

Genetic Diversity and Population Structure of Chinese  
*Cryptomeria* (*Cryptomeria japonica* var. *sinensis*) for  
Conservation Strategies

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CAI Mengying

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CAI Mengying

## Abstract

*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. Natural forests in China have, however, progressively reduced in extent over the centuries through a variety of unsustainable practices such as overgrazing, burning and exploitation. The area containing remnant forests has been occupied by new plantations and it is hard to distinguish natural stands from numerous artificial forests. In this context, ancient trees which originated from natural regeneration rather than planting will be valuable resources, and their genetic status needs to be understood. In addition, with the rapid spread of plantations, the genetic consequences of management practices need to be evaluated. However, unlike *Cryptomeria* in Japan, the genetics of Chinese *Cryptomeria* have seldom been studied, although there is ample evidence of the species' great ecological and economic value. In this study, I employed Restriction Site Associated DNA Sequence (RAD-seq) technology and investigated seven ancient populations, ten plantations and two seed orchards in order to reveal the genetic characteristics of ancient trees and the genetic background of plantations and seed orchards.

Lower genetic variation but higher genetic differentiation in ancient tree populations of Chinese *Cryptomeria* were found in comparison to natural populations of Japanese *Cryptomeria*. Structure analysis and dendrogram construction divided the seven ancient 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 using a Stairway Plot showed that the effective population size of Chinese *Cryptomeria* has experienced a continuing decline from the Ice Age to the present. My findings suggest that the strong genetic drift caused by climate fluctuation and intense anthropogenic disturbance together contributed to the current low diversity and strong genetic structure.

Most of the plantations examined in this study were derived from neighboring ancient populations or bow-vanished natural stands, since they show a similar genetic structure to these, and contain slightly higher genetic diversity. However, two relatively young plantations (YA and ZGS) in Sichuan province were found to have originated

from Tiantai mountain in Zhejiang province. Zhejiang and Fujian provinces have long been considered the two main gene pools of Chinese *Cryptomeria*. However, in my study, one plantation (LYS) and one seed orchard (HY) which were established in the 1950s and 1970s in Sichuan province, have a critically low diversity but are highly differentiated. My data combined with records in floras led me to hypothesize that Sichuan once supported a natural population of Chinese *Cryptomeria*, and although natural stands have declined sharply in recent decades, some genetic information is still present in some older plantations. More evidence is required to support this suggestion. In addition, the Xiapu seed orchard in Fujian province supports a high level of genetic diversity because of the multi-source provenances, but as a consequence, genetic structure of Chinese *Cryptomeria* might be disturbed if these seeds are widely used.

The findings highlight the precarious status of the genetic resource of Chinese *Cryptomeria*. For conservation purpose, firstly, forest zoning is recommended. Three zones should be divided: protected zone (ancient population), buffer zone (investigated plantations except ZGS and YA), and production zone (ZGS and YA). Secondly, three genetically differentiated seed zones are suggested in this study, seed orchards in each zone should be established soon in order to maintain the present genetic structure. More studies concerning population structure and gene flow will be conducted in more plantations and across a wider area, and more specific seed division and subdivision are expected in the future.

**Keywords:** Chinese *Cryptomeria*, ancient trees, plantation, seed orchard, genetic diversity, genetic structure, SNP, conservation, afforestation

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## Abbreviations

AMOVA	Analysis of Molecular Variance
<i>Ar</i>	Allelic richness
bp	Base pair
CTAB	Cetyl trimethylammonium bromide
DBH	Diameter at breast height
ddRAD-seq	Double Digest Restriction-site Associated DNA sequencing
FAO	The Food and Agriculture Organization of the United Nations
$F_{IS}$	Fixation index
FLI	The Forest Legality Initiative
$F_{ST}$	Differentiation coefficient
$H_e$	Expected heterozygosity
$H_o$	Observed heterozygosity
$H_T$	Expected heterozygosity
IBD	Isolation by distance
LGM	Last Glacial Maximum
LGP	Last Glacial Period
MCMC	Markov chain Monte Carlo
Mya	Million years ago
$N_a$	The number of alleles
$N_e$	The number of effective alleles
$N_e$	Effective population size
NGS	Next-generation sequencing
$N_m$	Gene flow
NPCP	National Forest Conservation program
$pAr$	Private allelic richness
RAD-seq	Restriction-site associated DNA sequencing
SDM	Species Distribution Modelling
SFS	Site frequency spectrum
SMP	Supplemental mass pollination
SNP	Single nucleotide polymorphisms

# Chapter 1 General introduction

## 1.1 Forest in China

### 1.1.1 Conservation of ancient trees in China

Ancient trees are keystone ecological species that play important roles in supporting natural community structure and dynamics, maintaining critical ecosystem functions, and providing habitat for a wide range of native organisms (Liu et al., 2018). They have high conservation, cultural and aesthetic values (Lindenmayer & Laurance, 2017). In many countries, traditional forestry practices support cutting large-diameter trees for timber resources and replacing them with younger trees regularly, and China is no exception. Before the 1950s, most of China's forests were naturally regenerated. Since then, demand for timber has resulted in extensive cutting of forests, and timber harvest increased from 20 million m<sup>3</sup>/year in the 1950s to 63 million m<sup>3</sup>/year in the 1990s (Zhang et al., 2000). Large-diameter trees are the first targets of timber extraction (Wu et al., 2020). Government policy did not require that native tree species be planted after logging, but promoted planting of fast-growing tree species, such as larch (*Larix* spp.), poplar (*Populus* spp.), and Chinese fir (*Cunninghamia lanceolata*). As a consequence, forest cover has increased substantially, but natural forest has declined to 30% of the total forest area in China (Zhang et al., 2000). In 1998, the Chinese government established the National Forest Conservation program (NPCP) to protect existing natural forest from excessive cutting. Nowadays, extremely large and old trees can be found only in remote, high altitude regions. Many trees have been given protection under national programs, however their distributions are fragmented and trees are disappearing under the pressure of both global climate change and human influence.

Under these circumstances, natural stands of Chinese *Cryptomeria* (*Cryptomeria japonica* var. *sinensis* Miquel) have become very rare, and the ancient populations which probably contain the ancestral genetic signatures are also limited, highlighting the precarious status of the genetic resource of the species.

### 1.1.2 Plantations in China

As mentioned above, with the implementation of the Natural Forests Conservation Program, the wood resource in China is shifting from natural forests to plantations. The

Chinese Government has launched another major program, the "China Fast-Growing and High-Yield Plantation Program". This project will invest 71.8 billion Yuan to establish 13.33 million hectares of plantation forests in China. Major plantation species include Masson pine (*Pinus massoniana*) and exotic pines (*Pinus* spp.), Chinese-fir, poplar (*Populus* spp.) and Eucalyptus (*Eucalyptus* spp.). Northern plantations have been a major supplier to the Chinese plywood industry, however, attention is shifting to the cultivation of eucalyptus and pine in the south to source fiber for use in pulp, for wood-based panels and lumber production (The Forest Legality Initiative, FLI, 2014).

In southern China, Chinese *Cryptomeria* is becoming a main species for afforestation and wood-production next to Chinese fir, Eucalyptus, Masson pine, *Cupressus funebris*, and *Pinus elliottii*, and in Fujian province in particular, it is dominant.

## **1.2 Botanical and ecological characteristics of Chinese *Cryptomeria***

### **1.2.1 Taxonomy of Chinese *Cryptomeria***

*Cryptomeria* is a monotypic relic genus that has been classified as belonging to the family Taxodiaceae before, while recent molecular studies have revealed that it actually belongs to the Cupressaceae (Tsumura, 2011). Extensive fossil records indicate that it was widely distributed throughout Eurasia during the Cenozoic. The earliest fossil record of *Cryptomeria* was described based on vegetative organs from the Paleogene in Kamchatka, in the far east of Russia (Chelebaeva, 1991; Budantsev, 1997). In the Neogene, *Cryptomeria* is well represented in the fossil records of Europe and Japan, including seed cones, leafy shoots and wood. However, there remains only one extant species, *Cryptomeria japonica* D. Don, which contains three recognized varieties that are found in Japan and southern China. *C. japonica* var. *japonica* (locally known as omote-sugi) and *C. japonica* var. *radicans* (locally known as ura-sugi), are found in the moist temperate region from Aomori Prefecture to Yakushima Island on the Japanese archipelago (Hayashi, 1960). Var. *japonica* mainly occurs on the Pacific Ocean side and has rough branchlets and hard needles, whilst var. *radicans* is found mainly on the Sea of Japan side and has slender branchlets and soft needles. Morphological differences between the two varieties in Japan are mainly attributed to local adaptation to climate, namely, a dry climate in winter on the Pacific Sea side and heavy snowfall during winter on the Sea of Japan side (Moriguchi et al., 2019). The third variety, var. *sinensis* (locally

known as liushan), is limited to southern China, with a few natural occurrences in Fujian (Nanping), Jiangxi (Lushan mountain), Sichuan, Yunnan and Zhejiang (Tianmu mountain) provinces (Fu et al., 1999). Compared to the two Japanese *Cryptomeria* varieties, this variety has slender overhanging branchlets with short needles (Tsumura et al., 2020).

