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Genetic Diversity and Differentiation of Chinese Fir around Karst Landform in Guangxi

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Abstract: The karst geo-ecosystems are fragile environments. The largest karst region in the world is located in southwestern China, within which the Guangxi province is one of the main areas. Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.), an evergreen species, is an important fast-growing timber tree in southern China. In the present study, we examined the genetic diversity and spatial genetic differentiation of Chinese fir in sampling localities around the karst landform region of Guangxi by genotyping 330 individuals from 11 sampling localities with 22 novel polymorphic microsatellite loci. High levels of gene flow have homogenized Chinese fir in Rongshui, Nandan, and Tiane sites, which are speculated to be the primary center of gene exchange and diversity for Chinese fir around the karst landform in Guangxi. Significant isolation by distance pattern was found among nine sampling localities. A moderate level of genetic differentiation (F_{ST} = 0.089, D_{est} = 0.139) between sampling localities was detected. Structure analysis divided Chinese fir into three subgroups (K = 3). With higher differentiation and less genetic variation than in the central population, marginal populations of Cangwu and Pubei were identified in the south of the karst landform. An effective conservation strategy focusing on the maintenance of genetic variation for marginal populations of the species was proposed.

Keywords: genetic diversity; central–marginal population; conservation strategy; karst landform; Chinese fir

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Citation: Li, K.; Chen, S.; Chen, X.; Lan, X.; Huang, K. Genetic Diversity and Differentiation of Chinese Fir around Karst Landform in Guangxi. *Forests* **2023**, *14*, 340. https://doi.org/10.3390/f14020340

Academic Editor: Bryce Richardson

Received: 23 December 2022
Revised: 31 January 2023
Accepted: 3 February 2023
Published: 9 February 2023

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1. Introduction

Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.), a diploid species (2n = 2x = 22), is an important fast-growing timber tree in southern China [1]. The species occupies approximately 25% of plantations in subtropical areas of southern China [2], and the wood of the Chinese fir is widely used in building, decoration, and furniture production [3]. Chinese fir forests provide considerable ecological benefits, including carbon sequestration, soil conservation, and increasing groundwater storage [4,5].

Karst landscapes represent an important facet of the Earth's geodiversity, and the karst geo-ecosystems are fragile environments. Guangxi province, with an area of 2.38×10^5 km^2, is home to one of the largest karst areas in China, and the distribution area of carbonate rock accounts for 41.57% of the total area [6]. The areas are rocky desertification, which is characterized by soil erosion and progressive degradation and is associated with very low land productivity [7]. Chinese fir cannot grow in karst areas which would fragment the distribution habitat of the species.

The effects of habitat fragmentation can cause severe consequences on trees, such as reduced effective population size, increased inbreeding, loss of genetic diversity, and a

discontinuous pattern of genetic variation, mainly due to the limited gene flow [8,9]. The central–marginal hypothesis states that marginal populations show higher differentiation and less genetic variation than central populations [10]. Marginal populations are often small, spatially isolated, and occur in different or extreme habitats. Marginal populations have long caught the attention of conservation biologists because they provide insights into marked genetic differentiation from populations and the roles of adaptation [11]. Current marginal populations may become future evolutionary units, especially faced with the problem of ongoing global climatic changes [12]. Guangxi is the southernmost province in China where Chinese fir grows. Marginal populations of Chinese fir identified in the area may be of great significance for the evolutionary process and special conservation interest for the species.

Successful conservation, management, and utilization strategies for a species require an accurate assessment of the genetic variation of the population. Here, we present the first survey of genetic variation of Chinese fir in sites around the karst landform in Guangxi, China, with novel and highly polymorphic SSR markers. We hypothesize that there is an increasing genetic differentiation of Chinese fir from the north to the south of the karst landform. A second hypothesis is that a marginal population of Chinese fir exists in the south of the karst landform in Guangxi. The main goals are to (a) develop neutral EST-SSR markers with low scoring error rates for Chinese fir, (b) assess the levels of genetic diversity and differentiation among sampling localities around the karst landform, and (c) identify marginal populations of Chinese fir around the landform in the southernmost province of China.

2. Methods

2.1. Plant Sample Collection

Chinese fir is a coniferous and indigenous tree species in subtropical southern China (Figure 1). The species is monoecious and predominantly outcrossing. From October to December 2019, 330 individuals of Chinese fir were collected from 11 counties (Table 1) around the karst area in Guangxi, with 30 trees being collected from each county. The age of each individual was greater than 30 years, and the distances between trees were at least 50 m. Tree age was estimated by counting growth rings of increment core sampled from breast height of Chinese firs. For each county, every group of 10 plants was considered a repeat, and the interval between three repeats was greater than 10 km. The geographical distribution of the 11 sampling localities is shown in Figure 2. Fresh leaves without diseases and insect pests were picked and immediately transported back to the laboratory in liquid nitrogen and stored at −80 °C until use.



Figure 1. (**A**) Twigs and cones of Chinese fir. (**B**) Trunks and tree canopies of Chinese fir.

Table 1. Information on samples and sampling locations.

Sampling Locations	Sample Size	Latitude/° (E)	Longitude/° (N)	Altitude/m
RS	30	108.25	25.07	500–1500
ND	30	107.52	24.97	500–1000
TE	30	107.17	25.85	800–1300
JX	30	109.67	23.97	115–500
LS	30	109.33	25.80	1200–1600
LL	30	105.32	24.77	800–1400
ZY	30	110.62	26.02	800–1200
PB	30	110.17	22.62	144–300
NP	30	105.82	23.42	260–794
PG	30	111.53	24.42	800–1000
CW	30	111.22	23.42	144–196

RS represents Rongshui, Liuzhou; ND, Nandan, Hechi; TE, Tiane, Hechi; JX, Jinxiu, Laibin; LS, Longsheng, Guilin; LL, Longlin, Baise; ZY, Ziyuan, Guilin; PB, Pubei, Yulin; NP, Napo, Baise; PG, Pinggui, Hezhou; and CW, Cangwu, Wuzhou.

