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GENOTYPING-BY-SEQUENCING REVEALS THE GENETIC DIVERSITY OF *CRYPTOMERIA JAPONICA* VAR. *SINENSIS* IN SOUTHEASTERN CHINA

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Abstract

Cryptomeria japonica var. *sinensis* is a valuable timber and ecological tree species in East Asia. Despite previous research that has revealed significant differences in tree height, diameter at breast height, and wood volume among *C. japonica* var. *sinensis* from different geographical sources, their genetic structure and phylogenetic relationship among different populations are still unable to accurately determined. Therefore, we extensively collected different geographical sources of *Cryptomeria* spp. in China (Fujian, Zhejiang area) and Japan, and performed their genetic structure and affinities by using simplified genome sequencing (GBS). A total of 306.5 Gb of sequence raw data and 875,329 high-quality SNPs were obtained, with an average of 4.26 Gb of raw data per sample. The results showed that the genetic variation of *C. japonica* var. *sinensis* populations was lower than that of *C. japonica* populations, and the genetic diversity of *C. japonica* var. *sinensis* in Fujian populations was slightly higher than that of Zhejiang populations. At the same time, molecular analysis of variance showed that the genetic variation of these two populations mainly came from within the population (99.6%). Furthermore, the cluster analysis results showed that *C. japonica* var. *sinensis* was clustered into five groups, and the clustering results were still consistent with the actual geographical distribution. All findings clarified the genetic background and structure of *Cryptomeria* spp. This will be a reference for the conservation, utilization, and genetic breeding improvement of *C. japonica* var. *sinensis* germplasm resources.

Key words: *Cryptomeria japonica* var. *sinensis*; *Cryptomeria japonica;* GBS; Genetic diversity; SNP.

Introduction

Cryptomeria (Cupressaceae) spp. are some ecologically and economically important coniferous species in East Asia (Zhao *et al*., 2014; Wen *et al*., 2022), and a relic genus of the Tertiary period and intermittently distributed in China and Japan (Yu *et al*., 1995). Up to date, three varieties of *Cryptomeria japonica* (Linn. f.) D. Don, are currently and extensively recognized worldwide, containing *Cryptomeria japonica* var. *japonica* and *Cryptomeria japonica* var. *radicans* from Japan,known as "the national tree" of Japan, and *Cryptomeria japonica* var. *sinensis* from southeast China (Hayashi, 1960), which plays a crucial part in the forestry industry in southern China. However, the frequent introduction and cultivation of *C. japonica* var. *sinensis* germplasm in East Asia has led to ambiguity in the historical origin and affinities of *C. japonica* var. *sinensis*.

Previous studies have been demonstrated morphological difference (Lu *et al*., 2023) and genetic structure variation (Xu *et al*., 2014) within different populations. Some superior families and varieties of *C. japonica* var. *sinensis* have been screened by using traditional breeding methods (Xie, 2008; Huang, 2010), and the next- generation sequencing (NGS) technologies have been widely applied to determine the genetic diversity of germplasm resources of several coniferous species (Jin *et al*., 2016; Duan *et al*., 2017; Ikeda *et al*., 2019; Jia *et al*., 2020; Santiniet *et al*., 2020; Lee *et al*., 2022; Klapste *et al*., 2022), including genetic diversity and relationship of *Cryptomeria* (Cupressaceae) spp. by using SSR markers (Liu *et al*., 2012), microsatellite markers, and constructing a RAD-seq database (Cai *et al*., 2023), while the population genetics of *C. japonica* in Japan have also been determined by using polymorphic STS marker (Tsumura & Tomaru, 1999), RAPD marker (Shimizu *et al*., 2002) and SNP marker (Uchiyama *et al*., 2014). All of these methods and findings will be helpful to reveal potentially desirable genes and deepen our understanding of the genetic diversity, ancestral origins and population microevolution of *C. japonica* var. *sinensis* (Guo *et al*., 2015).

Technically, Genotyping-by -sequencing (GBS) has gradually become an effective method for genome-wide identification of genetic gene variation, which was extensively adopted to obtain SNP markers and successfully analyzed the genetic diversity of 70 European blueberry samples (Campa & Ferreira, 2018), 320 peanut germplasm samples (Zheng *et al*., 2018), 270 Indian rice samples (Liang *et al*., 2018), 68 watermelon materials, and other species (Migicovsky *et al*., 2022). With the advantages of low cost, high density and coverage of molecular markers, high flexibility, and independence of reference genome (Elshire *et al*., 2011; Poland & Rife, 2012; Peterson *et al*., 2014; Wallace & Mitchell, 2017), it has great potential and broad prospects for application in forest genetic breeding and population research (Chung *et al*., 2017).