### 1.2.2 Morphology of Chinese *Cryptomeria*

*Cryptomeria japonica* var. *sinensis* is a monoecious conifer. Trees to 40 m tall; trunk to at least 2 m DBH (diameter at breast height); bark reddish brown, fibrous, peeling off in strips; crown pyramidal; main branches whorled, horizontally spreading or slightly pendulous; branchlets usually pendulous, those of first year green (flora of China). Morphological differences between *C. japonica* and *C. japonica* var. *sinensis* are as follows (Fu et al., 1999) (Figure 1.2) ,

1) Leaves. In *C. japonica*, they are more or less straight at least in the proximal half of the branch, often recurved apically on leader branchlets, arising at a 35 to 45° angles to the axis on leader branchlets, 45 to 55° on fertile branchlets, they are rigid and hard. In var *sinensis*, they are usually strongly incurved throughout, arising at 15 to 30° angles to the axis on leader branchlets, 30 to 40° on fertile branchlets, and are rigid but relatively soft.

2) Pollen cones. In *C. japonica*, they are longer than their subtending leaf; in var. *sinensis*, they are shorter than their subtending leaf.

3) Seed cones. In *C. japonica*, they have 20 to 30 cone scales, each bearing 6-9 seeds; the distal projections of bracts and cone scales are 2 to 3.5 mm long. In var. *sinensis*, they have ca. 20 cone scales, each bearing two seeds; distal projections of bracts and cone scales are 1-2 mm long.

This variety has been treated as a separate species by some authors, under the invalid name *Cryptomeria fortunei* Hooibrenk.

### 1.2.3 Ecology and habitat of Chinese *Cryptomeria*

Chinese *Cryptomeria* is native to China, Fujian (Nanping city), Jiangxi (Lu shan), Sichuan, Yunnan and northwestern Zhejiang (Tianmu mountain) provinces according to the “flora of China” (Fu et al., 1999). However, the native distribution range was

narrowed and concentrated in eastern China including Zhejiang, Fujian and Jiangxi provinces according to “Conifers around of the world” (Debreczy & Rácz, 2011) (Figure 1.1). At present, this species is also widely introduced for forestry in other provinces of China. It grows in forests on deep, well-drained soils subject to warm, moist conditions at elevations from below 1,100 m up to 2,500 m above sea level. It is fast-growing under these conditions, but intolerant of poor soils and cold, drier climate. The species usually reproduces by seeding in the wild, and is also easy to propagate from stem cuttings. Japanese *Cryptomeria*, var. *radicans* on the Japan Sea side of Japan reportedly reproduces both by seeding and layering (Kimura et al., 2013); the latter occurs because of the heavy snow pressure in specific environments. I did not observe this trait in Chinese *Cryptomeria*.

### **1.3 Importance of Chinese *Cryptomeria* in China**

*Cryptomeria* has long been cultivated both in Japan and China. As the tree has a straight bole with soft wood, for hundred years it has been used for house construction, wooden ships, wooden barrels and other daily items (Ohba, 1993). Currently, in Japan, *Cryptomeria* is the most important forest species and is present in 44% of all Japanese planted forests (Tsumura, 2011); it is known as “the national tree” of Japan. In China, planted forests are not as common as in Japan, but still play an important role in forestry in the south of the country.

Chinese *Cryptomeria* has long been valued for the beauty of both the tree shape and wood, and it is widely planted around temples. The earliest historical document referring to *Cryptomeria* can be traced back to 1279 and is from the Tianmu mountains area (Wang et al., 2007). Some ancient trees are still well preserved in villages in the Fengshui forest and they are supposed to bring good fortune and happiness, some trees are tourist attractions in the landscape dominated by humans.

After the establishment of the new China, the government launched a series of greening campaigns, as part of which var. *sinensis*, as a common and popular species, was widely planted throughout southern China in afforestation schemes, to be harvested in the future for timber. Now, extensive studies have revealed that Chinese *Cryptomeria* forests have great ecological benefit with respect to soil properties, water infiltration and biodiversity (Mo et al. 2012; Zhao et al. 2014; Yang et al. 2016), and also have substantial economic benefit in terms of wood output (Qin et al. 2016).

## 1.4 Research objectives

Because of the unresolved genetic background of Chinese *Cryptomeria* and its great importance for forestry in southern China, my study uses RAD-seq (Restriction-site Associated DNA sequencing) technology to gain insights into the following:

- 1) The genetic composition of the extant ancient tree population of Chinese *Cryptomeria*
- 2) The demographic history of Chinese *Cryptomeria*
- 3) Genetic characterizations of plantations in southern China
- 4) The origins of plantations in different provinces in southern China
- 5) Genetic consequences of plantation establishment of Chinese *Cryptomeria*

Chapter 1 of this thesis provides an overall introduction to *C. japonica* var. *sinensis* and the importance of this species. Objectives 1 and 2 are described in Chapter 2, which has been published in Cai et al. (Cai et al., 2020). Objective 3 is described in Chapter 3. Objectives 4 and 5 are described in Chapter 4. Chapter 5 discusses the conservation of the genetic resources of Chinese *Cryptomeria*, and also summarizes the overall results.

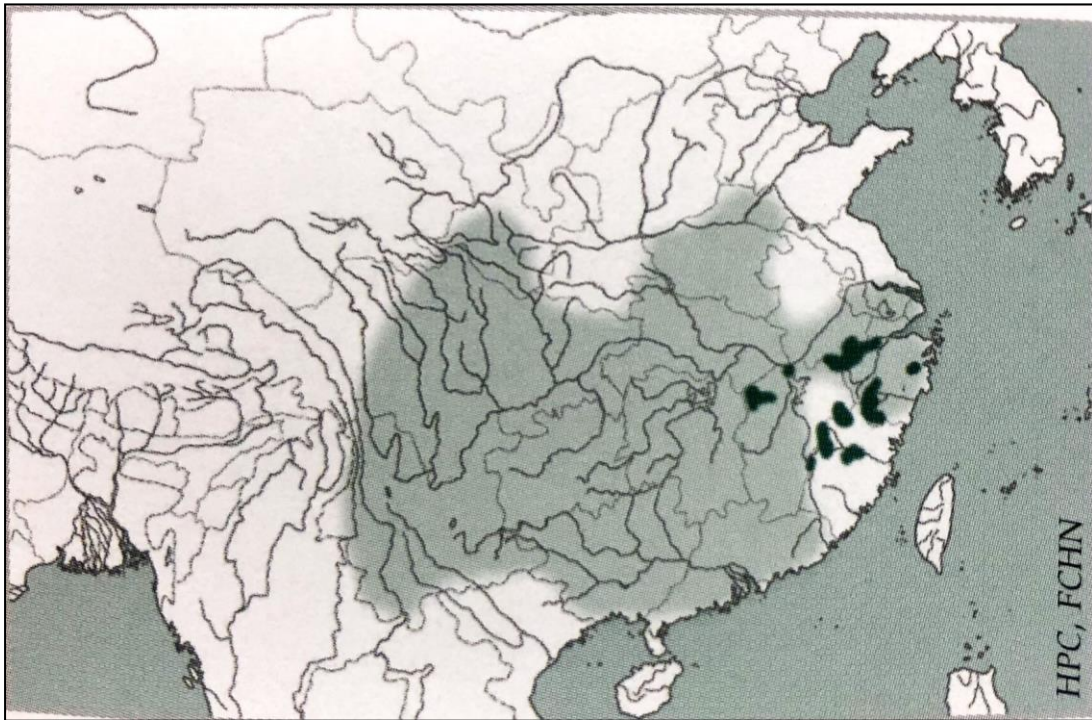


Figure 1. 1 Distribution of *C. japonica* var. *sinensis* reported by Debreczy et. al (Debreczy & Rácz, 2011). Green: mostly naturalized; Dark green: possibly natural forests.



Figure 68. 1-5. *Cryptomeria japonica* (Thunberg ex Linnaeus f.) D. Don var. *sinensis* Miquel, 柳杉 liu shan. (*C. fortunei* Hooibrenk). —1. Cone-bearing branchlet and seed cones. —2. Leaf. —3. Seed scale abaxial view and bract apex. —4. Seed scale adaxial view and bract apex. —5. Seed. 6-11. *C. japonica* var. *japonica*, 日本柳杉 (原变种) ri ben liu shan (yuan bian zhong). —6. Cone-bearing branchlet and seed cones. —7, 8. Leaves. —9. Seed scale abaxial view and bract apex. —10. Seed scale adaxial view and bract apex. —11. Seed. (FOC 57; FRPS 7: 296, pl. 68. 1978. —张荣厚 & 吴彰桦 Zhang Ronghou & Wu Zhanghua; redrawn by 李爱莉 Li Aili).