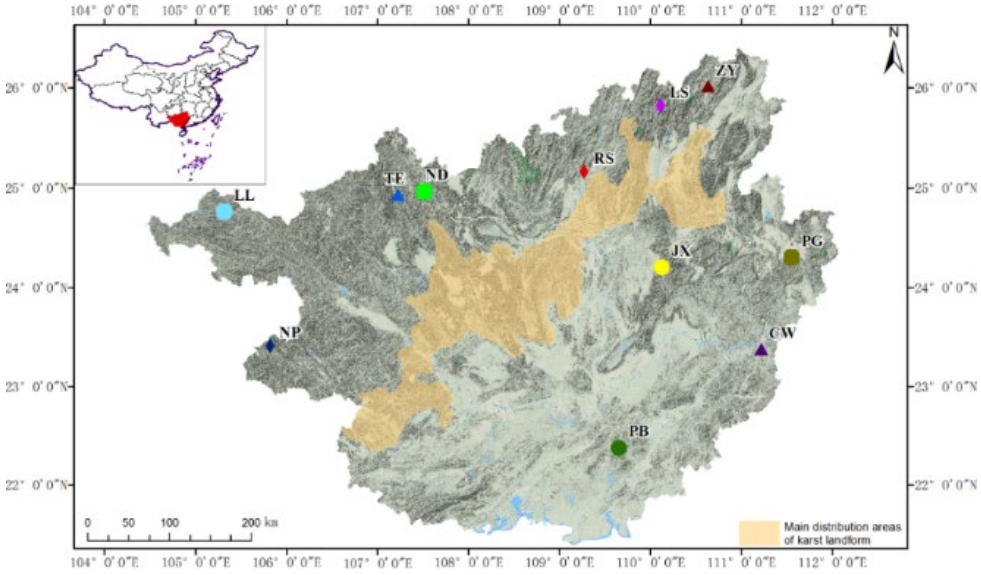


Figure 2. Map showing the geographical locations where samples were collected. Locations of the 11 sampled sites are shown by colorful markings (see Table 1 for the details of these sampled locations).

2.2. DNA Extraction and SSR Genotyping

Total DNA of Chinese fir leaves was extracted using a Rapid Plant Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The purity and integrity of the DNA were detected using 1% agarose gel. A Nanodrop-2000 ultramicro spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA) was used to detect the concentration of DNA. The DNA concentration was adjusted to 20 ng μL^{-1} with deionized water and stored at −20 °C.

The forward primers of 22 microsatellite markers were synthesized and labeled, respectively, with FAM, HEX, TAMRA, and ROX (RuiBiotech, Beijing, China). Single PCR reactions were performed over all the genotypes with 22 microsatellites. Polymerase chain reaction was performed on an Analytik Jena Biometra Tone 96G (Jena, Germany). The amplification system consisted of 5 µL 2× taqplus mix (5 U/µL, Takara, Dalian, China), 0.3 µL each of the forward (0.5 µM) and reverse primers (0.5 µM), 2 µL DNA template, and 2.4 µL deionized water. The annealing temperature was 55 °C for 40 cycles, and the amplified products were stored at 4 °C.

The amplified products were divided into five sets according to microsatellite markers. Set A includes SSR1(FAM), SSR2(HEX), SSR3(TAMRA), and SSR4(ROX). Set B includes SSR5(FAM), SSR6(HEX), SSR7(TAMRA), and SSR8(ROX). Set C includes SSR9(FAM), SSR10(HEX), SSR11(TAMRA), and SSR12(ROX). Set D includes SSR13(FAM), SSR14(HEX), SSR15(TAMRA), and SSR16(ROX). Set E includes SSR17(FAM), SSR18(HEX), and SSR19(TAMRA). Set F includes SSR20(FAM), SSR21(HEX), and SSR22(TAMRA). The amplified products of each set from the same genotype were mixed for capillary electrophoresis. Capillary electrophoresis was performed in ABI 3730xl (Applied Biosystems, Foster City, CA, USA), and the fragment size of each sampling locality was read using genemarker 2.2.0 software (SoftGenetics, State College, PA, USA).

2.3. SSR Loci Development

RNA was extracted from the roots, stems, and leaves of the Chinese fir and sequenced using Illumina Hiseq TM2000. The transcriptome sequence splicing software Trinity was used to splice transcriptome sequences [13]. SSR loci were identified using MISA software [14], and SSR primers were designed using Primer3 [15]. Primer3 options were default. Twenty-two novel SSR loci with the highest polymorphism and low null allele frequencies were selected from 1000 SSR loci. Identification method of the loci was described [16]. Twenty-two pairs of primers are shown in Table 2.

MICRO-CHECKER 2.2.3 was used to check for allelic dropout and scoring errors [1+]. The Ewens–Watterson test for neutrality and Hardy–Weinberg equilibrium (HWE) of SSR markers were conducted by POPGENE 1.32 [17] to test whether the loci were under selection pressures and the level for deviations from HWE. Linkage disequilibrium (LD) tests between pairs of microsatellite loci were performed in each sampling locality using the Arlequin 3.1 [18]. Then, the Bonferroni correction for multiple tests [19] was applied.

2.4. Genetic Diversity

Based on SSRs profiles, the polymorphism information content (PIC) was calculated using Cervus 3.0.7 [20]. The number of alleles (*Na*), number of effective alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), number of private alleles (*Np*), Shannon's diversity index (*I*), frequency of null alleles (F_{NA}), and inbreeding coefficient (F_{IS}) were calculated using GenAlEx6.5 [21,22]. The 95% confidence interval of the F_{IS} was calculated from 5000 bootstrap re-samplings using the package bootES of the software R to test whether F_{IS} was significantly different from zero [23].

2.5. Isolation-by-Distance

To analyze which sampling localities have a greater impact on IBD, the correlation between the matrix of genetic distances [$F_{ST}/(1 - F_{ST})$] and the matrix of geographic distances (Km) in Chinese fir among the different sampling localities were analyzed with Mantel tests (999 permutations) run in GenAlEx6.5.