In this work, 69 samples of *C. japonica* var. *sinensis* from Fujian and Zhejiang, China; were used to develop genetic markers and determine the genetic structure of *C. japonica* var. *sinensis* populations, and 3 samples from Japanese varieties were used as control to compare the genetic diversity of *C. japonica* and *C. japonica* var. *sinensis*, and historical origin and evolutionary relationship

of *C. japonica* var. *sinensis* populations in Fujian, Zhejiang, and Japan. The effectiveness and feasibility of GBS in the application of genetic diversity in *C. japonica* var. *sinensis* were also evaluated.

Material and Methods

Plant materials: The collected samples were preserved in liquid nitrogen and placed in an ultra-low temperature refrigerator at -80°C. The asexual lines were selected from nine different geographical sources, and one healthy *C. japonica* var. *sinensis* individual was randomly selected from each asexual line. The asexual lines of *C. japonica* var. *sinensis* in this study were provided by the Yangmeiling National Forest Farm in Fujian Province, including asexual accessions from three Japanese geographical sources, 34 from Fujian geographical sources, and from 35 Zhejiang geographical sources. Fujian sources were collected from the regions of Laizhou, Xikou, Huanglong Shuimen, Xiapu Yangmeiling, and Layang, with the Zhejiang were from Tianmu and Shiyang (Table 1).

DNA extraction: Genomic DNA was isolated from dried and young branches and leaves using the Plant Genomic DNA Kit (Tiangen, Beijing, China) following protocol. After extraction, DNA integrity was analyzed by agarose gel electrophoresis. DNA purity (OD260/OD280 ratio) was tested using Nanodrop. The DNA concentration for the library was quantified using Qubit. DNA samples were immediately stored at -80°C for later use after quality control.

Library Construction and Sequencing: The extracted genomic DNA was randomly broken into short DNA fragments by EcoRI and NIaIII (New England Biolabs, Ipswich, MA) enzymes and then repaired with flat ends; dA tails were attached to both ends of the DNA fragments and sequencing connectors were attached; the DNA fragments with connectors were purified by AMPure XP magnetic beads and fragments in the range of 300-400bp were selected for PCR amplification; finally, the constructed libraries were sequenced by Paired end 150 using the Illumina HiSeq sequencing platform.

SNP identification: Since there is currently no genomic information for *C. japonica* var. *sinensis*, the way to detect SNPs is different, and it is generally referred to as population SNP analysis. SNP development was performed by comparing the resulting sample sequence fragments to a reference genome according to the method of Julian *et al*., (2015). The processed alignment files were tested by the software GATK (3.4-46) for Variant detection of multiple samples, SNPs were filtered using GATK's Variant Filtration with proper standards (-Window 4， -filter "QD<2.0 ||FS>60.0|| MQ<40.0 "， -G_filter "GQ<20").

Genetic diversity analysis and population genetic distance: After excluding SNPs with MAF < 0.05 and deletion rate > 20% in each population, allele abundance (AR) was calculated by ADZE V1.0 software, polymorphism proportion (p), nucleotide diversity (Pi), polymorphic information content (PIC), observed heterozygosity (Ho) and expected heterozygosity (He) were calculated by PLINK software, and genetic distance between populations was calculated by PLINK and R software, where when the PIC value >0.5, it indicated high polymorphism, when 0.25 < PIC < 0.5, it indicated moderate polymorphism, and when the PIC value <0.25, it indicated low polymorphism (Botstein *et al*., 1980; Albert *et al*., 2009; Mark *et al*., 2011).

Population structure analysis: Three methods were used: (1) Based on the obtained SNP information, the Treebest software was used to calculate the inter-sample distances, and the phylogenetic tree was constructed using the neighbor-joining methods with 1000 calculations (bootstrap values) (Jian *et al*., 2013). (2) The eigenvectors and eigenvalues of PCA were calculated by GCTA according to the degree of individual differences of SNPs (Joshua & Akey, 2015). And the PCA distribution map was generated by R software. (3) Population structure analysis requires marker loci to be independent (Matthew & Peter, 2000), and the filtered high-quality SNPs, obtained after repeated filtering using LD (threshold R2≤0.05), were used for structure analysis. According to the calculation method of Bayesian model (Evanno *et al*., 2005), the structure analysis of SNPs was performed by Structure software (Francis, 2017).

Molecular variance analysis: To further understand the inter-group, intra-group, and inter-individual variation in performance, this study conducted an analysis of molecular variance (ANOVA, analysis of molecular variance) on two *C. japonica* var. *sinensis* populations in Fujian and Zhejiang using ANOVA software.

Table 1. The list of information about *Cryptomeria* populations collection.