Figure 1. 2 Morphology of branchlet, leaf and seed cones of *C. japonica* var. *sinensis* (1-5) and *C. japonica* var. *japonica* (6-11). (Flora of China, Vol. 4) (Fu et al., 1999)



## Chapter 2. Genetic diversity and genetic structure of ancient tree population

### 2.1 Introduction

Ancient trees are amongst the largest and oldest living organisms on the Earth and contribute to only a small percentage of the tree population; however they hold historical, cultural, traditional and ecological significance globally. Such trees can be studied on a local or national scale to allow us to gain insights into the history of the land and past climate change. Up to 2009, China had 2.847 million ancient trees across the country. These trees have been conserved as individual units by the Afforestation Committee and Forestry Bureau. Collection is not allowed except for research and educational purposes (FAO, 2010)

Most ancient Chinese *Cryptomeria* trees are found in Zhejiang and Fujian provinces. Tianmu mountain, which is located in Zhejiang province, is regarded as having the largest and most famous ancient population in China. As many as 664 individuals with a DBH greater than 1m were investigated in this area, among them, 12 individuals measuring more than 2m. However, a remarkable decline in growth was observed in this population and it is also under severe attack from burl disease. Chen et al. (Chen et al., 2008) investigated the demographic structure of Chinese *Cryptomeria* on Tianmu mountain using microsatellite markers, and found a low level of genetic diversity compared to Japanese *Cryptomeria*. Even though Tianmu mountain harbors the largest number of ancient trees, a recent bottleneck was detected in some younger stands. However, besides Tianmu mountain, ancient trees of Chinese *Cryptomeria* in other areas have seldom been studied due to the scattered distribution. (Figure 2.1). At the species level, we know little about its population genetics.

Single nucleotide polymorphisms (SNP) have proved to be the most abundant form of variation within a species at genome level and can provide detailed insights into the genetic basis of a population (Howe et al., 2013). Combined with Next-Generation Sequencing (NGS) technology, SNP markers are having substantial impacts on population genetics as well as plant breeding (Davey & Blaxter, 2010; Iwata et al., 2011). 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

(Brandrud et al., 2019; Miller et al., 2007; Peterson et al., 2012; Zhou et al., 2018). This approach has great advantages, including generating a large quantity of data across the genome, reasonable costs, relatively simple procedures for library construction, short experiment duration, no requirements for a reference genome, and a well-developed pipeline for data treatment and analysis (Eaton & Ree, 2013; McCormack et al., 2013).

Using ddRAD-seq (double digest RAD-seq) technology, based on 122 samples from seven ancient tree populations in China, the aims of the work described in this chapter are as follows:

- 1) To evaluate the level of genetic diversity,
- 2) To explore the genetic structure among current ancient tree populations
- 3) To estimate the demographic history.
- 4) To compare the genetic diversity between Japanese and Chinese *Cryptomeria*.

The intention was to gain insights into the genetic status of the ancient tree resource of Chinese *Cryptomeria*.

## **2.2 Materials and methods**

### **2.2.1 Population sampling**

I investigated the sites where natural forests supposedly occurred in China, according to previous studies (Wang et al., 2007). Unfortunately, most forests have been subject to severe disturbance as a result of human activities and the species is now found only in patches in villages and national forest parks. To avoid material from unknown sources, only ancient trees with DBH 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 2.2). The name, geographic location, altitude and sample size for each population are listed in Table 2.1. I 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 (Tsumura et al., 2020) (Table 2.2)

### **2.2.2 DNA extraction and RAD-seq library construction**

The total genomic DNA was extracted from fresh needles using a modified CTAB method (Zhang, 2014). Purified DNA was digested with *Pst*I and *Sph*I, ligated with Y-shaped adaptors, amplified by PCR with KAPA HiFi polymerase (KAPA BIOSYSTEMS). After PCR amplification with adapter-specific primer pairs (Access Array Barcode Library for Illumina, Fluidigm), 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. (Ueno et al., 2019). 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.

### **2.2.3 SNP calling and filtering**

SNPs were called by dDocent (version 2.17) (Puritz et al., 2014) which is a pipeline containing a series of statistical tools. Because no reference genome was available for *C. japonica*, a reference was constructed using the dDocent de novo assembly and optimized utilizing the reference optimization steps provided in the dDocent assembly tutorial. I followed the default settings of dDocent for mapping and SNP calling, and the resulting vcf-file was used for filtering by vcftools (Danecek et al., 2011) in the dDocent environment. Specifically, for the first filtering operation, sites with >50% missing data across all individuals, and sites with a minor allele count <3 and quality value <30 were excluded. Secondly, I removed individuals with >10% missing data, and further filtered SNPs with the following criteria: mean depth  $\geq 20$ , the proportion of missing data >95%, a Minor Allele Frequency (MAF)  $\geq 0.05$ . In addition, I removed sites that deviated greatly from Hardy-Weinberg equilibrium within populations and thinned sites that were tightly linked at < 1 kb intervals using VCFtools. For stairway plot analysis, I included all SNPs located less than 1 kb apart with no MAF filtering.

### **2.2.4 Genetic diversity and genetic differentiation**

Neutral loci tests of the genotype data for all populations and markers were conducted using BayeScan (Foll & Gaggiotti, 2008) and the 'Fsthet' package (Flanagan & Jones, 2017). Genetic indices, such as the number of alleles ( $N_a$ ), the number of

effective alleles ( $N_e$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ) and Fixation index ( $F_{IS}$ ) were estimated within each population using GenALEx 6.502 (Peakall & Smouse, 2012). HP-Rare v.1.1 (Kalinowski, 2005) was employed to calculate allelic richness ( $Ar$ ) and private allelic richness ( $pAr$ ) with the minimum sample size of eight.

To examine differences between populations, genetic differentiation coefficients were calculated following Weir & Cockerham's method (Weir & Cockerham, 1984) in the R 'hierfstat' package. Hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) 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 (ROUSSET, 2008). Genetic distance matrices of pairwise population  $F_{ST}$  and pairwise population gene flow were also calculated in GenALEx 6.502.

### 2.2.5 Population structure

I inferred the most likely number of genetic clusters using Structure v.2.3.4 (Pritchard et al., 2000): 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 (Earl, 2012) to determine the most likely number of clusters according to  $\Delta K$  (Evanno et al., 2005) and mean  $\text{LnP}(K)$  (Pritchard et al., 2000). CLUMPP v.1.1 (Jakobsson & Rosenberg, 2007) was then used to calculate the average pairwise similarity of runs based on the Greedy method, finally the outputs of CLUMPP were visualized in Distruct v.1.1 (Rosenberg, 2004).

The pairwise  $F_{ST}$  distance matrix was used to generate a dendrogram in MEGA v.7.0.26 (Kumar et al., 2016) using the Neighbor-joining method (Saitou & Nei, 1987) and a network in SplitsTree v.4.14.8 (Huson & Bryant, 2006) using the Neighbor-Net method (Bryant & Moulton, 2004). In addition, I 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, implemented in GenAlEx.

In order to assess the relationship structure within each population, I employed the COANCESTRY software to calculate the pairwise relatedness for all individuals with a Wang estimator (Wang, 2017). These relatedness coefficients ( $r$ ) vary from 0 to 1, and a value of 0.5 indicates that individuals are first-order relatives, such as parent-

offspring or full-siblings. A value of 0.25 indicates second-order relatedness, such as half-sibling, grandparent-grandchildren, avuncular, or double first cousins.

### 2.2.6. Demographic history

The variation in effective population size ( $N_e$ ) over time was inferred using the composite likelihood approach with a multi-epoch model implemented in the Stairway plot software (Liu & Fu, 2015). This method evaluates the difference between the observed site frequency spectrum (SFS) and its expectation under a specific demographic history (Khimoun et al., 2020). The software was run using the two-epoch method, following the recommended 67% of sites for training and 200 bootstraps on the folded SFS. I excluded singletons from the estimation to minimize errors due to genotype calling. I assumed a mutation rate per generation of  $1.50 \times 10^{-9}$  based on previous studies by Moriguchi et al. (Moriguchi et al., 2019) and  $0.59 \times 10^{-7}$  by Kusumi et al. (Kusumi et al., 2015). *Cryptomeria japonica* is a long-lived species and there are many ancient trees older than 1000 years in the wild. Suzuki and Susukida (Suzuki & Susukida, 1989) estimated that 100 to 300 years were necessary for regeneration of natural forest on Yakushima Island, thus I set the generation time to 150, 200 or 300 years in different runs.

## 2.3. Results

### 2.3.1 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 loci did not depart from neutrality according to the result of 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  $H_o = 0.187$  to  $0.307$  and  $H_e = 0.174$  to  $0.316$  with an average of  $H_o = 0.269$  and  $H_e = 0.253$ . 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 in WT (Table 2.3).

The overall population differentiation coefficient ( $F_{ST}$ ) among all Chinese

populations for the 922 loci was 0.134 (Weir and Cockerham's method). AMOVA results (Table 2.4) showed that the proportion of variation between populations was 12%, between individuals it was 12% and within individuals it was 76%; the majority of variation occurred within individuals. Pairwise  $F_{ST}$  and pairwise  $Nm$  for each population in this study suggested significant differentiation in every pair of populations, and gene flow ( $Nm$ ) varied widely between different pairs. The greatest gene flow occurred between populations TBY and WYS (7.846) and the lowest between populations YTG and WT (0.696) (Table 2.5).

Six Japanese populations were also sequenced and merged with the Chinese populations into a CHN-JPN dataset. I 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 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 2.6). In addition, higher genetic differentiation among Chinese populations ( $F_{ST}=0.1204$ ) was detected than among Japanese populations ( $F_{ST}=0.0455$ ).

### 2.3.2. Genetic structure

I explored the genetic structure of Chinese and Japanese populations based on 183 SNPs. Two groups were clearly identified, but population LS was placed with the Japanese group in the network (Figure 2.3).

I subsequently analyzed the genetic structure of Chinese populations using the 922 SNPs. The Bayesian cluster analysis assigned the seven populations into four distinct clusters (Figure 2.4). The results based on  $\Delta K$  and mean  $\text{LnP}(K)$  indicated optimal values of 4 and 5, respectively (Figure 2.5). 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. 2.6).