2.6. Genetic Structure

The population genetic structure of Chinese fir in the study area was investigated with STRUCTURE version 2.3.1 software [24]. The parameters were set as follows: group number *K*, 1–10; length of burn-in period and MCMC value, 100,000 and 200,000 times; admixture and correlated allele frequencies models, respectively; and each *K* value was repeated 20 times. The results were uploaded to STRUCTURE Harvester [25], and the optimum *K* value was obtained. STRUCTURE graphical bar plot was generated with DISTRUCT program to show membership coefficients [26].

Table 2. Characterization of novel 22 EST-SSR primers pairs in Chinese fir.

Primer ID	Motif	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Allele Range (bp)	Annealing Temperature (°C)	Genebank ID
SSR1	(GGA)6	GCCTTGTGCAAAGCGGTAAA	AGGAAACTGCACTGTACGCA	198–222	58	MZ027666
SSR2	(AT)8	CCCGGCCCAAGCTTTTAGAA	ACAGGACAACATCAGGACCC	169–185	56	MZ027660
SSR3	(TCTGCC)7	TGTTGTCTTGTGTTGGCCCT	ACTCCAACACAGGTGACAGC	215–245	58	MZ027649
SSR4	(TC)9	ACCGTAATTGGCTGCACTGT	TCGAGACAGCCATGGTCATG	247–273	58	MZ027654
SSR5	(ATG)10	CTCTGTGCAAAAGTCACCGC	CGAGGACAAGAGAGGGTTTGA	159–186	60	MZ027653
SSR6	(TTGGAG)5	GCGGTAGTCCCTGCTTTTCT	TGGATGGGGAGTGTCTCGAT	199–229	56	MZ027650
SSR7	(GGC)7	GTGAGCGATCGAGAGACTGG	ACCACTTCGGCGACTTTCAT	228–261	56	MZ027658
SSR8	(TGC)6	ATTGTGCCACTGGTTGCATT	GCTCAACAACAACAGCTGCA	222–264	58	MZ027662
SSR9	(GGA)7	CATCAACAGCCCACAAAGCC	ACATGGTAGCGAGTCCAAGC	178–193	58	MZ027663
SSR10	(TAG)7	TCACTGCCGATCAACCAACA	TCCACTCTTACCCTGCCTGA	209–236	60	MZ027661
SSR11	(TAT)6	AACATTTGGCCGAGAGATCG	TGCCGCTTGACTTGTGCTTA	239–275	60	MZ027668
SSR12	(TGGGCA)7	TGTTGCAGCATTTGGGCTTC	ACCCATGCTCAACTTACGGG	259–331	56	MZ027656
SSR13	(CA)6	GCGCGCGCACATATACATAC	TTCACCTTGGCCAAGATTGT	90–106	58	MZ027669
SSR14	(GAT)8	TGGCCTTTCCATGACTGCTT	CTAGGCGGCAGAGTGAACAA	197–161	58	MZ027664
SSR15	(TGCC)5	GTCTCTGGTGTGGGGTTGTG	CGGCAGCTGTACGTACTCAT	215–235	57	MZ027651
SSR16	(CAGG)5	GCACATGAAAAGCTCCGTCG	GACAGACGGGCAAAGGAAGA	246–278	56	MZ027652
SSR17	(GTG)5	TACAAGTGCTCCATGGCCAG	TGCATCCACGCAAGATACGT	114–153	60	MZ027655
SSR18	(AGGC)6	CGGCAGCTGTACGTACTCAT	CTGTCTCTGGTGTGGGGTTG	208–232	60	MZ027665
SSR19	(CTCCAT)5	GTCCCTGTTTCTGCCAGTGA	ACTCCATCTCCTAGAGCACCA	244–286	58	MZ027659
SSR20	(AATG)5	GGCAATCTAGCGAGCTTCCT	CTTGCTCTCCCTGTATGCGT	109–173	58	MZ027670
SSR21	(CAG)7	GCAAGAGCATCAGCATCAGC	CAAAGTCAGGCATGCCCCTA	189–210	56	MZ027667
SSR22	(CACCAA)8	AGGAAACCCCACCGCATATG	CACTGCTCGTTGGCATTGTC	219–261	56	MZ027657

Isolation by distance pattern can cause the algorithm of STRUCTURE to overestimate the number of genetic clusters for increased genetic differentiation among individuals with geographical distance [27]. Pairwise population differentiation coefficient F_{ST} and R_{ST} between pairs of sampling localities were computed using SPAGeDi 1.3 to explore the relationship of genetic differentiation among sampling localities [28]. If R_{ST} is > F_{ST}, then there is phylogeographic signal. Jost's *D* values (D_{est}), Nei's genetic distance (*D*), and gene flow (*Nm*) were calculated using GenAlEx6.5. To estimate the genetic differences between and within sampling localities, an analysis of molecular variance (AMOVA) was performed using Arlequin 3.1 software. In addition, NTSYS pc 2.10e [29] software was used to draw the dendrogram with Nei's genetic distance based on the unweighted group average method (UPGMA). To further confirm genetic discontinuities, structure, and cluster analysis, principal coordinate analysis (PCoA) was performed using GenAlEx6.5.

3. Results

3.1. Diversity Analysis of SSR

Among the 22 loci, 195 alleles were obtained. The highest *Na* in the loci was 15, and the highest *Ne* was 5.457 (Table 3). On average, 8.864 alleles and 2.824 effective alleles were amplified per locus. The values of *Ho* and *He* ranged from 0.290 to 0.785 and 0.255 to 0.816, and the mean values were 0.603 and 0.596, respectively. The PIC of SSR loci ranged from 0.265 to 0.831, with an average value of 0.589, indicating that the loci were rich in polymorphism. The variation range of *I* was 0.530–1.901, with an average of 1.146. Fifteen loci had a positive F_{NA} value, and the mean value of 22 loci was 0.03. Eleven loci had a positive F_{IS} value, with an average value of −0.019. No large allelic dropout or scoring errors due to stuttering were detected in all the loci. All the observed fixation indexes were within the 95% confidence interval between the lower and upper limits for the test, suggesting that each SSR loci were not under positive selection. No significant LD was detected between the analyzed pairs of loci after Bonferroni correction for multiple tests.

Table 3. Genetic parameters of 22 simple sequence repeat markers in all tested Chinese fir.