Provenance	County	Sample collection site ID	Clones ID	Coordinates		Altitude (m)
				Latitude (°N)	Longitude (°W)	
Zhejiang	Tianmu	TM	22、23、24、25、26、27、29、30、31、32、33、34、35、36、37、38、39、40、YS1	30°19' 42"	119°27' 37"	548
	Shiyang	SY	41、42、44、45、46、47、51、52、53、54、55、56、57、58、59、60	27°52' 11"	119°50' 25"	546
Fujian	Laizhou	LZ	1	26°38' 16"	118°0' 60"	547
	Xikou	XK	2、3、4、5、6、7、8、9	25°22' 46"	118°32' 20"	558
	Huang Long	HL	10、11、12、13、14、15、16、17、18	25°43' 51"	118°58' 31"	548
	Shuimen	SM	61	26°53' 12"	120°0' 42"	549
	Xiapu	XPBC	63、64、65、66、67、68、69、70、71、72、73、74	26°52' 19"	119°56' 37"	553
	Layang	LY	19、20、21	27°0' 30"	119°22' 23"	545
Japan	-	-	J1、J2、YS2	-	-	-

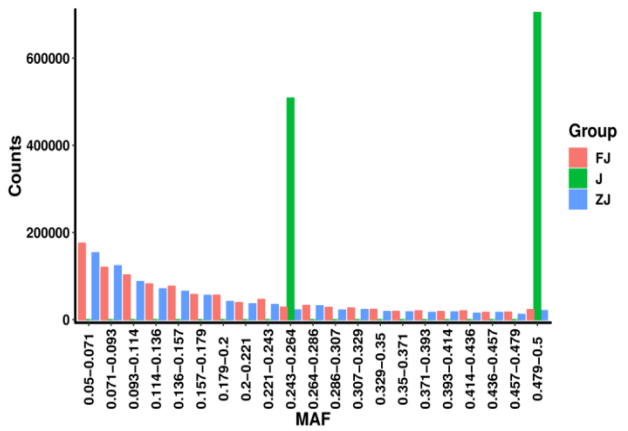

Fig. 1. Distribution of Minimum allele frequency.
Note: FJ-Fujian provenance, ZJ-Zhejiang provenance, J-Japanese provenance (CK).

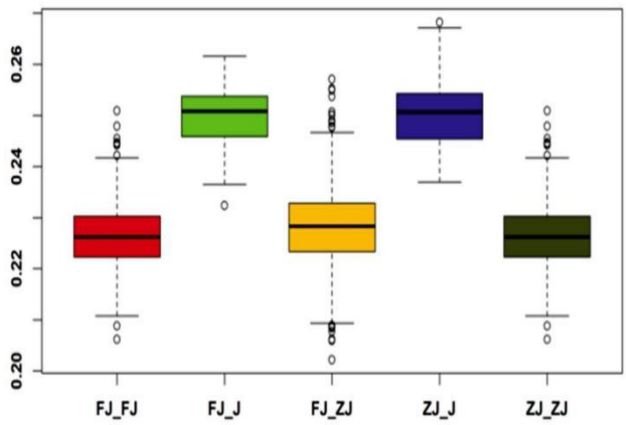

Fig. 2. Distribution of gene distance between and within different populations.
Note: FJ- Fujian provenance, ZJ-Zhejiang provenance, J-Japanese provenance (CK).

Table 2. Genetic diversity estimation of *Cryptomeria* population.

Group	NSNP	P	AR	Ho	He	Pi	PIC
Fujian	1029372	100	1.421	0.203	0.271	0.276	0.226
Zhejiang	901235	100	1.413	0.214	0.267	0.272	0.223
Japan(CK)	1213141	100	1.795	0.349	0.436	0.581	0.339

Note: NSNP is SNP number; P is polymorphism proportion

Results

SNP Discovery and Characterization: A total of 306.5 Gb sequencing data volume containing 2,164,805,208 reads was generated in 72 samples of *C. japonica* var. *sinensis* from China (Fujian, Zhejiang) and Japan, with an average of 30,066,739 reads and 4.26 Gb bases per sample. First, to ensure the accuracy and reliability of SNPs, SNPs with MAF < 0.05 and deletion rate > 0.2 were filtered out, and finally, 875,329 high-quality SNPs were obtained.

In terms of the minimum allele frequencies of SNPs in each population, the MAF distribution characteristics of SNPs in Fujian and Zhejiang populations were more consistent, mainly between 0.05-0.2, with about 65% and 66% of SNPs having MAF distribution in this range. The MAF of SNPs in the Japanese population mainly ranged from 0.243-0.64 and 0.479-0.5, accounting for 42% and 58%, respectively, indicating that the distribution of the minimal alleles of SNPs in the Japanese population differed greatly from that of the two *C. japonica* var. *sinensis* populations from China (Fig. 1).

