Genetic characterization of red-colored heartwood genotypes of Chinese fir using simple sequence repeat (SSR) markers

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ABSTRACT. The present study investigated the genetic characterization of red-colored heartwood Chinese fir [*Cunninghamia lanceolata* (Lamb.) Hook.] in Guangxi using 21 simple sequence repeat (SSR) markers and analyzes of the genetic variation (N = 149) in samples obtained from five sites in Guangxi Province, China. The number of different alleles and the Shannon's information index per locus ranged from 3 to 12 and from 0.398 to 2.258 with average values of 6 and 1.211, respectively, indicating moderate levels of genetic diversity within this germplasm collection. The observed and expected heterozygosity ranged from 0.199 to 0.827 and from 0.198 to 0.878 with an average of 0.562 and 0.584, respectively. Although, the mean fixation index was 0.044, indicative of a low level of genetic differentiation among germplasms, analysis of molecular variance revealed considerable differentiation (99%) within the

samples. The neighbor-joining dendrogram revealed that the majority of red-colored Chinese fir genotypes were apparently not associated with their geographic origins. Further analysis by STRUCTURE showed that this Guangxi germplasm collection could be divided into three genetic groups comprising 76, 37, and 36 members, respectively; these were classified into mixed groups with no obvious population structure. These results were consistent with those of the cluster analysis. On the whole, our data provide a starting point for the management and conservation of the current Guangxi germplasm collection as well as for their efficient use in Chinese fir-breeding programs.

Key words: Red-colored heartwood; Chinese fir; Genetic diversity; Genetic differentiation; Molecular marker

INTRODUCTION

Chinese fir [*Cunninghamia lanceolata* (Lamb.) Hook] is an important indigenous tree species in China with many industrial applications due to its high growth rate, wood quality, and the versatility of its wood (Shi et al., 2010). It is widely cultivated in southern China. According to the multiple provenance testing results of 1976, a set of stands of Chinese fir were identified as elite provenance resources (Zheng et al., 2013). Of these, red-colored heartwood (a unique variety of Chinese fir) is particularly important because of its high wood quality and a higher ratio of hearts that improve the wood density and material properties, yielding commercial benefits and providing aesthetic value to solid wood utilization (Zhang, 2009).

Genetic variability is important for this tree species, especially for those varieties with high economic value for conservation and breeding. Abundant genetic variation allows the development of broad forest varieties adapted to multiple environmental conditions and enhances the genetic gain for several useful traits (Hoffmann and Sgrò, 2011; Matuszewski et al., 2015; Lopes et al., 2015). The biological factors inherent in a particular species, such as gene flow, divergence between populations, and reduction in diversity, can be elucidated based on genetic diversity and population structure, measured using molecular markers (Frankham et al., 2002). Previous studies on the Chinese fir have focused on characterizing genetic variation (Chung et al., 2009; Huang, 2010; Ou et al., 2014; Hao et al., 2014; Xu et al., 2014; Zheng et al., 2015), which could be used for germplasm conservation programs as well as for increasing our understanding of their biological characteristics (Eizirik, 1996). As appropriate evaluation of red-colored heartwood Chinese fir is required for understanding their genetic background, red-colored heartwood Chinese fir samples were collected from Guangxi province and conserved in the *ex situ* gene bank of Rongshui State-run Bei River Forest Farm (Guangxi, China). Understanding the genetic diversity of red-colored heartwood Chinese fir in Guangxi is important for effective utilization of its germplasm resources and for broadening the genetic base of this species to achieve continuous improvement.

Genetic diversity analysis requires suitable markers. Among the various molecular markers available for Chinese fir (You and Hong, 1998; Shen et al., 2011; Wen et al., 2013), simple sequence repeats (SSR) are the most popular because they are highly co-dominant, informative, and hypervariable (Ellis et al., 2007). Therefore, we used SSR markers to discern the DNA variation of this germplasm collection and to better understand the level of genetic variability as well as the

relationship and population structure of this species distributed within the selected regions with the aim of enhancing the efficiency of Chinese fir-breeding programs.