In summary, I 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*.

I 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. 2.7). No isolation by distance (IBD) was found. Here I considered six populations, excluding LS because this population appears to be an old plantation derived from Japanese stock.

### **2.3.3. Demographic history**

I obtained very similar trends in the three different scenarios (generation time=150, 200 and 300 years) and considering two different possible mutation rates (mutation rate/per generation =  $1.59 \times 10^{-9}$  and  $0.59 \times 10^{-7}$ ), namely that the effective population size of Chinese *Cryptomeria* has experienced a continuous decline from the mid-Pleistocene to the present. Based on a mutation rate of  $1.59 \times 10^{-9}$ , 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 (Figure 2.8). The second decline began ca. 0.1 Mya to 0.06 Mya BP when the Last Glacial Period (LGP) commenced. Based on a mutation rate of  $0.59 \times 10^{-7}$ , the first decline occurred in the period of the Last Glacial Maximum (LGM), and the second decline was recent, i.e. within the last 2000 years (Figure 2.9). In both simulations, the range of *Cryptomeria* in China did not increase, but continued to decline through the Holocene.

## **2.4. Discussion**

### **2.4.1. An old plantation of Japanese origin**

The NeighborNet based on  $F_{ST}$  revealed a clear separation between Chinese and Japanese *Cryptomeria* (Figure 2.3), 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 1<sup>st</sup> century

BC, there was trade and the movement of people between Japan and China. Indeed, this kind of relationship has continued to the present. 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 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. I, therefore, consider population LS to be a Japanese *Cryptomeria* population based on my 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 Bayesian clustering (Figure. 2.4). 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 2.1a). The *Cryptomeria* forest on Lushan mountain is, therefore, a mix of Chinese and Japanese *Cryptomeria*, and I found no significant phenotypic difference between them during my field investigation.

#### **2.4.2. Low genetic diversity and high genetic differentiation in Chinese *Cryptomeria***

Ancient tree populations of *C. japonica* var. *sinensis* in China harbor very low genetic diversity but there is high genetic differentiation between populations compared to its congener, *C. japonica* in Japan. Here I excluded the LS population when discussing genetic diversity and differentiation of Chinese *Cryptomeria*. Previous studies such as those by Chen et al. (Chen et al., 2008) and Tsumura et al. (Tsumura et al., 2020) also indicated 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 (Furlan et al., 2012). In this study, I found evidence of a continuous decline in effective population size since the Last Glacial Period. 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. I speculate that habitat loss or degradation and artificial selection caused by intense human activities have further accelerated the decline of 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, I



detected restricted gene flow ( $Nm=1.858$ ) between all the ancient tree populations, lower than the normal value of  $Nm > 3$  in conifers (Ledig, 1998). Given that 76% of variation occurred within populations, I consider that limited gene flow is also a factor accounting for the low genetic diversity. However, I found no sign of inbreeding in any population except WT. Relatedness analysis also revealed a low proportion of kinship in most cases except populations WT and YTG (Figure 2.10). The low diversity in WT may be caused by family relationships, while in YTG, even with the small sample size (only 8), no inbreeding was detected ( $F_{IS} = -0.123$ ). As for the other Chinese *Cryptomeria* populations, I believe that the low level of genetic diversity can be attributed to climate change and intense human activity. Interestingly, three populations (WYS, YTG and XTM) showed signs of significant excess heterozygosity, which I consider probably to be the consequence of selection. However,  $F_{IS}$  values of the three populations are not particularly high (WYS=-0.087, TBY=-0.023 and XTM=-0.082, Table 2.3) 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 2.11).

Similar situations, with low genetic variation and high genetic differentiation have been found in isolated and threatened species such as *Tsuga caroliniana* (Potter et al., 2017), *Podocarpus sellowii* (Dantas et al., 2015) and *Lupinus alopecuroides* (Vásquez et al., 2016). 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.

#### **2.4.3. Specific genetic structure with an absence of IBD**

I 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 2.6). A certain mixing of the two groups occurred in the contact population, WYS.  $\text{Ln}(K)$  provided an alternative optimal structure that showed

differentiation within the Fujian province group when  $K=5$  (Figure 2.4, 2.5). I noticed that it was YTG, not the contact population of WYS, that was separated. As mentioned before, this that may relate to the small sample size of that population.

Even though clear genetic structure was found, I did not detect a significant correlation between geographical distance and genetic distance, although Japanese *Cryptomeria* does exhibit such a correlation (Tsumura et al., 2007). Generally, most tree species exhibit clear isolation by distance (IBD) if there is no strong human disturbance and selection (Aoki et al., 2014; Tsumura et al., 2007). 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, 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. (Tsumura et al., 2020) and Mori et al. (Mori et al., 2020). On the other hand, the resulting habitat fragmentation also led to great genetic differentiation. I think that the reason for the unexpected absence of IBD is probably human disturbance. The topography of the range investigated, southern China, is characterized by numerous plains and basins between low hills (Cao et al., 2018). 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 (Warmuth et al., 2013). Thus, I 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 I 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 – I 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, I found a similar structure pattern, namely an absence of IBD, as for other species in southern China, including *Miscanthus lutarioriparius* (Yang et al., 2019), *Houpoea*

*officinalis* (Yang et al., 2018) and *Brasenia schreberi* (Li et al., 2018). This may indicate similar evolutionary history under anthropogenic pressure.

#### **2.4.4. Continuous decline of population size without postglacial recolonization**

Climate oscillations throughout the Late Quaternary had a dramatic effect on species ranges of both plants and animals in subtropical mainland Asia and the Japanese Archipelago (Harrison et al., 2001). The same goes for Chinese *Cryptomeria*: a remarkable decline in effective population size was detected since the LGM. However, unlike many widely spread temperate plant species in Japan and East China (Qiu et al., 2009), there was no recolonization after contractions during the LGM, but the population kept declining through the Holocene. Tsumura et al. (Tsumura et al., 2020) also did not find an obvious range expansion in the mid-Holocene and at present using Species Distribution Modelling (SDM). There are two possible explanations for the continuous decline in population size after the LGM. First, the  $N_e$  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 (Yang et al., 2018). Similar cases can be seen in other plant species in eastern China, including the genus *Croomia* (Li et al., 2008), *Davidia involucrate* (Chen et al., 2020), *Ostrya rehderiana* (Yang et al., 2018) and *Kalopanax septemlobus* (Sakaguchi et al., 2012).

In Japan, the range of *Cryptomeria* contracted to several refugia, mainly concentrated in the southwestern part of the country during the last glaciation (Tsukada, 1986), and some natural stands have been retained up to the present. Japan may have had a favorable environment, with sufficient precipitation and fertile soil (Tsumura et al., 2020), 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.