Genetic diversity analysis: In general, higher allele abundance (AR), expected heterozygosity (He), observed heterozygosity (Ho), and nucleotide diversity (Pi) indicated richer genetic diversity (Li *et al*., 2006; Luo *et al*., 2023). The analysis revealed that the polymorphism ratios of all three populations reached 100%, with the highest degree of genetic diversity in Japanese geographic origin *C. japonica* var. *sinensis* (AR=1.795, He=0.436, Pi=0.581), followed by Fujian (AR=1.421, He=0.271, Pi=0.276) and the lowest in Zhejiang (AR=1.413, He=0.267, Pi=0.272). Among them, the Japanese population was moderately polymorphic seat (PIC > 0.25), and both Fujian and Zhejiang populations were low polymorphic seat (PIC<0.25) (Table 2).

Population genetic distance: The genetic distances between and within populations were further calculated as shown in figure 2: the two populations from Fujian and Zhejiang, China, were close to each other in both inter- and intra-population genetic distances, while the genetic distances with the Japanese population were both relatively distant (Fig. 2.). This is also coincide with the actual geographic distribution location of *C. japonica* var. *sinensis*.

Phylogenetic analysis: In the phylogenetic trees of nine geographic sources of *C. japonica* var. *sinensis*, Japanese and Chinese geographic sources were clustered into two separate major classes (Fig. 3a.), indicating that Chinese and Japanese populations are more distantly related to each other. Although there are some genetic differences among the Chinese geographic source groups, the genetic distances are closer to each other than to those of Japanese *C. japonica* var. *sinensis*, which suggests some interbreeding among them. Still, there have not been significant differences in their genetic composition.

Considering that the phylogenetic clustering of *C. japonica* var. *sinensis* from Japanese and Chinese geographic sources are separated, and the clustering of *C. japonica* var. *sinensis* from Chinese geographic sources have more interleaved relationships, a phylogenetic tree was constructed for *C. japonica* var. *sinensis* from two natural forests in Fujian and Zhejiang, China, and all *C. japonica* var. *sinensis* could be clustered into five classes (Fig. 3b.). Group 1 (blue branch) contains 19 samples, including 12 from SY, 5 from XPBC, 1 from SM, and 1 from XK. Group 2 (purple branch) contains 9 samples, including 5 from XPBC, 2 from SY, 1 from XK, and 1 from HL. Group 3 (green branch) contains 21 samples, including 8 from HL, 6 from XK, 4 from TM, 1 from XPBC, 1 from LY, and 1 from LZ. Group 4 (yellow branch) contains 12 samples, 11 from TM and 1 from SY. Group 5 (red branch) contains 8 samples, including 4 from TM, 2 from LY, 1 from SY, and 1 from XPBC. Despite some overlaps between groups, the clustering results were highly consistent with the geographic distribution.