MATERIAL AND METHODS

Plant material and DNA extraction

In the present study, 149 genetic resources of red-colored heartwood Chinese fir were analyzed using five samples collected from the Guangxi province. These genetic resources have been well-conserved in the *ex situ* gene bank of Rongshui State-run Bei River Forest Farm (Guangxi, China). Genomic DNA was extracted from mature leaves of the Chinese fir using a Plant Genomic DNA Kit (TIANGEN, Beijing, China). The quality and concentration of the extracted DNA was determined by electrophoresing the DNA samples on a 1% agarose gel.

SSR analysis

Genomic-SSR polymerase chain reaction (PCR) was performed in a 25-µL reaction volume containing 1.0 µL genomic DNA (about 100 ng), 1.0 µL forward 10 µM primer, 1.0 µL 10 µM reverse primer, 12.5 µL 2X Taq Plus PCR MasterMix (TIANGEN, Beijing, China), and 9.5 µL double distilled water. The reaction was performed in the T100™ thermal cycler made by BIO RAD in the Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants with an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 56/58/62°C (annealing temperature depending on the primer used in the reaction) for 30 s, 72°C for 30 s, 72°C (final extension) for 10 min. Twenty-one microsatellite loci from the 35 samples investigated earlier (Wen et al., 2013) were selected for analysis (Table 1), based on the degree of polymorphism as well as the clarity and reproducibility of the amplified DNA fragments. The forward primer of each pair was labeled with a fluorescent dye (ROX, FAM, or HEX) during the synthesis. The PCR products were separated by capillary electrophoresis using an ABI3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). The amplicons were analyzed using Gene-Marker 2.2.0 software (SoftGenetics LLC, State College, PA, USA).

Data analysis

Convert 1.3.1 (Du et al., 2012) was used to convert microsatellite data to various formats for further analysis. Polymorphism of the SSR markers was evaluated by analyzing the genotypes from the five areas. Genetic diversity, including the number of alleles (N_A), effective number of alleles (N_E), Shannon's Information Index (I), observed heterozygosity (H_O), expected heterozygosity (H_E), Gene Flow (Nm), and F-statistics calculations (F_{IS}, F_{IT}, and F_{ST}) were calculated using GenAlEx 6.5 software (Peakall and Smouse, 2006). Polymorphism information content (PIC) was calculated using PowerMarker v3.25 (Liu and Muse, 2005). AMOVA (analysis of molecular variance) was conducted using GenAlEx 6.5 combined with Microsoft Excel 2010 software (Peakall and Smouse, 2006). A neighbor-joining (NJ) tree was generated based on pairwise genetic distances among individuals using PowerMarker 3.25 (Liu and Muse, 2005). The program FigTree v. 1.3.1 (Rambaut, 2009) was employed to visualize and edit the resulting tree.

Table 1. EST-SSR markers used in the study.