Loci	N_a	N_e	*I*	*Ho*	H_e	PIC	F_{NA}	F_{IS}	Obs.F	L95	U95
SSR1	8	2.581	1.080	0.600	0.609	0.565	0.015	0.015	0.371	0.219	0.806
SSR2	7	2.866	1.157	0.640	0.648 *	0.680	0.105	0.012	0.323	0.259	0.909
SSR3	10	3.280	1.364	0.663	0.690	0.733	0.097	0.040	0.262	0.208	0.774
SSR4	5	1.965	0.808	0.476	0.480	0.421	0.020	0.010	0.509	0.288	0.950
SSR5	11	3.357	1.394	0.781	0.695 *	0.673	−0.051	−0.123	0.288	0.188	0.741
SSR6	5	2.607	1.063	0.785	0.610 *	0.544	−0.130	−0.287	0.380	0.304	0.947
SSR7	11	3.307	1.343	0.735	0.693 *	0.721	0.042	−0.062	0.272	0.190	0.732
SSR8	15	5.457	1.871	0.784	0.814	0.816	0.035	0.038	0.166	0.147	0.554
SSR9	6	2.622	1.120	0.533	0.569 *	0.583	0.082	0.063	0.382	0.265	0.912
SSR10	9	3.270	1.356	0.680	0.689	0.679	0.036	0.013	0.287	0.224	0.818
SSR11	6	2.491	1.017	0.581	0.594 *	0.576	0.057	0.021	0.379	0.305	0.945
SSR12	14	5.594	1.901	0.739	0.816 *	0.831	0.082	0.094	0.160	0.154	0.624
SSR13	7	2.448	0.970	0.399	0.567 *	0.607	0.267	0.296	0.391	0.255	0.918
SSR14	9	2.929	1.207	0.699	0.657	0.637	−0.001	−0.064	0.318	0.217	0.826
SSR15	7	2.397	1.004	0.631	0.580 *	0.523	−0.037	−0.088	0.408	0.267	0.909
SSR16	8	1.935	0.841	0.511	0.473	0.434	−0.013	−0.081	0.505	0.220	0.811
SSR17	12	1.810	0.818	0.427	0.393	0.401	−0.013	−0.086	0.574	0.167	0.632
SSR18	8	2.308	0.968	0.590	0.557	0.531	0.014	−0.059	0.402	0.247	0.877
SSR19	9	2.489	1.162	0.583	0.582	0.577	0.032	−0.002	0.406	0.214	0.819
SSR20	11	3.012	1.289	0.620	0.647 *	0.651	0.058	0.041	0.314	0.192	0.734
SSR21	8	1.371	0.530	0.290	0.255	0.265	−0.046	−0.138	0.735	0.238	0.866
SSR22	9	2.024	0.938	0.527	0.491	0.502	0.014	−0.074	0.492	0.222	0.833
Mean	8.864	2.824	1.146	0.603	0.596	0.589	0.030	−0.019			

Na, number of alleles; *Ne*, number of effective alleles; *I*, Shannon's diversity index; *Ho*, observed heterozygosity; *He*, expected heterozygosity; PIC, the polymorphism information content; F_{NA}, frequency of null alleles; F_{IS}, inbreeding coefficient; Obs.F, observed frequency of marker; L95, lower 95% confidence limit; U95, upper 95% confidence limit. *, the significant level for deviations from HWE ($p \leq 0.01$).

3.2. Genetic Diversity of Chinese Fir in Sampling Localities around the Karst Landform

Chinese fir in 11 sampling localities showed a higher level of *Ho* (overall mean 0.603) than of *He* (overall mean 0.596), and F_{IS} (overall mean −0.001) indicated an excess of heterozygotes in the 330 individuals (Table 4). The *He* values ranged from 0.521 to 0.635, with an average value of 0.571. The mean value of *I* was 1.146. Heterozygote deficiency was observed in five sampling localities (RS, ND, TE, JX, and LL; *Ho* < *He*). The F_{IS} value of the 11 sampling localities ranged from −0.013 to 0.041, with an average value of −0.064. JX showed a higher F_{IS} value (0.058) than other sampling localities, and the value was statistically different from zero ($p < 0.05$). However, Chinese fir in JX contained the highest number of private alleles. Except for the PB and NP, Chinese fir of the remaining nine sampling localities harbored private alleles. Chinese fir in RS had the highest *I* value (1.234), and Chinese fir in CW had the lowest value (0.976). With the highest *I*, *Ho*, and *He* values, Chinese fir in RS were in possession of the highest genetic diversity among 11 sampling localities.

Table 4. Genetic diversity of Chinese fir in 11 sampling localities.

Sampling Locations	*Np*	*Na*	*Ne*	*I*	*Ho*	*He*	F_{IS}	*CI*95%
RS	4	5.318	3.004	1.234	0.628	0.635	0.023	−0.028~0.076
ND	3	5.455	2.958	1.196	0.602	0.614	0.032	−0.021~0.075
TE	2	5.227	2.923	1.193	0.608	0.615	0.018	−0.038~0.122
JX	6	5.591	3.009	1.231	0.600	0.628	0.058	0.008~0.118
LS	3	5.182	2.893	1.181	0.617	0.612	0.010	−0.046~0.069
ZY	4	5.091	2.732	1.120	0.603	0.585	−0.019	−0.079~0.054
LL	2	5.091	2.865	1.146	0.589	0.596	0.022	−0.041~0.073
PG	3	5.000	2.630	1.090	0.597	0.574	−0.022	−0.105~0.043
NP	0	4.909	2.791	1.125	0.632	0.589	−0.060	−0.188~0.005
PB	0	4.818	2.707	1.109	0.587	0.586	0.013	−0.037~0.094
CW	1	4.364	2.548	0.976	0.574	0.521	−0.088	−0.240~0.048
Mean	3	5.095	2.824	1.146	0.603	0.596	−0.001	−0.029~0.019

Np, number of private alleles; *Na*, number of alleles; *Ne*, number of effective alleles; *I*, Shannon's diversity index; *Ho*, observed heterozygosity; *He*, expected heterozygosity; F_{IS}, inbreeding coefficient; *CI*95%, the 95% confidence interval of the F_{IS}.