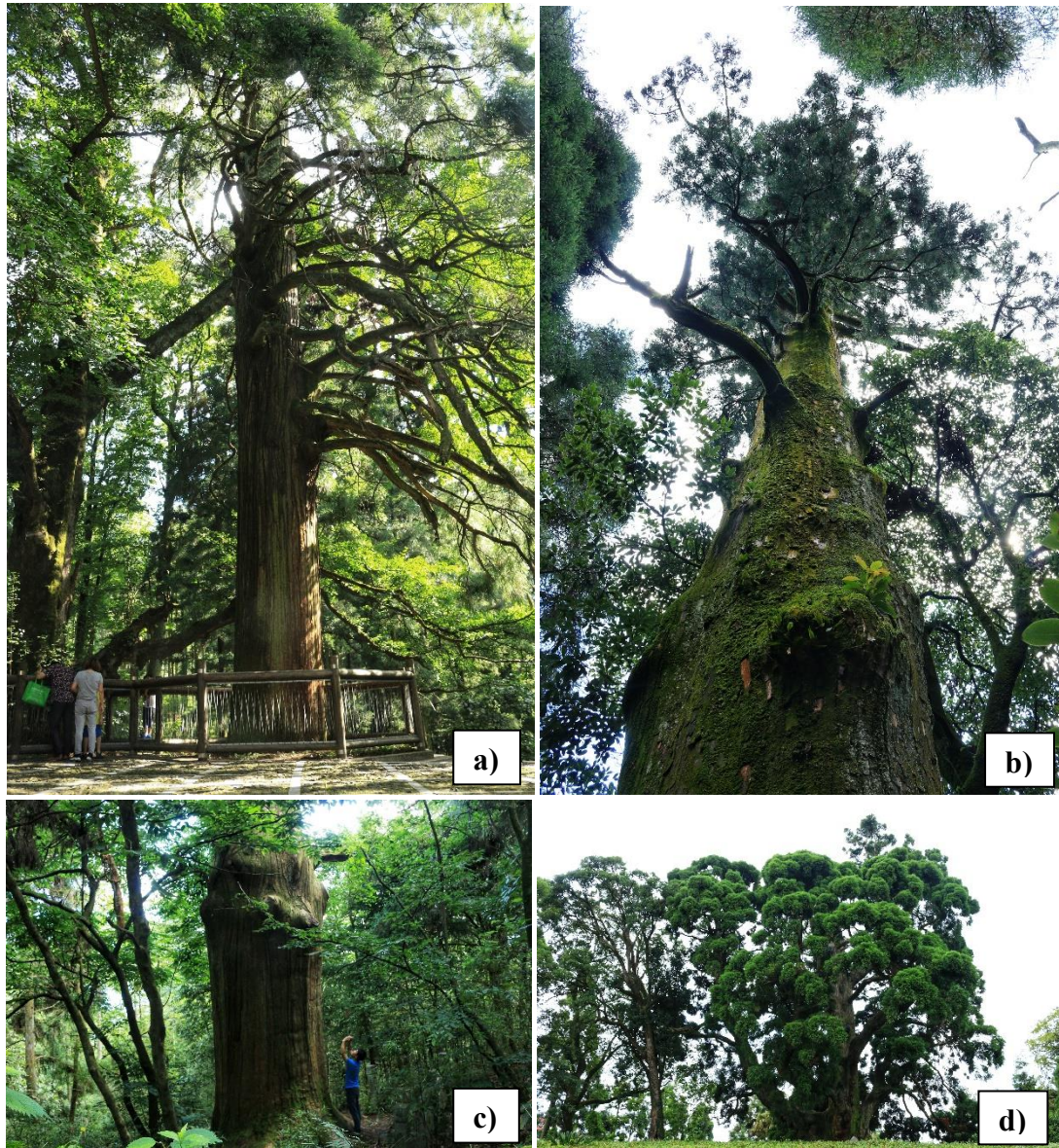


Figure 2. 1 Ancient trees of Chinese *Cryptomeria* investigated in our study; these are located in a) Lushan Mountain, Jiangxi province, b) Mangdang Mountain, Fujian province, c) Tianmu mountain, Zhejiang province, d) Guling forest park, Fujian province.

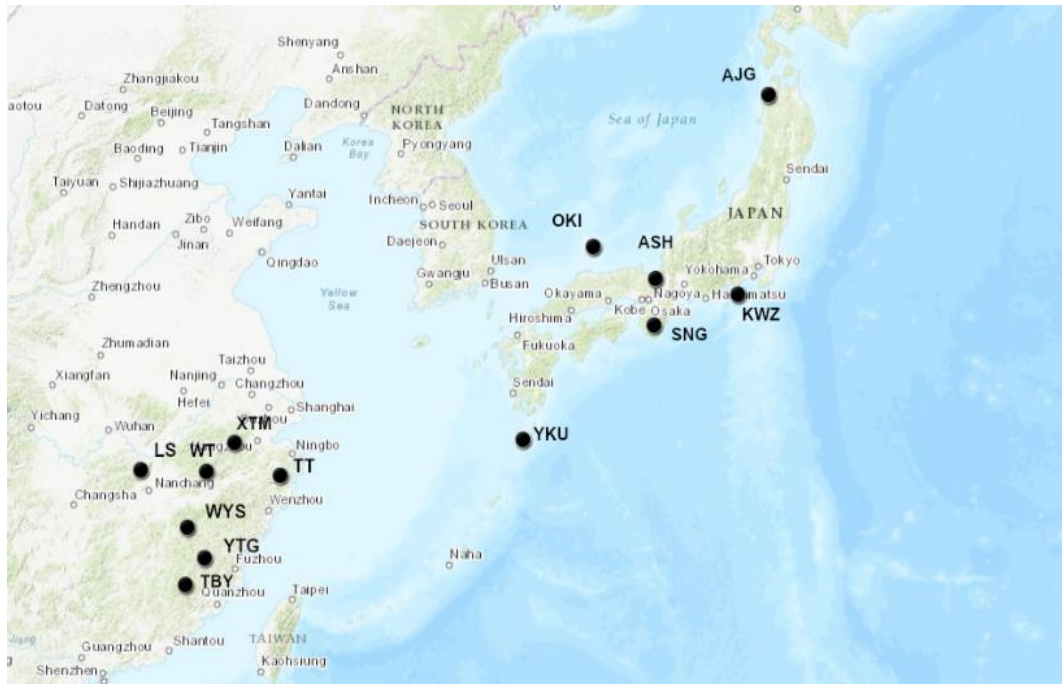


Figure 2. 2 Geographic locations of the seven Chinese *Cryptomeria* populations and six Japanese *Cryptomeria* populations.

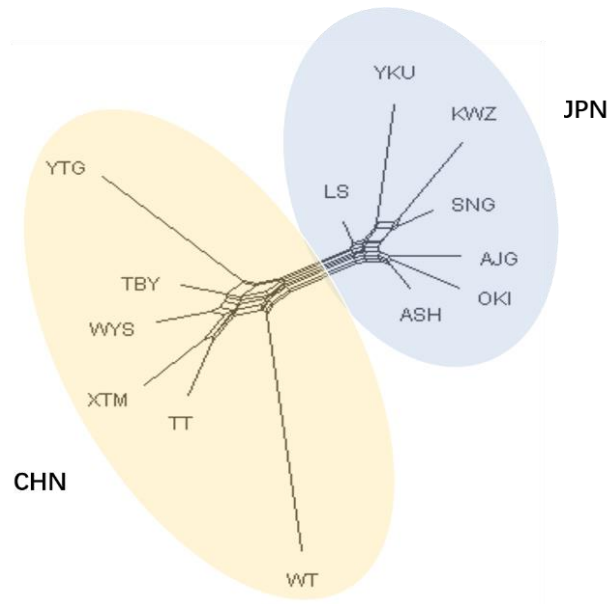


Figure 2. 3 NeighborNet based on the pairwise  $F_{ST}$  matrix of Chinese and Japanese populations

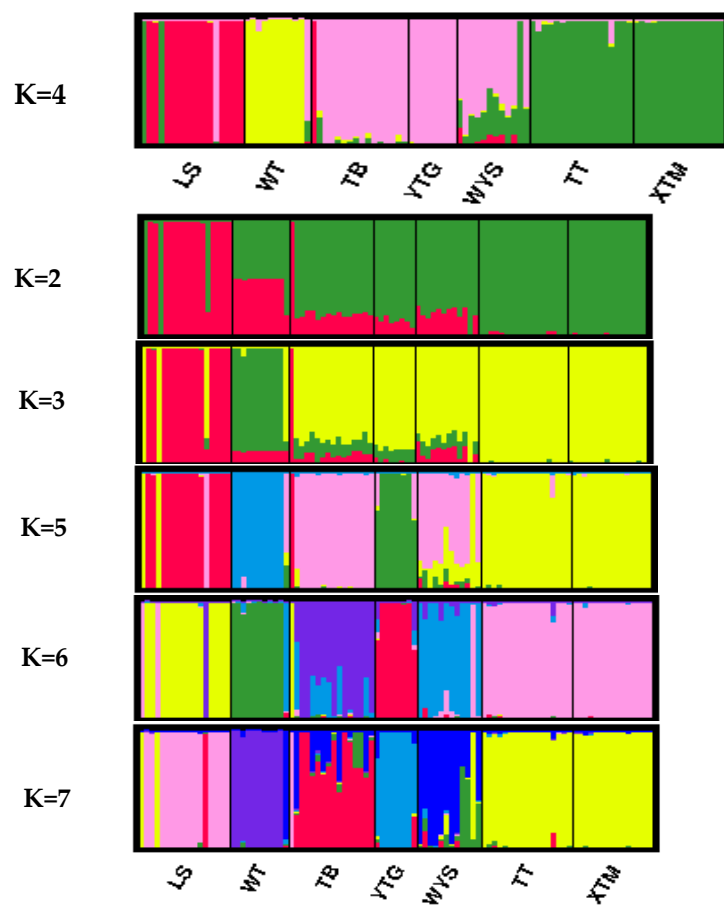


Figure 2. 4 Population genetic structure of seven populations of *C. japonica* var. *sinensis* according to Structure.

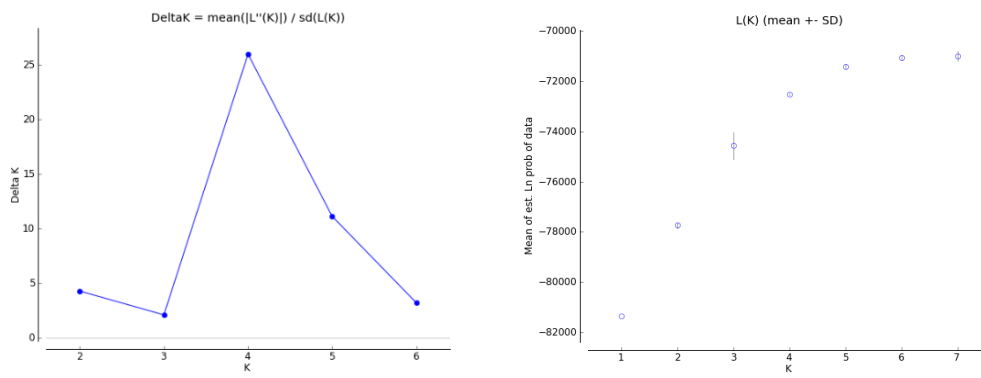


Figure 2. 5 The number of inferred clusters  $K$  based on  $\Delta K$  and mean  $\text{LnP}(K)$  obtained from Structure Harvester.