Locus	Primer sequence (5' - 3')	Repeat motif	GenBank accession no	Anneal temperature (°C)	Size Rang (bp)
SSR1	F: CAATCAGCCAAGTTGTACAGGC	(AT)8(AG)18	AB757708	62	343-375
	R: CATACCTTAGCAAAGCCCTCAGC		AB757709		
SSR2	F: TGAAATTGCGTTGTACCGAAGG	(GA)13	AB749554	56	273-297
	R: TAACGAGACGAGCGACAATCTCC		AB749555		
SSR3	F: GATCCTCTGGTACTTGGTGCCC	(AT)9	AB749556	56	186-190
	R: TGCAAAGTCATGTCATCTCTGGC		AB749557		
SSR4	F: TGAATGGACTGCCACAAATTCC	(AG)11	AB749550	56	288-298
	R: TTCTTTGCAGGAAAGCCAACAAG		AB749551		
SSR5	F: CTTAAGATAGCAGCGGGAATGG	(CT)11	AB749562	56	258-279
	R: CTTGCTCGATTTCTTGCATCTGG		AB749563		
SSR6	F: AGAAATGTATACGGACCCTGCG	(TA)9	AB749564	62	247-255
	R: AAATCAAAGCACACGGTGAGAGC		AB749565		
SSR7	F: AAGAGAAGAGGAGGAGGTCCAAG	(AG)9	AB749570	56	269-283
	R: CAGGAGCAGGTGCAGTAGCATTC		AB749571		
SSR8	F: ATTATCCGAGGCAGATACGCAC	(GGA)10	AB749572	56	336-354
	R: CTTCTCCGTATTTGATCCATCGC		AB749573		
SSR9	F: GAGCCGTGAAGAACGAAGGTCTC	(GAA)12	AB749574	56	260-281
	R: ACGATCGGATTGTCTCAGAAACG		AB749575		
SSR10	F: TGATCTTGGCATGTCAGTCTGG	(AT)9	AB749576	56	144-158
	R: TGTCTGTCTGCCTGCAGTTATGC		AB749577		
SSR11	F: TCATCAGCCTCAGTTTGTACTTGC	(AT)9	AB749584	56	348-384
	R: GCAATCATGGGCTCTCTGCAC		AB749585		
SSR12	F: AATGCGACTTGCAAATTTCTGG	(AGA)10	AB749582	56	240-258
	R: CGAATTCCTCAATCACTTGGCTG		AB749583		
SSR13	F: GCGGCCATTTATATCATCTTC	(GAA)9	AB749588	56	126-135
	R: CACGCCTGTAATTCATCTCCGTC		AB749589		
SSR14	F: GGTACTGCGAATCTTCAAATCC	(TC)9	AB749580	56	293-297
	R: TGTTCAAGAAAGGAAGCAAACGG		AB749581		
SSR15	F: TTTGGGACCTTATGGAGGTGGAG	(GGA)9	AB749602	56	153-159
	R: AAACCACCAGGTTGAGAAGCAGC		AB749603		
SSR16	F: TTTCGGCTCTCCGACTCCTTAAC	(CT)11	AB749594	56	129-147
	R: AGAATCGCGTCCAGAACACAGAG		AB749595		
SSR17	F: TCCAGGAGTCTGTGAATCCGAAG	(CTG)9	AB749600	56	223-238
	R: CAGTACCAATTCAACCCAGCAGC		AB749601		
SSR18	F: TCAAGAAGTTCCGCCATTGAGAG	(CTT)10	AB749590	56	354-375
	R: CCCATGAGGATTCAGAAACATGC		AB749591		
SSR19	F: TTAATGGTGCAAGGTGGAATTGG	(GAA)10	AB749592	56	280-286
	R: TTGAATCCCACTGATCACACTGC		AB749593		
SSR20	F: CTGTTTGTACATTGGCCTCGAC	(CTC)9	AB749596	58	136-148
	R: TCACAAACCACTGTGCTGGAATG		AB749597		
SSR21	F: GTTGGGATGTCATCAAGATTGG	(TC)11	AB749598	58	226-270
	R: TGAAAGAGGCGGAAATTGGTAGG		AB749599		

The population structure was analyzed using the Bayesian model-based clustering approach with STRUCTURE v2.3.1 (Pritchard et al., 2000). A burn-in period was used; the number of Markov chain Monte Carlo (MCMC) reps after burn-in was 100,000, with a 100,000-run length, with 10 replicate runs for each analysis, and subpopulations (K) ranging from 1 to 10. The approach to maximum likelihood with Ln probability of data ± SD [LnP (D); Pritchard et al., 2000] and the calculation of ΔK by Evanno et al. (2005) was employed to determine the most probable value for K using Structure Harvester (Earl and Vonholdt, 2012).