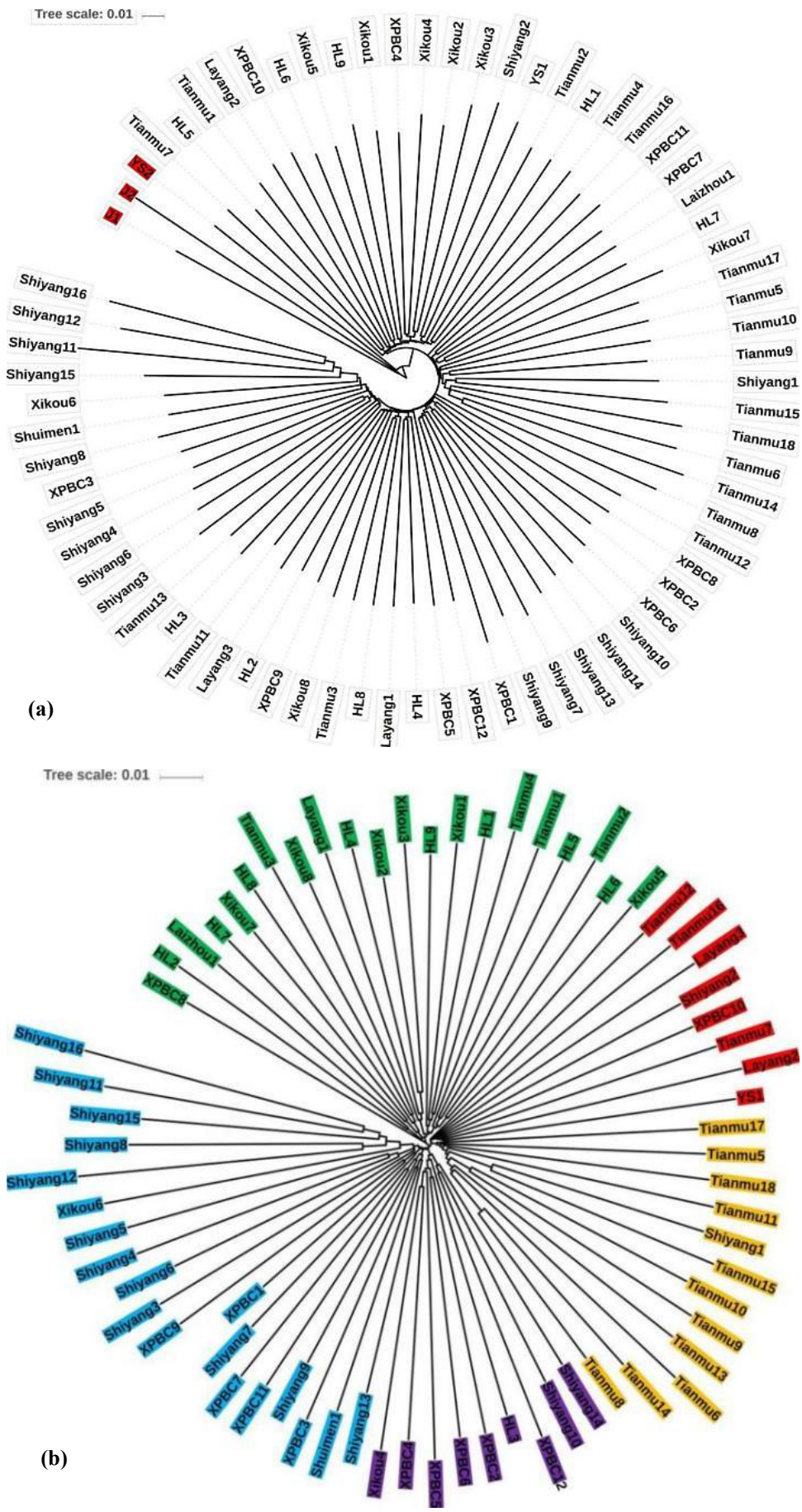

Fig. 3. The phylogenetic tree of *Cryptomeria* populations: (a) The phylogenetic tree of *Cryptomeria* populations from China and Japan (CK); (b) The phylogenetic tree of the *C. japonica* var. *sinensis* populations from Fujian and Zhejiang.

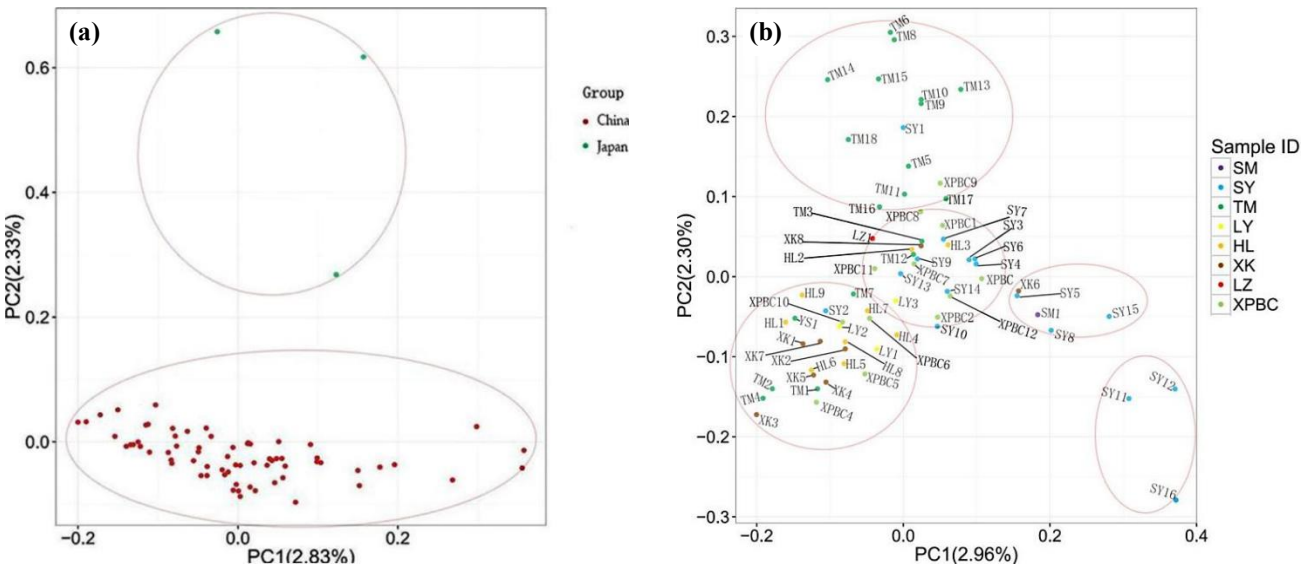

Fig. 4. PCA analysis diagram of the *Cryptomeria* populations: (a) PCA analysis diagram of the *Cryptomeria* populations in China and Japan; (b) PCA analysis diagram of the *C. japonica* var. *sinensis* populations from Fujian and Zhejiang.