3.3. Genetic Differentiation of Chinese Fir in Sampling Localities around the Karst Landform

Owing to the high heterozygosity of Chinese fir, the primary genetic variability exists within individuals (Table 5). AMOVA revealed that a variation within populations was 4.44%, and only 3.12% of the total molecular variance was attributable to among-population variation. Among the 11 sampling localities, the F_{ST} value ranged from −0.002 to 0.089 (Table 6), and the D_{est} values ranged from −0.003 to 0.139 (Table 7). F_{ST} and D_{est} values in these sampling localities indicated similar results of pairwise sampling localities differentiation. Both F_{ST} and D_{est} values between ND and TE were the lowest (F_{ST} = −0.002, D_{est} = −0.003), followed by that between the RS and ND groups (F_{ST} = 0.001, D_{est} = 0.002). Both values between RS and CW were the highest (F_{ST} = 0.089, D_{est} = 0.139), followed by that between CW and ZY (F_{ST} = 0.086, D_{est} = 0.120). No evidence of a significant phylogeographic signal was found in pairwise comparisons between 11 sampling localities (Table 7). The *Nm* value ranged from 4.100 to 33.041, with an average number of 12.671. The largest gene flow was observed between ND and other sampling localities. The maximum *Nm* value was 33.041 for ND and TE. Among them, the minimum value was 4.100 between RS and CW.

Table 5. AMOVA partitioning of molecular variance of Chinese fir in 11 sampling localities.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variance (%)	*p*
Among populations	10	202.34	0.22	3.12	<0.001
Among individuals within populations	319	2266.33	0.31	4.44	<0.001
Within individuals	330	2139.00	6.48	92.44	<0.001
Total	659	4607.67	7.01		

d.f., degrees of freedom. *p*, the significance of the covariance tested using non-parametric permutation procedures.

Table 6. Pairwise population differentiation based on F_{ST} values and gene flow among Chinese fir in 11 sampling localities. Abbreviations for sampling localities are given in Table 1. Pairwise F_{ST} below diagonal; Pairwise *Nm* above diagonal. Statistically significant results of allele size permutation tests to assess differences in F_{ST} ($p < 0.05$) are shown in bold type.

	RS	ND	TE	JX	LS	ZY	LL	PG	NP	PB	CW
RS		27.298	21.755	16.127	15.114	11.538	9.990	8.525	8.494	6.170	4.100
ND	0.001		33.041	20.445	19.875	16.359	15.382	14.306	11.517	8.697	5.391
TE	0.005	−0.002		19.622	20.526	15.551	12.108	12.521	10.130	9.480	5.143
JX	**0.013**	0.007	0.007		20.206	12.079	16.208	16.218	12.622	13.289	5.917
LS	**0.015**	0.008	0.006	0.006		15.710	11.247	11.000	9.803	10.169	4.648
ZY	**0.024**	**0.013**	**0.015**	**0.023**	**0.015**		9.423	9.649	7.581	8.756	4.184
LL	**0.028**	**0.011**	**0.018**	**0.010**	**0.022**	**0.029**		19.692	17.893	10.433	6.637
PG	**0.040**	**0.017**	**0.020**	**0.013**	**0.026**	**0.033**	**0.008**		16.447	13.649	12.176
NP	**0.037**	**0.023**	**0.028**	**0.022**	**0.030**	**0.043**	**0.009**	**0.015**		7.623	8.900
PB	**0.062**	**0.040**	**0.036**	**0.020**	**0.032**	**0.041**	**0.029**	**0.021**	**0.049**		5.547
CW	**0.089**	**0.063**	**0.066**	**0.058**	**0.076**	**0.086**	**0.051**	**0.021**	**0.040**	**0.067**	

Table 7. Pairwise population differentiation based on Jost's *D* and R_{ST} values among 11 sampling localities on Chinese firs. Abbreviations for gene pools are given in Table 1. Pairwise Jost's *D* values below diagonal; Pairwise R_{ST} above diagonal.

	RS	ND	TE	JX	LS	ZY	LL	PG	NP	PB	CW
RS		0.015	−0.005	−0.003	0.016	0.021	0.043	0.039	0.021	0.069	0.098
ND	0.002		0.003	0.000	0.002	−0.001	−0.001	−0.008	−0.005	0.009	0.048
TE	0.009	−0.003		−0.014	0.010	0.013	0.028	0.011	−0.001	0.037	0.058
JX	0.024	0.012	0.012		0.013	0.015	0.023	0.007	−0.004	0.031	0.052
LS	0.026	0.012	0.011	0.011		0.000	0.003	0.018	0.024	0.039	0.096
ZY	0.041	0.020	0.024	0.039	0.016		0.009	0.018	0.020	0.029	0.086
LL	0.048	0.017	0.029	0.017	0.030	0.045		0.008	0.019	0.021	0.067
PG	0.066	0.026	0.031	0.021	0.041	0.049	0.012		−0.009	−0.002	0.018
NP	0.064	0.037	0.045	0.036	0.040	0.067	0.013	0.022		0.016	0.029
PB	0.107	0.066	0.058	0.033	0.047	0.062	0.045	0.031	0.077		0.037
CW	0.139	0.092	0.096	0.086	0.102	0.120	0.070	0.027	0.053	0.092	

STRUCTURE was used to analyze the genetic structure of the Chinese fir. The maximum ΔK value (43.694) was observed for $K = 3$ (Figure 3A), which indicated that the 330 individuals of Chinese fir could be divided into three different subgroups. With the help of the structure bar plot (Figure 3C), the genotypes were clearly grouped. There is a certain degree of genetic introgression among populations, but there is still differentiation. Cluster one included RS, ND, TE, JX, LS, LL, ZY, NP, and PG, which are mainly located in the north and west of Guangxi. Cluster two was PB, and cluster three was CW, which were located in the southern coastal areas and eastern areas of Guangxi, respectively. When PB and CW were excluding, an investigation of the substructure was conducted among the remaining nine sampling localities. ΔK value peaked at $K = 4$, and the maximum value was only 3.458 (Figure 3B). However, Chinese fir in all sampling localities had different genetic

backgrounds (Figure 3D). Most sampling localities are composed of admixed individuals, and only PB and CW are clearly differentiated.