RESULTS

Assessment of genetic diversity using SSR markers

A total of 149 red-colored heartwood Chinese fir accessions from Guangxi province,

including Sirong (SR:89 accessions), Yongle (YL:36 accessions), Xiadong (X:13 accessions), Gaoling (G:5 accessions), and a set of Gandong (GD:6 accessions), were analyzed using 21 SSR markers. The genetic profiles detected 122 alleles ranging from 3 to 12 per locus, with an average of 6 (Table 2). The mean N_E was 3.422, ranging from 1.272 to 8.895. Shannon's information index (I) ranged from 0.398 to 2.258, with an average of 1.211. The H_O in the total sample was 0.562, which was lower than the H_E, at 10 SSR loci, in accordance with the mean fixation index (F_{IS} = 0.014; P < 0.05). A moderate inbreeding coefficient (0.056) using the F_{IT} and an F_{ST} value of 0.044 across all loci suggested genetic subdivision, indicative of low genetic differentiation among the sites. With an average of 6.968, the values of gene flow (Nm) ranged from 3.456 to 19.750. The majority of loci conformed to Hardy-Weinberg equilibrium (HWE). Furthermore, mean PIC loci was 0.75, ranging from 0.42 to 0.97 (Table 2), which was reasonably informative. AMOVA revealed a low (1%) but significant (P < 0.01) variation among samples, and 99% variation was attributed to differences within samples (Table 3).

Table 2. Summary statistics of genetic variation in Chinese fir accessions included in this study using SSR markers.

locus	N_A	N_E	I	H_O	H_E	F_{IS}	F_{IT}	F_{ST}	N_M	PIC
SSR1	12	8.895	2.258	0.494	0.878	0.438	0.467	0.051	4.609	0.97
SSR2	10	7.037	2.013	0.781	0.838	0.069	0.107	0.042	5.749	0.97
SSR3	3	1.365	0.478	0.290	0.263	-0.105	-0.091	0.013	19.75	0.42
SSR4	8	4.300	1.669	0.811	0.762	-0.064	-0.002	0.059	3.989	0.92
SSR5	6	2.616	1.206	0.589	0.606	0.028	0.070	0.043	5.520	0.82
SSR6	5	2.406	1.088	0.515	0.537	0.043	0.107	0.067	3.456	0.82
SSR7	5	3.328	1.345	0.758	0.692	-0.095	-0.022	0.067	3.504	0.85
SSR8	5	2.240	0.980	0.426	0.519	0.180	0.231	0.062	3.798	0.72
SSR9	7	4.690	1.680	0.827	0.786	-0.053	-0.024	0.028	8.724	0.93
SSR10	4	2.476	1.009	0.561	0.579	0.031	0.089	0.060	3.918	0.78
SSR11	10	7.329	2.056	0.644	0.845	0.238	0.278	0.053	4.476	0.97
SSR12	3	1.272	0.398	0.199	0.198	-0.005	0.047	0.052	4.553	0.41
SSR13	4	2.288	0.931	0.583	0.546	-0.069	-0.014	0.052	4.586	0.70
SSR14	4	2.341	0.965	0.552	0.569	0.031	0.047	0.017	14.349	0.71
SSR15	5	2.089	0.968	0.532	0.501	-0.062	-0.015	0.044	5.391	0.72
SSR16	6	2.184	1.022	0.622	0.535	-0.163	-0.097	0.057	4.133	0.75
SSR17	4	1.373	0.553	0.264	0.267	0.013	0.041	0.029	8.504	0.45
SSR18	5	3.556	1.421	0.724	0.715	-0.013	0.004	0.016	15.150	0.89
SSR19	3	1.595	0.641	0.401	0.362	-0.108	-0.071	0.033	7.262	0.53
SSR20	3	1.720	0.701	0.476	0.415	-0.148	-0.121	0.024	10.286	0.54
SSR21	10	6.763	2.038	0.756	0.841	0.101	0.147	0.051	4.623	0.97
Mean	6	3.422	1.211	0.562	0.584	0.014	0.056	0.044	6.968	0.75
C.V	44.91	65.63	45.93	0.03	34.98					
Total	122	-	-							

N_A: Number of Different Alleles; N_E: Number of Effective Alleles; I: Shannon's Information Index; H_O: Observed Heterozygosity; H_E: Expected Heterozygosity; F_{IS}: inbreeding coefficient; F_{IT}: over inbreeding coefficient; F_{ST}: fixation index; N_M: Gene Flow; PIC: polymorphic information content.

Table 3. Analysis of molecular variance (AMOVA).