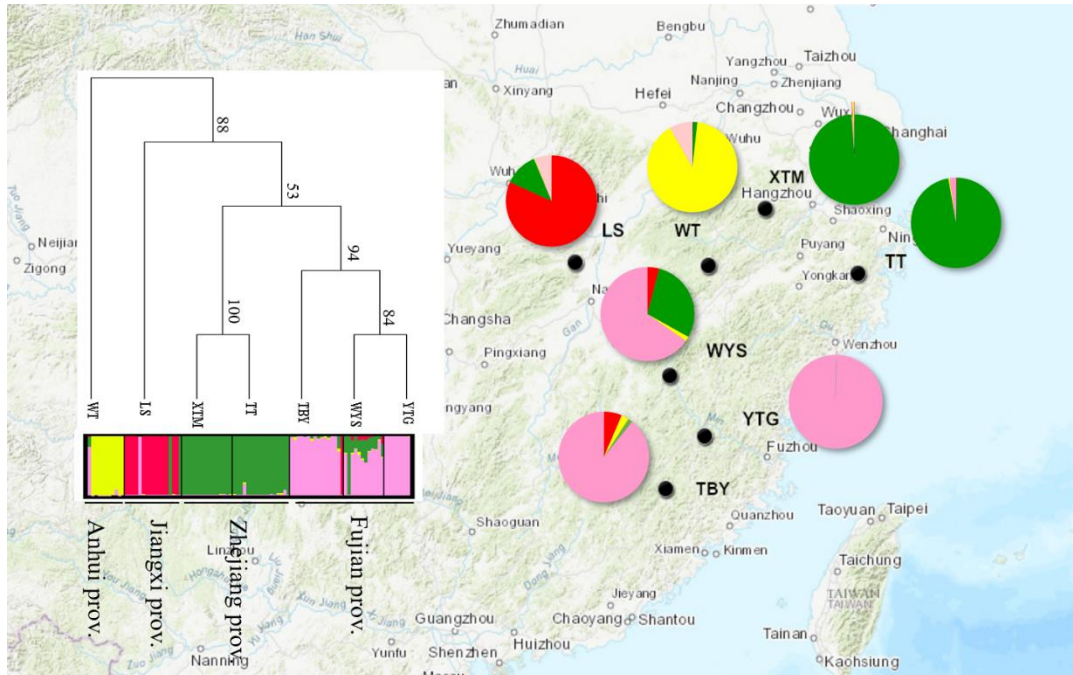


Figure 2. 6 Genetic structure of *C. japonica* var. *sinensis* based on 922 SNPs. The pie charts on the map represent the membership coefficients of the four clusters inferred in Structure. The Neighbor-joining dendrogram is based on pairwise  $F_{ST}$  values with 1000 bootstraps.

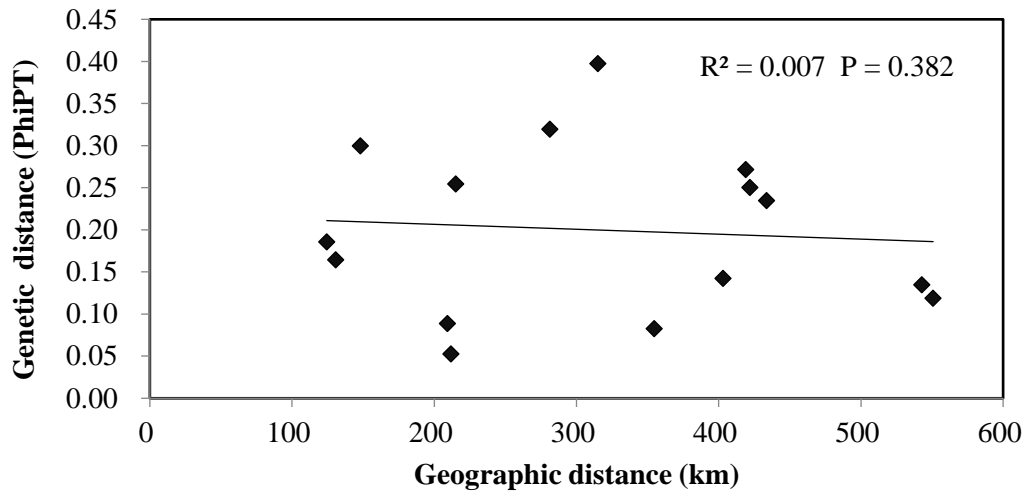


Figure 2. 7 Mantel test between geographical distance (km) and genetic distance of six ancient populations of Chinese *Cryptomeria* based on 922 SNPs. Suspected Japanese population of LS was excluded.

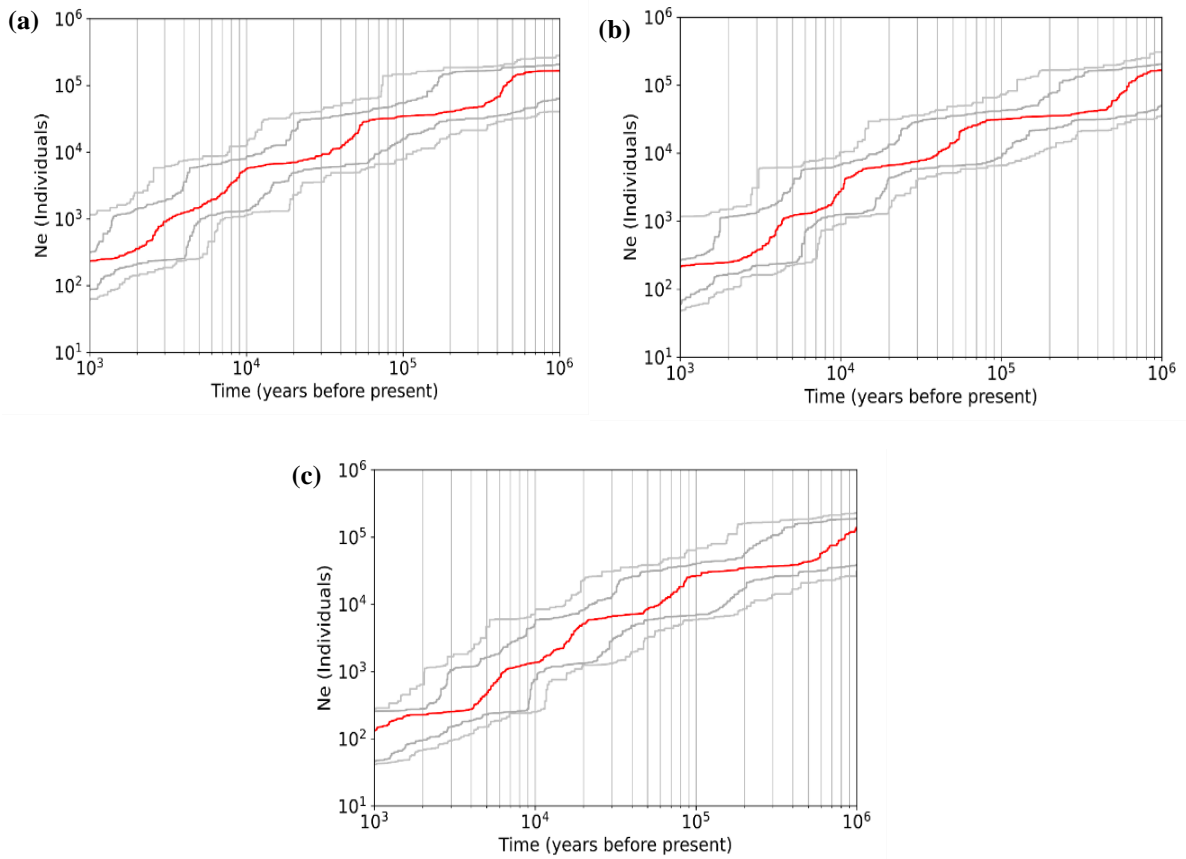


Figure 2. 8 Effective population size ( $N_e$ ) estimated based on folded SFS with a generation time of a) 150 years, b) 200 years and c) 300 years with a mutation rate of  $1.50 \times 10^{-9}$ , according to Stairway Plot software. Red and grey lines represent the medians and the 2.5 and 97.5 percentiles, respectively.