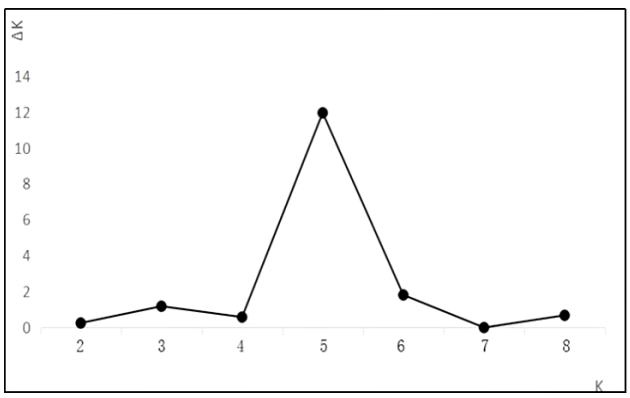

Fig. 5. ΔK based on the rate of change of LnP(D) between successive K using STRUCTURE analysis.

Principal components analysis: Principal component analysis showed that all Cryptomeria populations were very clearly clustered into two major groups, *C. japonica* and *C. japonica* var. *sinensis* (Fig. 4a). The distances between *C. japonica* and *C. japonica* var. *sinensis* were distant. Most *C. japonica* var. *sinensis* samples were distributed in clusters between them, with a few individuals slightly distant from each other, indicating that *C. japonica* var. *sinensis* of geographic origin in Fujian and Zhejiang, China, did not differentiate into new populations, in high agreement with the results of the phylogenetic tree. The first and second principal components explained 2.83% and 2.33% of the genetic variation, respectively.

Similarly, a principal component analysis of only the geographic sources of *C. japonica* var. *sinensis* in Fujian and Zhejiang, China (Fig. 4b.), showed that the first and second principal components explained 2.96% and 2.30% of the variation, respectively. Sixty-nine samples were partitioned into five genetic clusters. Most of the TM samples were placed together into same cluster. Most XPBC and SY samples were staggered to form a set. At the same time, some SY samples were grouped into the same cluster. Most of the XK and HL samples are clustered together. Besides, SY11, SY12, and SY16 showed a sparse distribution, indicating differentiation in the genetic background of the SY population, and the genetic variation is rich. The PCA results were consistent with the results from phylogenetic tree analysis by using the neighbor-joining methods, and the two samples of SY16 and SY12 showed a sparse distribution in the genetic background than other SY samples in both the phylogenetic tree and PCA results.

Population genetic structure analysis: The genetic structure of the *C. japonica* var. *sinensis* collection investigated in the software STRUCTURE analysis revealed 5 optimal clusters with strong support from a change in △k variance with the largest △k value (Fig. 5). The details are shown in figure 6. At K = 2, most of the germplasm materials from XK and HL were grouped, indicating that the population genetic structure of XK and HL were similar. Since samples from these 2 populations originated from Putian, it was considered that these two populations are a local variety, namely Putian strain. Meanwhile, most of the SY samples were organized in groups, with SY 12 and SY 16 the purest breed. There was certain gene flow between other SY samples and other subgroups. At K = 3, most of the TM individuals were grouped. At K = 4, the SY 16 and 12 samples form a group. At K = 5, most of the XP samples were independent. In addition, SY 12 and SY16 belonged to different subgroups from other SY samples, which was consistent with the phylogenetic tree and PCA. Samples from XP and SY s had a highly mixed genetic structure, indicating relatively frequent gene flow occurred between these two populations. The LZ samples appeared in XK and HL subgroups, while the SM and LY samples were mixed in the SY subgroup, which indicated that there was a certain gene flow among these samples. In sum, the subgroups were highly consistent with their geographical distribution.

Molecular variance analysis: The analysis of variance (ANOVA) revealed that the variance among populations was 337.1602, with 0.34% variance, and the variance within populations was 99672.98321, with 99.66% variance ($p<0.0001$), indicating that the main genetic variation of *C. japonica* var. *sinensis* populations in Fujian and Zhejiang is intra-population variation (Table 3).