Source	df	SS	MS	Est. Var.	%	P value
Among the populations from Guangxi	4	63.045	15.761	0.072	1	<0.01
Within a population from Guangxi	144	2046.224	14.210	14.210	99	<0.01
Total	148	2109.268		14.282	100	

Genetic differentiation among five regions

Further analyses of N_A, N_E, I, H_O, and H_E (Table 4) were effected to estimate the variations among the samples obtained from five different regions. The highest degree of genetic diversity was observed in SR (N_A = 8.333, N_E = 4.077, I = 1.388, H_O = 0.576, and H_E = 0.624) and Y (N_A = 7.571, N_E = 4.009, I = 1.392, H_O = 0.595, and H_E = 0.627), whereas the diversity was lowest in GD (N_A = 3.810, N_E = 2.707, I = 0.992, H_O = 0.484, and H_E = 0.521) and G (N_A = 3.952, N_E = 3.109, I = 1.085, H_O = 0.610, and H_E = 0.568). The H_O value was significantly lower than the H_E value in all the populations, except G. This result is consistent with the high value of the fixation index. The value of N_A and the number of private alleles in SR was far higher than those in other samples, possibly because of the large disparities in sample size (Santos, 2015). The pairwise genetic differentiation values (F_{ST}) calculated for the samples from five regions showed genetic differentiation between each region (Table 5), with the values ranging from -0.00642 to 0.02862. Among the samples from five populations, those from GD population had higher F_{ST} value, possibly due to a history of genetic drift. Rousset's genetic distance values [F_{ST} / (1-F_{ST})] (Rousset, 1997) indicated that the most closely related regions were G and SR, whereas the largest Rousset's distance (0.0295) was between G and GD.

Table 4. Genetic diversity parameters for the Guangxi Chinese fir samples.

Pop	N	SN	N_A	N_E	I	H_O	H_E	F	Private alleles
SR	89	1-89	8.333	4.077	1.388	0.576	0.624	0.059	23
GD	6	90-95	3.810	2.707	0.992	0.484	0.521	0.021	1
X	13	96-108	5.286	3.209	1.195	0.546	0.578	0.021	3
Y	36	109-144	7.571	4.009	1.392	0.595	0.627	0.037	13
G	5	145-149	3.952	3.109	1.085	0.610	0.568	-0.095	1

SN: Serial Number.

Table 5. Pairwise F_{ST} values in five populations.

	SR	GD	X	Y	G
SR	0.00000				
GD	0.02043	0.00000			
X	0.00381	0.01170	0.00000		
Y	0.00261	0.01420	0.00834	0.00000	
G	-0.00642	0.02862	0.00111	-0.00086	0.00000

Cluster analysis

An unrooted neighbor-joining (NJ) tree (Figure 1) was constructed based on the pairwise genetic distances using PowerMarker V3.25 (Liu and Muse, 2005). Broadly, 149 Chinese fir germplasms could be divided into three main clusters (A, B, and C), among which B and C were further divided into two subclusters (B1 and B2; C1 and C2). Genotypes from SR were distributed in all the clusters, with maximum percentage in cluster B; 63.89% of genotypes from Y belonged to subcluster B2, and the others to cluster C, of which only one belonged to subcluster C1. Only one genotype originated from G belonged to cluster C, and the other four belonged to cluster B. Genotypes from X belonged to cluster B and C and did not cluster into the same class. Thus, we redefined the relationship between the resources not related to their natural regional distributions. Of these subclusters, subcluster B2 contained the maximum (N = 82) members from divergent populations.
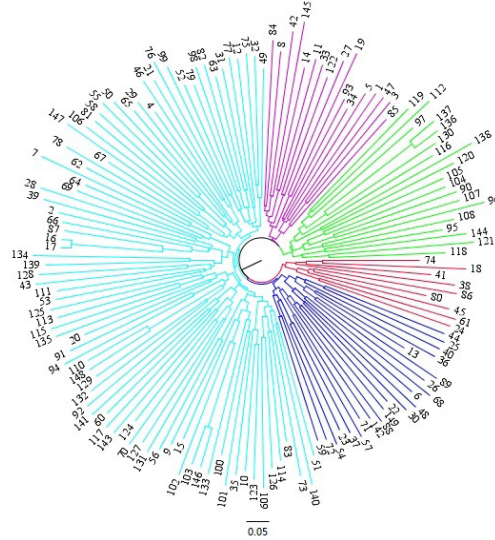
Figure 1. Neighbor joining analysis of the genetic similarity among 149 red-colored Chinese fir genotypes. Red represents group A, deep blue represents group B1, Cambridge blue represents group B2, purple represents group C1, and green represents group C2.