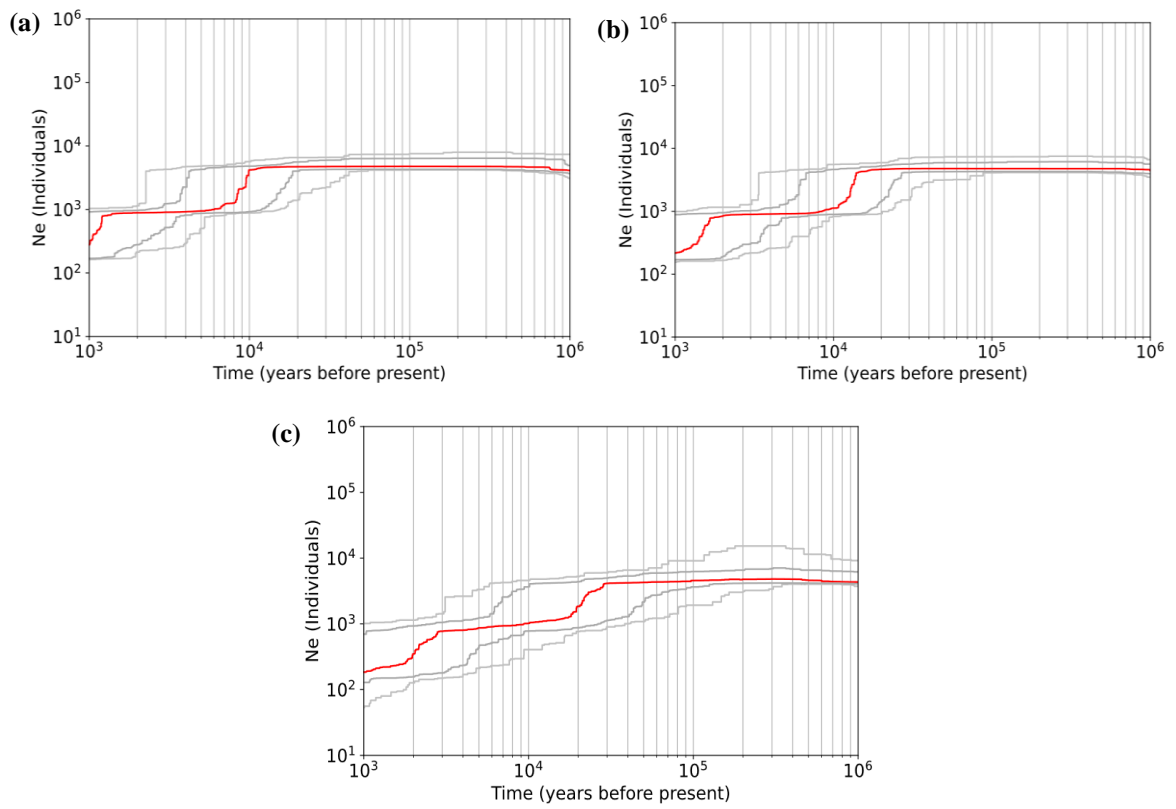


Figure 2. 9 Effective population size ( $N_e$ ) estimated based on folded SFS with a generation time of a) 150 years, b) 200 years and c) 300 years with a mutation rate of  $0.59 \times 10^{-7}$ , according to Stairway Plot software. Red and grey lines represent the medians and the 2.5 and 97.5 percentiles, respectively.

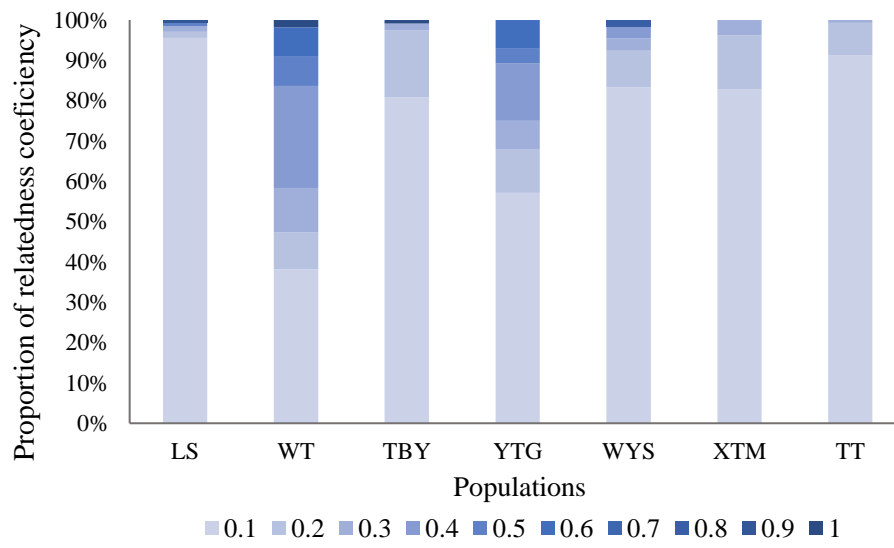


Figure 2. 10 Relatedness analysis within seven ancient populations. Darker blue indicates a higher relatedness coefficient.

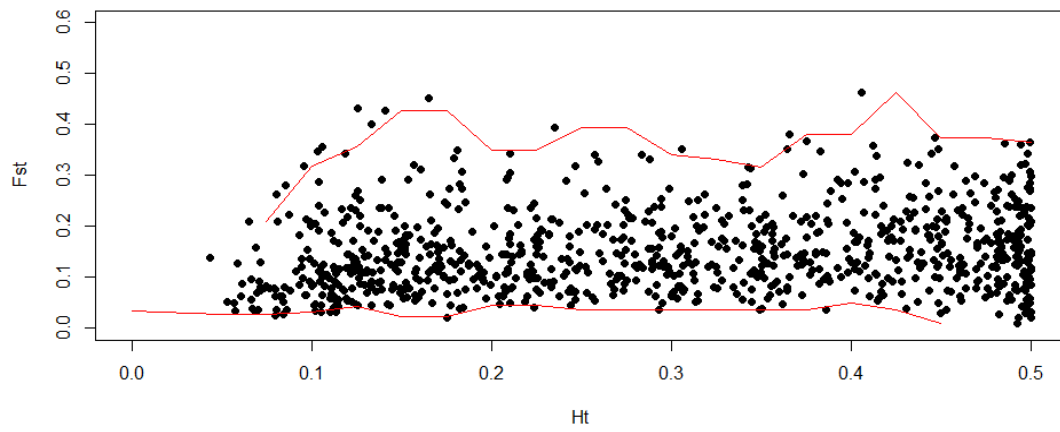


Figure 2. 11 Distribution of the  $F_{ST}$ - $H_T$  (expected heterozygosity) relationship based on 922 loci in *C. japonica* var *sinensis*. The two red lines delimit the confidence interval. A total of 20 outlier loci were detected among 922 loci.

Table 2. 1 Geographic locations and sample sizes ( $N$ ) of the seven ancient populations of Chinese *Cryptomeria*

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

Table 2. 2 Geographic locations and sample sizes (*N*) of the six Japanese *Cryptomeria* populations

Population					
Prefecture	Name	Longitude (E)	Latitude (N)	Altitude (m)	<i>N</i>
Amori	Ajigasawa (AJG)	140.2053	40.6757	319	13
Akata	Nibetsu (NBT)	140.26	39.8061	315	15
Shizuoka	Kawazu (KWZ)	139	34.8314	664	8
Kyoto	Ashu (ASH)	135.7739	35.3078	802	18
Shimane	Oki (OKI)	133.3292	36.2683	363	9
Wakayama	Shingu (SNG)	135.71	33.89	592	15
Kagoshima	Yakushima (YKU)	130.5731	30.3035	1071	5
Total					83



Table 2. 3 Genetic diversity indices of the seven ancient populations of *C. japonica* var. *sinensis* based on 922 loci.

<b>Population</b>	<b><i>N</i></b>	<b><i>Na</i></b>	<b><i>Ne</i></b>	<b><i>Ho</i></b>	<b><i>He</i></b>	<b><i>F<sub>IS</sub></i></b>	<b><i>Ar</i></b>	<b><i>pAr</i></b>
LS	16	1.939	1.532	0.307	0.316	0.023*	1.77	0.03
WT	11	1.55	1.309	0.187	0.174	0.023*	1.42	0
TBY	15	1.9	1.458	0.286	0.277	-0.023	1.7	0.01
YTG	8	1.614	1.351	0.239	0.209	-0.123*	1.53	0
WYS	11	1.871	1.466	0.306	0.279	-0.087*	1.71	0
TT	17	1.838	1.429	0.266	0.257	-0.033	1.64	0.01
XTM	15	1.807	1.427	0.279	0.257	-0.082*	1.64	0.01
Total	13.3	1.789	1.424	0.267	0.253	-0.042	1.63	0.0086

*N*: Sample Size, *Na*: No. Alleles, *Ne*: No. Effective Alleles, *Ho*: Observed Heterozygosity, *He*: Expected Heterozygosity, *F<sub>IS</sub>*: Fixation Index, *Ar*: Allelic richness, *pAr*: private allelic richness

\*Significance (> confidence Interval 99%)

Table 2. 4 Analysis of Molecular Variance (AMOVA) for the seven ancient populations of *C. japonica* var. *sinensis*.

<b>Source</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>Est. Var.</b>	<b>%</b>
Between Populations	6	4033.190	672.198	18.885	12%
Between Individuals	89	14142.633	158.906	18.552	12%
Within Individual	96	11693.000	121.802	121.802	76%
Total	191	29868.823		159.239	100%
<i>F<sub>ST</sub></i>	0.119				
<i>N<sub>m</sub></i>	1.858				

df = degrees of freedom, SS = sum of squares, MS mean squares, Est. var. = estimate of variance, % = percentage of total variation.

Table 2. 5 Pairwise  $Nm$  (above the diagonal) and pairwise genetic differentiation ( $F_{ST}$ ) (below the diagonal) of the seven ancient 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

Table 2. 6 Genetic diversity indices of six populations of *C. japonica* and seven populations of *C. japonica* var. *sinensis* based on 183 loci.