Table 3. Analysis of molecular variance on *C. japonica* var. *sinensis* populations from Fujian and Zhejiang.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	1	138261.48	337.16	0.34
Among individuals within populations	67	7705155.06	15329.33	15.3
Within individuals	69	5819712.00	84343.65	84.34
Total	137	13663128.54	100010.14	100

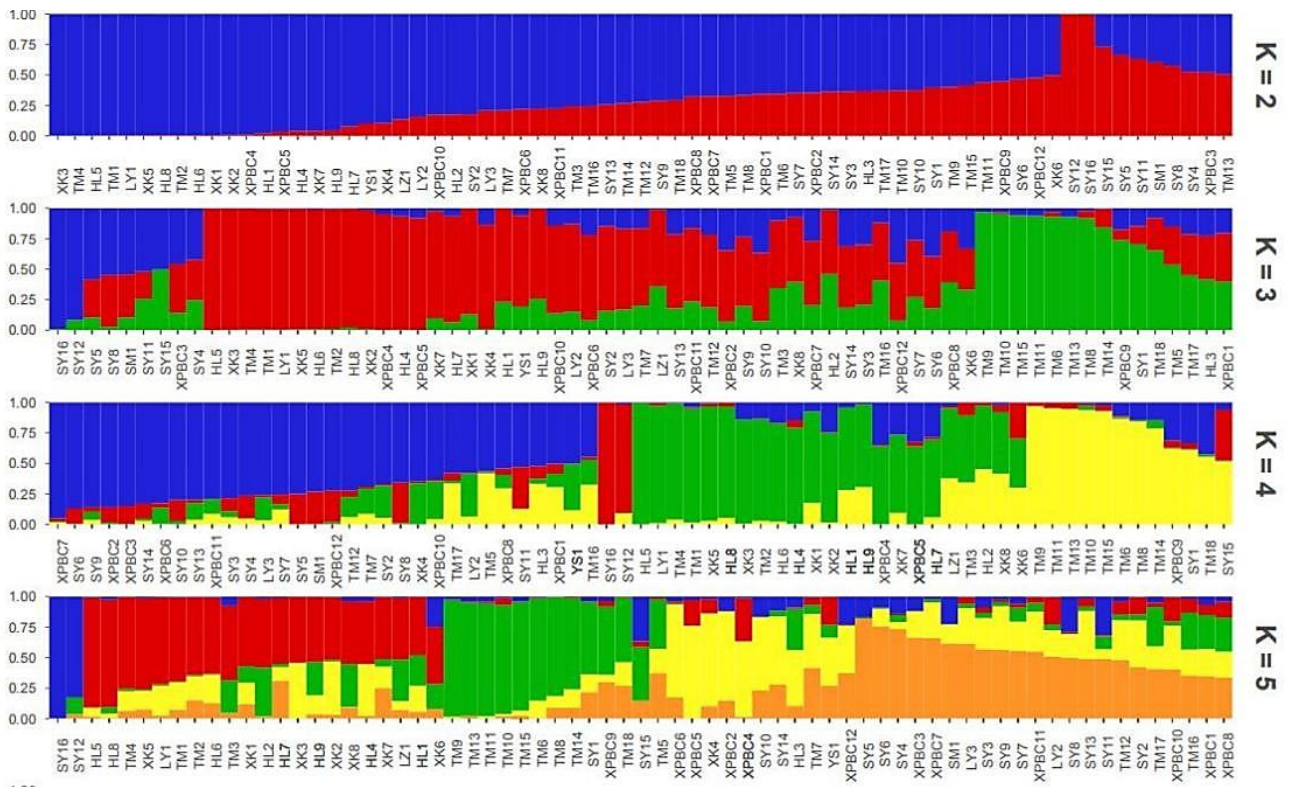

Fig. 6. The population structure analysis on the *C. japonica* var. *sinensis* from Fujian and Zhejiang.

Discussion

SNPs are the most basic component of population genetic variation, which is found throughout the entire genome of each species, and is suitable for rapid and large-scale screening, and has become a powerful tool for studying the genetic diversity of populations (Wang *et al*., 2019). In this work, the first SNP development using GBS simplified genome sequencing was performed on *C. japonica* var. *sinensis* from Japan and China (Fujian and Zhejiang). 875,329 high-quality SNPs were obtained, and the phylogenetic and genetic structure of *C. japonica* var. *sinensis* populations were discussed. The results supported and corroborated the feasibility of this population genetic analysis by using GBS simplified genome sequencing.

