting the precarious status of genetic resources of Chinese *Cryptomeria*.

During our field investigation, we found that some ancient trees occur sporadically in secondary forest and they are vulnerable to habitat fragmentation, atmospheric drought, and other competing species such as moso bamboo (*Phyllostachys edulis* (Carrière) J.Houz) [70]. However, some ancient trees in human-dominated landscapes are known for their high cultural and socioeconomic value, so citizens are willing to pay for their conservation [69]. Overall, the fate of ancient trees of *Cryptomeria* in China still hangs in the balance.

Under these circumstances, a more profound understanding and effective measurement are urgently needed. Here we propose both the in situ and ex situ conservation of this species. First, we appeal for stricter implementation of regulations to protect existing ancient trees in the wild. We suggest a

complete ban on cutting and grazing; in addition, the thinning of dense, competitive bamboos or other problem species in the habitat is required. Even those trees that are worshipped as "sacred" still face some threats from industrial pollution, urbanization, and other forms of economic development. Sightseeing activities should be properly restricted. Compared to other populations, measures for the conservation of population WT, which is located on Huangshan mountain in Anhui province, are urgently required because it has the lowest diversity and diverges most from the other populations. Secondly, ex situ collections, which represent the highest genetic variation in the wild, need to be established. So far, two large germplasm gardens of Chinese *Cryptomeria* have been constructed and abundant sources have been collected from Fujian province and Zhejiang province, but we recommend a wider range of collection, targeting every single ancient tree in China. In view of afforestation, even though this species has been planted widely across southern China, many plantations lack clear provenance information, thus obscuring the genetic composition of planted forests; better data is required in order to develop a better afforestation strategy. As revealed in our study, populations from Tianmu mountain (XTM) in Zhejiang province and Tianbaoyan nature reserve (TBY) in Fujian province have a relatively high diversity and harbor some private alleles; these populations should be considered core resources.

5. Conclusions

Although the Chinese *Cryptomeria* population in Tianmu mountain has been studied and its low genetic diversity has been reported before, our study is the first study to explore the genetic composition of ancient *Cryptomeria* trees across a distribution range in China. The findings confirm that the ancient *Cryptomeria* population in China contains a low level of genetic diversity and high differentiation. Populations in different provinces were genetically differentiated, but no IBD was detected. We infer that the genetic drift caused by climate oscillations during the Last Glacial Period greatly reduced the population size of Chinese *Cryptomeria*, and this was followed by intense anthropogenic disturbance, which accelerated the loss of diversity and also led to a clear differentiation between regions. In addition, we present some theoretical guidance for conservation work in the future. Our study is the first to explore the genetic composition of ancient *Cryptomeria* trees in China and lays a firm foundation for further molecular research.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/11/1192/s1: Figure S1: NeighborNet based on pairwise F_{ST} matrix of Chinese and Japanese populations; Figure S2: The number of inferred cluster K based on ΔK and mean LnP(K) obtained from Structure Harvester; Figure S3 Neighbor-joining dendrogram based on F_{ST} pairwise matrix of *C. japonica* var. *sinensis* after removing the suspected Japanese population of LS; Figure S4: NeighborNet based on pairwise F_{ST} matrix of all individuals. Individuals in red color indicated two trees in population of LS that belong to Chinese group; Figure S5: A ancient tree growing in Lushan mountain, coded LS001 in this study, was estimated more than 600 years old; Figure S6: The result of relatedness analysis of 7 populations, the darker blue indicated the higher relatedness coefficiency. Figure S7: Distribution of F_{ST}–H_T (expected heterozygosity) relationship based on 922 loci in *C. japonica* var *sinensis*. Two red line indicated the confidence interval of high and low value. A total of 20 outlier loci detected among 922 loci. Table S1: Geographic locations and sample size (N) of the 6 Japanese *Cryptomeria* populations; Table S2: Genetic diversity indices of six populations of *C. japonica* and seven populations of *C. japonica* var. *sinensis* based on 183 loci.

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