Population genetic structure

To further explore the genetic structure of these germplasm resources, population structure analysis was performed. When K = 3, ∆K showed a clear peak, suggesting that there were three major clusters in the above provenance (Figure 2 and Figure 3). Except the individuals of the GD population, members of all others populations were present in each cluster. About 51% of individuals belonged to Cluster I, which consisted of 47 and 72% of individuals of populations SR and Y, respectively. Cluster II and III consisted of 37 and 36 individuals, respectively, from all populations. The three clusters were dominated by different gene pools but showed similar ancestry that caused non-conspicuous genetic differentiation from one another.
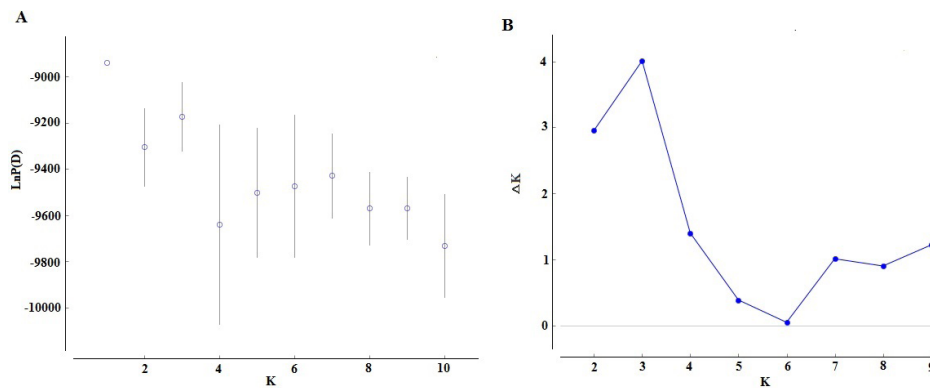

Figure 2. Plot of the Ln P(D) ± SD and delta K (DK). **A.** The mean of LnP (D) was based on 10 repetitions for each K value. **B.** Plot of ∆K according to K.
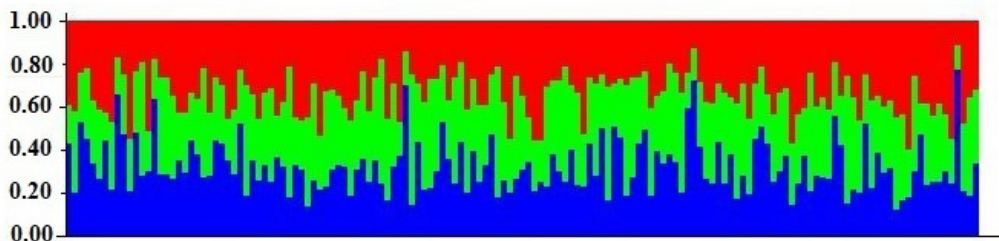
Figure 3. Genetic structure of the Guangxi Chinese fir germplasm collection. Graphic representation of the genetic structure of the Guangxi Chinese fir germplasm collection (N = 149) for K = 3. Each individual is represented by a vertical bar divided into segments representing the estimated member ship proportion in the three cluster inferred with STRUCTURE.