<b>Population</b>	<b><i>N</i></b>	<b><i>Na</i></b>	<b><i>Ne</i></b>	<b><i>Ho</i></b>	<b><i>He</i></b>	<b><i>F<sub>IS</sub></i></b>
AJG	12.366	1.847	1.390	0.254	0.243	-0.047
ASH	17.918	1.934	1.413	0.279	0.257	-0.067
OKI	8.940	1.858	1.391	0.278	0.248	-0.101
KWZ	7.967	1.787	1.406	0.277	0.248	-0.101
SNG	14.503	1.902	1.404	0.274	0.253	-0.087
YKU	5.874	1.721	1.355	0.239	0.221	-0.088
<b>Mean</b>	11.261	1.842	1.393	0.267	0.245	-0.082
LS	13.836	1.891	1.379	0.233	0.240	0.015
WT	10.623	1.240	1.128	0.076	0.073	0.056
TBY	14.393	1.623	1.241	0.155	0.152	-0.014
YTG	7.705	1.355	1.213	0.147	0.126	-0.131
WYS	11.120	1.530	1.244	0.163	0.152	-0.070
TT	16.787	1.464	1.202	0.128	0.125	-0.021
XTM	13.377	1.470	1.219	0.149	0.134	-0.092
<b>Mean</b>	12.549	1.511	1.232	0.150	0.143	-0.037

*N*: Sample Size, *Na*: No. Alleles, *Ne*: No. Effective Alleles, *Ho*: Observed Heterozygosity, *He*: Expected Heterozygosity, *F<sub>IS</sub>*: Fixation Index, *Ar*: Allelic richness, *pAr*: private allelic richness

### **Chapter 3. Genetic diversity and genetic structure of artificial populations and seed orchards**

In this chapter, a total of 248 samples that collected from ten plantations in four provinces and a total of 168 samples that collected from two seed orchards are used to examine the genetic composition of plantations and seed orchard of Chinese *Cryptomeria*. experiments and data treatment are same with the methods presented in Chapter2. In summary, I detect a low level of genetic diversity and specific genetic structure with that in ancient populations, namely, clustered by geographic provinces. However, I find two Sichuan population, plantation YA and plantation ZGS were likely introduced from Tiantai mountain, Zhejiang province. Two seed orchards, HY and XP, show the different level of genetic diversity with pretty high in XP and critically low in HY. That is because of the different genetic base. Finally, I proposal that plantations investigated in this study (except YA and ZGS) also should be conserved like ancient trees. Even though seed orchard XP holds high genetic diversity, it'll lead to disturbance of genetic structure due to its multi-source provenances. seed orchard HY should collect more genetic resources especially materials within Sichuan province.

## **Chapter 4. Genetic differences between ancient trees and artificial populations**

In this Chapter, two datasets that were used in Chapter 2 and Chapter 3 are combined and used to produce new SNPs data. In order to evaluate the genetic consequence of afforestation practice on Chinese *Cryptomeria*, two strongly suspected Japanese populations are excluded, and a total of six ancient populations, nine plantations and two seed orchards are examined in this Chapter. In summary, the similar level of genetic diversity and the similar pattern of genetic structure are detected in ancient populations and plantations, namely, clustered by geographic provinces, indicating that those plantations were derived from neighboring ancient trees or now-vanished natural stands, rather than seed orchards. The most important practice in the future is conserving as high as possible genetic diversity in wild and maintaining the current genetic structure.

## Chapter 5. General discussion and conclusion

### 5.1 General discussion

#### 5.1.1 Conservation strategies on genetic resources

This study highlights the less than optimistic situation in relation to the genetic resources of Chinese *Cryptomeria*. With the disappearance of natural forests, preserving genetic diversity and spatial structure has become particularly important. Herein, two methods are proposed to conserve the extant resource while meeting the demand for wood production.

##### 5.1.1.1 *Cryptomeria* forest zoning

To deliver ecological and economic sustainability of *Cryptomeria* forests, three zones with different management objectives are suggested.

- 1) Protected zone. The goal is conservation of the resource. Populations of ancient *Cryptomeria* trees are allocated to this zone. The ancient trees located in remote mountainous areas, such as Tianmu mountain, Tianbaoyan nature reserve and Wuyi mountain should be strictly monitored and supplementary actions undertaken to maintain vigorous growth. These actions would include thinning competing bamboo and pest control. Some ancient trees are located in human-dominated landscapes. Cutting and grazing should be completely prohibited in these areas and sightseeing activities should also be limited. In addition, propagation material from ancient trees should be collected and preserved *ex situ*. As revealed in my study, populations from Tianmu mountain (XTM) in Zhejiang province and Tianbaoyan nature reserve (TBY) in Fujian province have relatively high diversity and harbor some private alleles, these populations should be considered core resources.
- 2) Buffer zone. The goal is to extend the flexibility and adaptability of the protected zone while accommodating human use. Buffer zones are usually around the periphery of centrally protected zones and should have a genetic composition similar to the protected populations to prevent pollen contamination. The plantations investigated in this study (with the exception of YA and ZGS) are allocated to this zone. Logging is permitted, as long as it is in keeping with the natural regeneration of forests. Hunting, grazing and

agroforestry activities are also allowed to benefit local people in this area. However, the investigations of plantations presented in this study are insufficient and more buffer stands should be designated in the future.

- 3) Production zone. The main goal is timber production; generally, genetic structure can be ignored in this area. The more timber that can be extracted from this zone, the larger area that can be set aside for conservation. Thus, to achieve productivity gains, plus tree and genetically improved trees should be planted and silvicultural practices such as thinning and vegetation management implemented (Messier et al., 2009). In this study, a clear genetic structure was detected in both ancient populations and plantations of Chinese *Cryptomeria*. Therefore, to be safe, I suggest using seeds from the relevant seed division to create commercial plantations in the future.

#### **5.1.1.2 Seed zone division of Chinese *Cryptomeria***

Seed zones are essential tools in reforestation to ensure that seedlings are well adapted to the growing conditions of the planting site. Traditionally, seed zones are developed by series of provenance tests, aimed at maximizing tree growth and minimizing risk in different environments. For example, Japanese *Cryptomeria* has been separated into seven seed zones across the distribution range in Japan (Miura et al., 2009), and Chinese fir has been separated into nine zones in China (Chinese-Fir, 1988) by this method. Recently, genetic characteristics have also been applied for seed division in species such as *Pinus sylvestris* (Li et al., 2005) and *Picea asperata* (Luo et al., 2005). As the result of genetic structure analysis suggested in this study, and considering historical records, three separate provenances can be identified in southern China: the eastern region -- Zhejiang province (Tianmu mountain, Tiantai mountain); the southern region – Fujian province (Nanping area, Wuyi mountain, tianbaoyan area etc.); and the western region – Sichuan province (the western edge of Sichuan basin). Based on this division, seed orchards of Chinese *Cryptomeria* in each seed zone should be created as soon as possible, and the transfer of seeds between zones should be prohibited in order to maintain the highest level of adaptation and the genetic structure of this species. Additionally, Seed orchard XP contains very diverse material from the three regions (see Figure 4.5). To represent the provenance from Fujian, only materials



from Fujian province should be retained, and superior clones derived from other lineages should be used to establish the new seed orchards.

The data collected in this study, however, are still insufficient. Plantations in central areas such as Hubei, Jiangxi province, and in the southwest, such as Guangxi, Yunnan and Guizhou province were not examined. Most of the plantations in these areas lack clear provenance information. New divisions or subdivisions of the seed zones can be expected in the future.

### **5.1.2 Genetic resources status in other forest species in Southern China**

Apart from Chinese *Cryptomeria*, Chinese fir and Masson pine are the two main planted species in southern China, and occupied 19.01% and 6.51% of the area of all plantations in China according to the eighth national forest inventory data published in 2013. High genetic diversity and low genetic differentiation have been detected in both Chinese fir and Masson pine populations (Li et al., 2017; Peng et al., 2003). Li et al. (Li et al., 2017) found a clear genetic structure indicative of the geographic origins of ancient populations of Chinese fir, while Duan et al. (Duan et al., 2017) did not find any distinct structure in 700 clones of Chinese fir from six provenances. For Masson pine, research has revealed no significant correlation between genetic distance and geographic distance, but rather a significant positive correlation between genetic distance and latitude in both natural populations (Peng et al., 2003) and seed orchard collections (Xu et al., 2016). Compared to these two species, Chinese *Cryptomeria* has a low level of genetic diversity but a well-conserved genetic structure in ancient and artificial populations. It, therefore, follows that the top priority must be to protect genetic diversity while maintaining the current genetic structure. Great care is required in the management of the Chinese *Cryptomeria* genetic resource.

There have been studies identifying expressed genes associated with different stresses and pest resistance (Fan et al., 2020; Liu et al., 2017) as well as yield of oleoresin (Liu et al., 2015) in Masson pine, and studies on candidate genes involved in cambial and xylem activities (Huang et al., 2012; Wang et al., 2013; Zhang et al., 2016) in Chinese fir have been widely conducted. These studies provide a sound basis for future genomic research into Chinese *Cryptomeria*.

## 5.2 General conclusions

Although the Chinese *Cryptomeria* population on Tianmu mountain has been studied and its low genetic diversity has been reported before, as far as I know, this is the first study looking at the genetic diversity of Chinese *Cryptomeria* including ancient trees, plantations and seed orchards across the whole distribution range. The findings confirm that the ancient *Cryptomeria* population in China contains a low level of genetic diversity and high differentiation. Populations in different provinces are genetically differentiated, but no IBD was detected. I postulate that 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 clear differentiation between regions. Plantations in southern China have relatively high genetic diversity and similar genetic structure to that of ancient tree populations. The seed orchard in Xiapu state-owned forest nursery contains a high level of genetic diversity, while the seed stand in Hongya state-owned forest nursery has critically low diversity but is highly differentiated. The theoretical guidance for conservation and afforestation work in the future suggested here should assist in preserving the genetic resource of this valuable species.

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