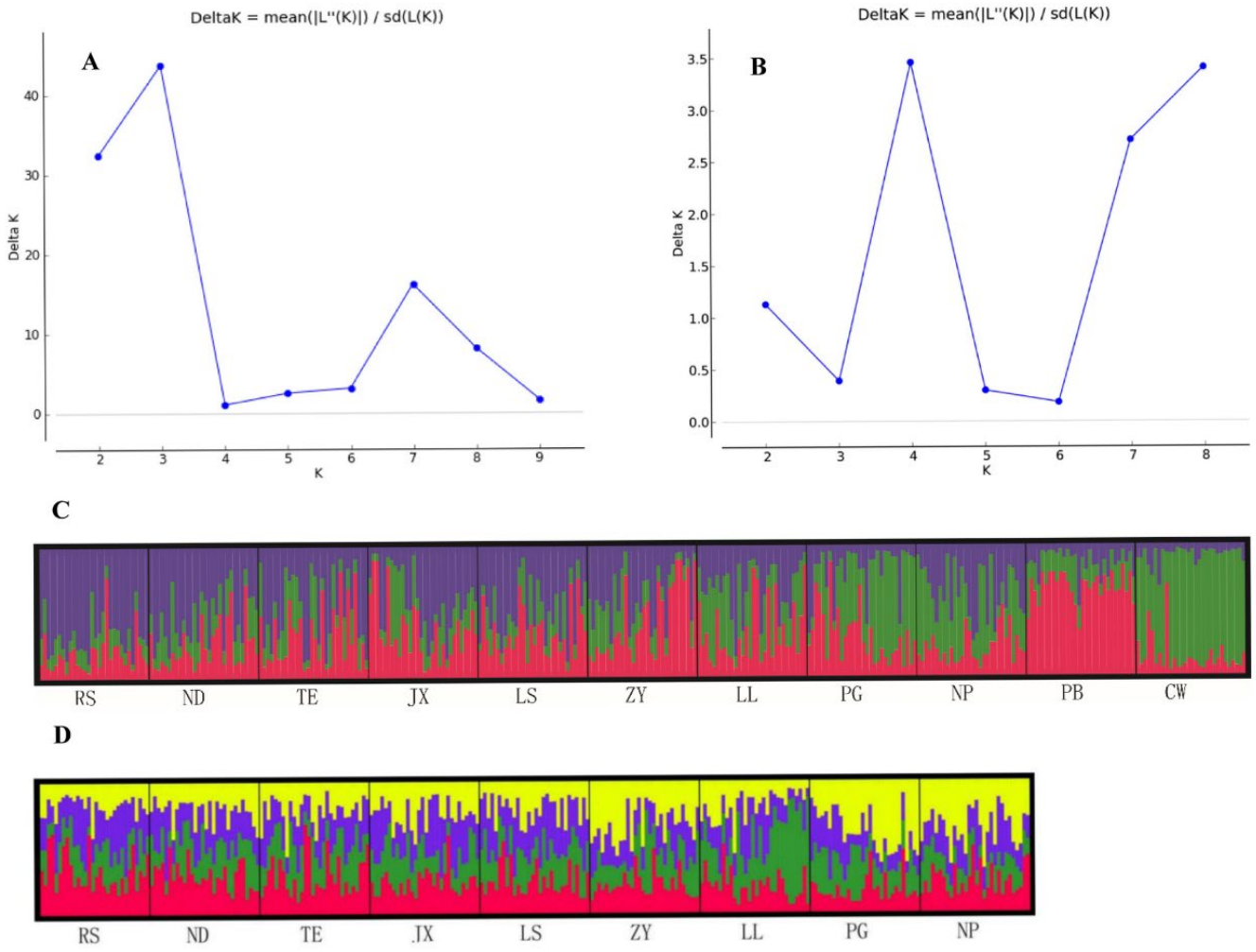


Figure 3. Population structure analysis for 11 and 9 sampling localities. (**A**) The best *K* value estimated by ln likelihood for 11 sampling localities. (**B**) The best *K* value estimated by ln likelihood for 9 sampling localities. (**C**) Bar plot of individual ancestral composition for the genetic clusters, using *K* = 3 and the dataset of 330 individuals from 11 sampling localities. (**D**) Bar plot of individual ancestral composition for the genetic clusters, using *K* = 4 and the dataset of 270 individuals from 9 sampling localities.

To further explore the genetic relationship among Chinese firs in different sampling localities, the Nei's genetic distance of the 11 sampling localities was used for PCoA (Figure 4A) and UPGMA cluster analysis (Figure 4B). The clustering result of PCoA was generally consistent with that of the STRUCTURE partition. Chinese firs in PB and CW were separated into a single cluster, and Chinese firs in another nine sampling localities were clustered together. According to the F_{ST} and *Nm* values and D_{est} values in different sampling localities, the same genetic group had smaller genetic differentiation and more frequent gene exchange, whereas Chinese fir from different groups had larger inheritance differentiation and less gene exchange.