DISCUSSION

Genetic evaluation is important for germplasm utilization in tree breeding. In the present study, 21 SSR primer combinations yielded 122 alleles with a mean of 6 per locus in 149 genotypes of red-colored heartwood Chinese fir. This value was equal to the N_A (6) obtained from 16 Chinese fir individuals based on 28 microsatellite markers (Wen et al., 2013). Among these, SSR6 had the highest diversity, with 12 alleles. Such a significant number of alleles may be related to the co-dominant SSR markers (Du et al., 2012) and was higher than the values that have been reported for other populations of Chinese fir identified using SSR markers (Ou et al., 2014; Xu et al., 2014) as well as the obtained PIC (0.42-0.97) and Shannon's diversity index values (0.398-2.258). Long life, outcrossing, and wind-pollination are prevalent in this species (Zhang and Lin, 1990), with only a narrow geographical range showing similar genetic backgrounds. This may explain the considerable level of genetic diversity observed in this species. The mean value of F_{ST} was 0.044, which is indicative of a low level of population differentiation (Wright, 1978). It was confirmed by AMOVA, where only 1% of the total molecular variance was attributable to the interpopulation level (Table 3).

Heterozygosity is an important indicator of gene diversity (Slatkin and Barton, 1989). In this study, the mean H_O and H_E levels of heterozygosity were 0.562 and 0.584, respectively. Lower values were reported for other populations of Chinese fir using SSR markers (Ou et al., 2014; Xu et al., 2014). H_E (0.584) obtained using SSR markers was also higher for some conifers, including *Pinus bungeana* (H_E: 0.249; Zhao et al., 2014), *Pinus massoniana* (H_E: 0.536; Bai et al., 2014), and *Pinus dabeshanensis* (H_E: 0.458; Xiang et al., 2015). However, it is lower than that of *Abies chensiensis* (H_E: 0.803; Li et al., 2012), and *Austrocedrus chilensis* (H_E: 0.865; Colabella et al., 2014). Further studies have indicated that the genetic diversity of this germplasm resource is moderate. In this study, H_O obtained was higher than the H_E at 11 SSR loci, which is indicative of an excess of heterozygosity at these loci. Hybridization of multiple related species at different geographic locations, over a long period of time, may have caused the excess heterozygotes across the entire natural distribution range (Li et al., 1997). However, the H_O was lower than the H_E for the other 10 SSR loci and for the mean value of all 21 sites. Fewer heterozygotes were observed at the population level, except in G population. Similar findings have been reported in many other tree species (Veron et al., 2005; Hadziabdic et al., 2012; Wang et al., 2014). A process of inbreeding in these trees, such as partial selfing (Veron et al., 2005), effect of biparental breeding because of limited pollen dispersal among relatives (Hadziabdic et al., 2012), and the result of half-sibling mating occurring over a small geographical area, may have caused the heterozygote deficiency

(Wang et al., 2014). Self-pollination found in Chinese fir (Zhang and Lin, 1990) effecting low levels of inbreeding may result in the deficiency of heterozygotes in red Chinese fir, observed in our study. Further studies are required to decipher the precise cause of the heterozygote deficiency.

In the neighbor-joining (NJ) dendrogram, it was observed that the majority of red-colored Chinese fir genotypes were not obviously related to the geographic origins. The genetic structure results further supported that the majority of genotypes were classified into a mixed group without ascertaining their geographic origins. This may be because germplasms collected from closer geographic distances showed non-conspicuous genetic differentiation or because they have a history of genetic drift. However, the moderate levels of genetic diversity but low degrees of genetic differentiation of the tested germplasms may be because of the limited number of SSR primers, not evenly distributed throughout the genome. More SSR primers or other markers, such as single-nucleotide polymorphisms (SNPs), may be applicable (Las et al., 2014) for effective genetic characterization and population structure analysis of Chinese fir. Genetic variations in red-colored heartwood Chinese fir have been analyzed in a variety of species (Zhang, 2009); however, further experiments are required to study Chinese fir genetic diversity. Further understanding of population structure is required to perform association analyses. In future, more unique shape control genes and diverse genotypes should be selected to improve the exploitation of available Chinese fir resources and augment the breeding process based on morphological traits and genetic diversity. However, based on the genetic relationships of the tested germplasms, it is possible to improve the germplasm resources and select appropriate materials for breeding.

Conflicts of interest

The authors declare no conflicts of interest.

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