Morpho-biochemicals and molecular based diversity help in the identification of unique genotypes within different populations (Shinwari *et al.,* 2018; Khan *et al.,* 2019; Anjum *et al.,* 2019; Sardar *et al.,* 2021; Jan *et al.,* 2024). The genetic diversity of two *C. japonica* var. *sinensis* populations from China was significantly lower than that of control (*C. japonica*) populations (AR=1.795, He=0.436, Ho=0.349, Pi=0.581),The genetic diversity of *C. japonica* populations was significantly higher than that of the two *C. japonica* var. *sinensis* populations, which was consistent with the results of previous researches (Yuan *et al*., 2019; Cai *et al*., 2020; Li *et al*., 2022; Cai *et al*., 2023). In recent years, climate fluctuations and human disturbance have led to increasing fragmentation of *C. japonica* var. *sinensis*, decreasing *C. japonica* var. *Sinensis* populations, and might leads to a inadvertent decrease in genetic diversity due to inbreeding. This findings about the genetic distance of the population also showed that the genetic distance within and between populations was very close, and 99.66% of the variation occurred within the populations, which further supported the speculation that the genetic diversity of *C. japonica* var. *sinensis* may be reduced due to inbreeding. Like the *C. japonica* population, it has a relatively higher proportion of forests in Japan, with a relatively scattered distribution pattern and few introductory plantations. Additionally, there is a long coastline and different microclimatic conditions in Japan, which might promote the differentiation of *C. japonica* populations, probably causing the higher genetic diversity, too.

In addition, we obtained consistent findings by using phylogenetic tree, PCA and population genetic structure analysis, respectively, namely, 69 *C. japonica* var. *sinensis* samples could be clustered into 5 groups. which is similar to previous research but different geographical distribution by using NJ (Liu *et al*., 2012). Despite minor genetic exchange occurrence between individuals among different populations, the clustering results were still highly

consistent with the actual geographical distribution. Meanwhile, although two *C. japonica* var. *sinensis* populations in Fujian and Zhejiang have not completely differentiated to form new populations, they have gradually formed local subpopulations through long-term adaptation and evolution to different environmental phenology.

Although both SY and TM samples were from Zhejiang area, they have not shown a high degree of gene flow between them, probably caused by geographical isolation and microclimate due to several mountains isolation (including Tianmu, Longmen, Dapan, Kuocang, and Donggong moutain) between these two sampling sites., playing a critical role in the evolution and specific differentiation of *C. japonica* var. *sinensis*. However, gene flow occurred frequently among samples from the SM, LY, and XPBC in Xiapu region and samples from SY, partly because the two localities were too close together, or some *C. japonica* var. *sinensis* were mutually introduced, or there might be historical hybridization events. Samples from HL and XK were mixed by clustering, and geographically originated from Putian, Fujian China, which is consistent with the evolutionary tree analysis, indicating that no new subgroups have formed between these two groups. Additionally, LZ1 was clustered into the group containing most of the HL and XK samples, possibly because Laizhou is geographically close to Huanglong and Xikou, despite isolation by the Daiyun mountains, which ensures the gene flow with each other.

Interestingly, the No. 16 and No. 12 from SY (Shiyang) were clustered into a separate subgroup rather than SY group by simultaneously using three methods, we assumed that SY 16 might be the most primitive native germplasm in Shiyang. The latter Shiyang population we tested might be a newly formed subgroup after a long introduction and cross-breeding with SY, XPBC and Tianmu populations, and SY12 is probably the hybridization result between Shiyang and Tianmu. In general, XPBC and SY samples were more highly admixed than other populations, and most genetic variation of *C.japonica* var. *sinensis* populations in Fujian and Zhejiang started from the interior of population, which was consistent with the genetic diversity analysis of 96 *C. japonica* var. *sinensis* clones using SSR molecular markers by Xu Jin *et al*., (2014). This also validates that SY samples were divided into three subgroups in the NJ tree. Thus, in the process of core germplasm construction, forest tree genetic breeding and diversity protection, we should give more attention to collect more and more germplasm resources.

Conclusions

In this study, genetic sequencing typing (GBS) was performed for the first time for genetic marker development in *Cryptomeria* spp. populations. A total of 875,329 high-quality SNPs was obtained, revealing the genetic structural differences and genetic relationships among 72 populations of *Cryptomeria* spp. in China (Fujian, Zhejiang area) and Japan. All samples of *Cryptomeria* germplasm resources could be divided into two geographical populations, *C. japonica* var. *sinensis* and *C. japonica*, and *C. japonica* var. *sinensis* populations from Fujian and Zhejiang area were clustered into five genetic lineages. The genetic diversity of *C. japonica* var. *sinensis* was significantly lower than that of *C. japonica*, and although there was a certain amount of gene exchange among individuals within *C. japonica* var. *sinensis* populations, the overall genetic diversity was still low, and the natural forest populations were seriously damaged. This will be a reference for the conservation, utilization, and genetic breeding improvement of *C. japonica var. sinensis* germplasm resources.

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