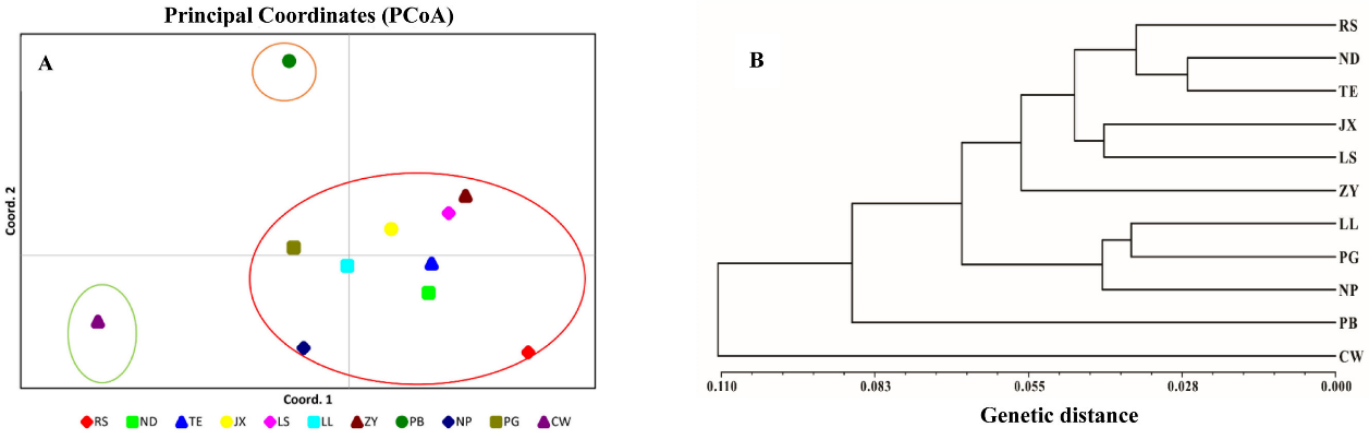


Figure 4. (**A**) Principal coordinate analysis for Chinese firs in 11 sampling localities. The first and second axis explained 57.05% and 23.33% of the genetic variation. For sampling localities abbreviations, see Table 1. (**B**) Unweighted group average method (UPGMA) cluster analysis of 11 sampling localities based on genetic distance.

3.4. IBD Pattern of Chinese Fir in Sampling Localities around the Karst Landform

The results of the Mantel test showed that there was a positive correlation between genetic distance and geographical distance for Chinese firs in 11 sampling localities, but this correlation was not significant (r = 0.15, p = 0.22) (Figure 5A). PB and CW were the most differentiated sampling localities. In order to analyze which localities have a greater impact on IBD, Mantel tests were applied in nine sampling localities, excluding PB and CW. The correlation was positive, and the Mantel tests were significant (r = 0.33, p = 0.04) (Figure 5B). It indicated significant IBD was found in the nine sampling localities. PG, JX, PB, and CW are located in the south of the karst landform. When Mantel tests were applied in seven sampling localities, excluding PG, JX, CW, and PB, the correlation coefficient reached 0.68, and the Mantel tests were significant (p = 0.01) (Figure 5C).

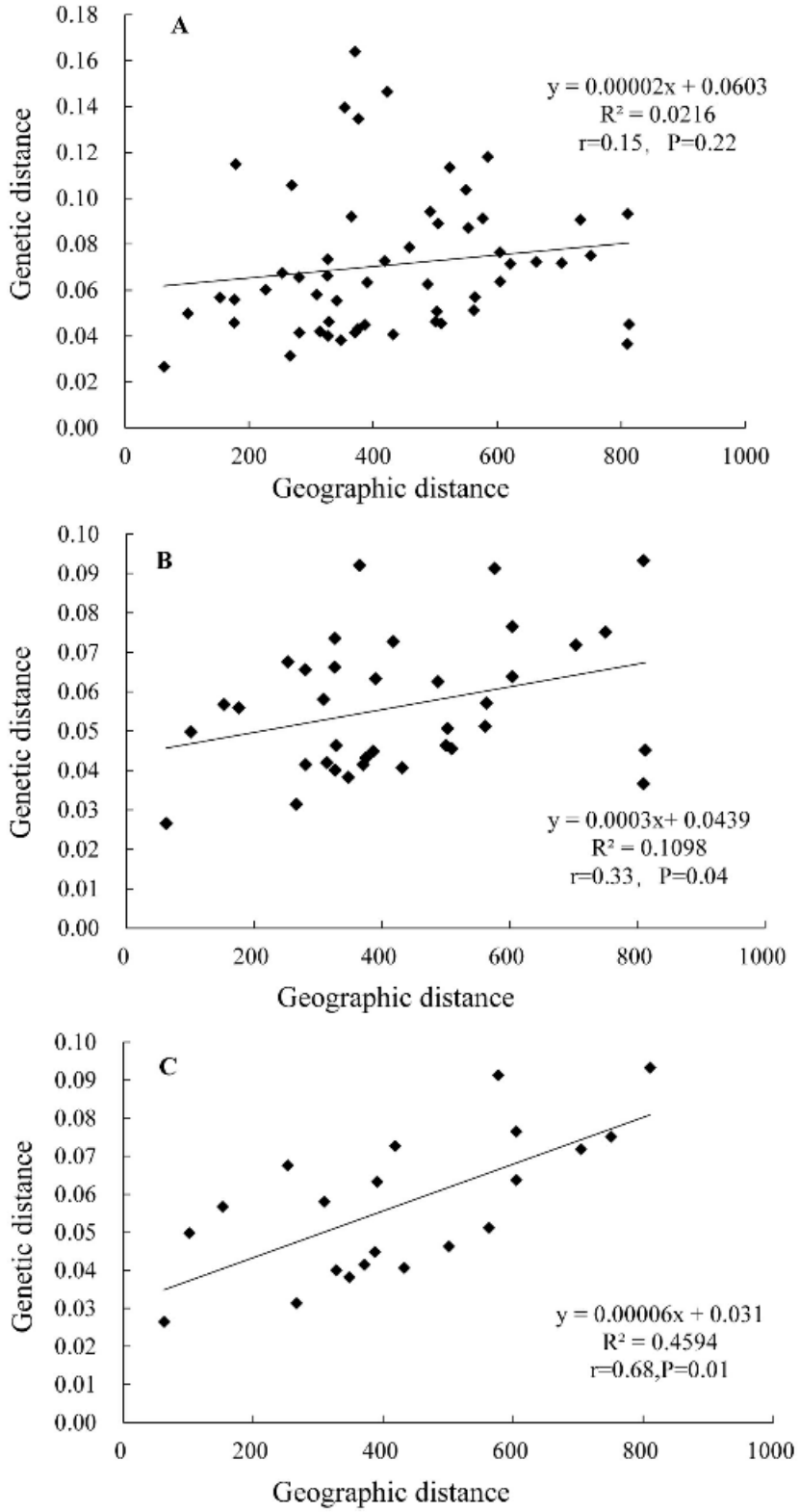


Figure 5. Correlation of genetic distance $[F_{ST}/(1 - F_{ST})]$ with geographical distance (Km) of Chinese firs in different sampling localities. (**A**) The correlation of all 11 sampling localities. (**B**) The correlation of 9 sampling localities, excluding sampling localities of CW and PB. (**C**) The correlation of 7 sampling localities, excluding sampling localities of PG, JX, CW, and PB.

4. Discussion

Highly variable microsatellite loci are a useful tool for studying genetic diversity and genetic structure [30,31]. However, stuttering, large-allele dropout, and null alleles are three typical sources of scoring errors capable of biasing biological conclusions with microsatellite data [32]. In addition, a small percentage of EST-SSR loci could be under selective pressure [33]. Because population genetics applications are based on neutral assumptions, SSR loci under selective pressure should be excluded from analyses [34]. Neutral markers could reflect population processes, including patterns of recent gene flow and historical connectivity, as well as changes in population size [35]. A locus with a polymorphism information content (PIC) value over 0.5 could be regarded as highly polymorphic [36]. The frequency of null alleles below 8% was found to have little effect on population differentiation [37]. In this study, we developed 22 novel SSR markers, and all the loci were suitable for population analyses.

Karst areas occupy approximately 15% of continental terrains [38]. Southwestern China, where karst areas cover nearly 4.26×10^5 km^2, contains the largest and most well-developed karst region in the world [6,39]. Guangxi province is home to one of the largest karst areas in China. The effects of habitat fragmentation may lead to a discontinuous pattern of genetic variation and reduce the genetic diversity of the populations, mainly due to the limited gene flow [9]. The genetic diversity of Chinese firs in RS was higher than that of the other sampling localities. RS population is one of the superior provenances of the species with a fast-growing phenotype in China [40]. ND had the highest rates of gene flow with other sampling localities. No significant F_{ST} differentiation was detected in pairwise comparisons among RS, ND, and TE sampling localities, which could be the consequence of high levels of gene flow homogenizing RS, ND, and TE sampling localities. The three sampling localities are speculated to be the central source of gene flow and genetic diversity for Chinese firs around the karst landform.

Marginal populations occur near the outer boundary of the geographic range and in atypical habitats of the species [41]. By reason of genetic drift and natural selection, marginal populations will diverge from central populations [41]. Compared to central populations, marginal populations show reduced neutral genetic diversity and increased genetic differentiation [10]. CW and PB counties are located near the southern tip of China. Pairwise differentiation based on F_{ST} and D_{est} values confirmed the results analyzed by PCoA and NJ tree that CW had the most genetic differentiation in all sampling localities, and PB came second. With higher differentiation and less genetic variation than in the central population, populations of CW and PB were identified to be the marginal populations in the south of the karst landform.

Tree species dynamics at range edges are frequently driven by climatic factors [42]. Many low-latitude populations of cold-adapted plant species will be negatively affected by global warming [43]. Cold-adapted populations at low-latitude range edges are important because they may harbor the combination of alleles that foster persistence in a warmer climate [44]. PB county is located in the south of the Tropic of Cancer, near the southern tip of China, with the maximum temperature for Chinese fir growth. The climate of CW and PB is similar to that of the nearby area, where the annual highest temperature is about 40 °C [45]. Chinese firs are not resistant to overly high summer temperatures [46]. Under a warmer climate, genetic diversity would be subjected to selective pressure [47]. Evolutionary adaptation is an important way for natural populations to cope with threats, including extreme climate [48]. Because neutral molecular variation rarely predicts adaptive and quantitative genetic variation, it is common to fail distinguishing between differentiation due to drift and differentiation due to adaptation to different environmental conditions [49,50]. The association between environmental gradients and genotypes could be analyzed by landscape genomic approach in the future to identify potential loci from Chinese fir underlying extreme climate adaptation.

The ability of a species to adapt to peripheral habitats in marginal populations plays a major role in the evolution of species ranges and adaptation in the face of environmental change [10,41]. Marginal populations are worthy of special conservation interest [12,51,52]. Large-scale afforestation and reforestation of other tree species have occurred in Guangxi [53]. Habitat loss has been most extensive for CW and PB populations. Translocations are among the most powerful tools for biodiversity conservation [54]. Augmentation (movement of individuals into a population of conspecifics) is recognized as one of the three different types of translocations by the International Union for Conservation of Nature [55]. Augmentation can be beneficial to increasing population size [56]. By promoting the adaptive potential for evolutionary change, individuals mixing from several source populations will enhance the establishment and persistence of the translocated populations [57,58]. To preserve the genetic diversity of CW and PB populations of Chinese fir, we propose the following measures. First, core germplasm individuals from CW and PB could be collected, as previous research demonstrated [59]. In the second place, considering that locally adapted populations are most likely to establish and persist under similar ecological conditions, it is proposed that the core germplasm individuals could be freely released into nature reserves in nearby counties such as JX. In the end, a mixture of individuals from several gene pools in the north of the karst landform could be freely released into nature reserves of CW and PB.

Author Contributions: Conceptualization, K.L.; formal analysis, K.L.; methodology, K.L.; resources, K.L.; writing—original draft, K.L.; writing—review and editing, K.L.; funding acquisition, K.L. and K.H.; software K.L. and S.C.; investigation, K.L., X.C. and S.C.; supervision, K.L. and S.C.; validation, K.L. and S.C.; visualization, K.L. and S.C.; data curation, X.C. and K.H.; Project administration, X.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the National Key Research and Development Program Sub-project, China (2022YFD2200201-5), National Natural Science Foundation of China (32060352), Department of Human Resources and Social Security of Guangxi Zhuang Autonomous Region, China (GuiCaiSheHan[2018]112) and Guangxi Science and Technology Program (GK AB22035083).

Data Availability Statement: Sampling locations and microsatellite genotypes: https://doi.org/10.5061/dryad.bk3j9kdb5 (accessed on 18 February 2021).

Acknowledgments: We thank the reviewers and editor for all the suggestions and improvements on the manuscript. The authors would like to thank Siming Gan from the Research Institute of Tropical Forestry of the Chinese Academy of Forestry for technical assistance.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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