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Genetic Diversity and Population Structure of *Pinus yunnanensis* by Simple Sequence Repeat Markers

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Pinus yunnanensis is the main species used for reforestation in Yunnan province, China. To manage the genetic resources of this economically and ecologically important species efficiently, greater knowledge of the distribution of its genetic diversity is needed. In this study, the genetic diversity of 20 natural populations representing most of the *P. yunnanensis* range was examined using seven simple sequence repeats. No loci showed significant departure from Hardy-Weinberg equilibrium. Most genetic diversity was found within populations, and overall genetic differentiation was low ($F_{ST} = 0.045$). There was no sign of isolation by distance. Clustering of populations using both neighbor joining pair groups and principal coordinates analysis based on Nei's genetic distance showed the presence of two major clusters. This result was reinforced using the Bayesian software STRUCTURE, which identified two southern populations as clearly separate from the others. There were no signs of genetic erosion, but some populations should be a focus of conservation efforts because of their higher level of genetic diversity and the presence of private alleles. These results will inform conservation and management of *P. yunnanensis* and will guide future studies of population genetics and breeding programs.

Keywords: microsatellite, Yunnan pine, genetic structure, genetic diversity

Southwest China is one of 25 biodiversity hotspots (Myers et al. 2000). Pine forests there are dominated by *Pinus yunnanensis* French, a species of great economic and ecological importance (Li et al. 2008, Liu et al. 2009) that grows at altitudes of 600–3,200 m and ranges from 23 to 30° N and 96–108° E (Jin and Peng 2004, Chen et al. 2012). Resources of *P. yunnanensis* are most threatened by deforestation and natural disasters such as fires and drought. Recently, Chen et al. (2014) suggested that the main vegetation types in Yunnan province that are prone to fires are forests and woodlands dominated by *P. yunnanensis*. Because *P. yunnanensis* favors steep mountainside habitats, most regeneration is natural, but this has been limited by competition from herbaceous plants and predation of seeds by birds (Huang 1993, Zhou et al. 2010).

Concerns about the high volume of removal from natural forests and high frequency of forked, crooked, and twisted trees in regenerated forests (He 1997) led to renewed interest in germ plasm assessment and conservation (Xu et al. 2012). Studies of the phenotypic variation of a small number of sources of *P. yunnanensis* demonstrated significant morphological diversity in needle traits based on habitat (Wang et al. 2003, 2004). A study of a large number of needle and cone traits of 18 populations of Yunnan pine showed that total variation was equally divided among and within provenances and that some needle traits showed clinal variation in response to latitudinal and altitudinal gradients (Xu et al. 2015).

Provenance trials of this species began in 1980. Provenances were remarkably variable in seed weight, seed germination percentage, seedling root length and needle length, and 10-year-old seedling

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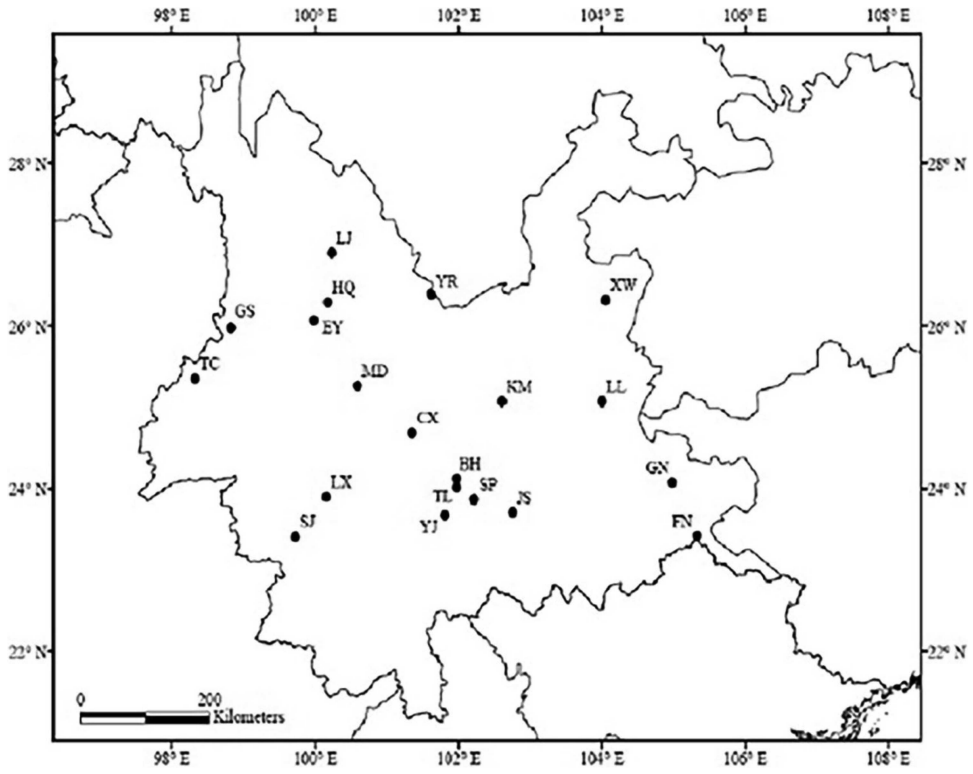

Figure 1. Distribution of *P. yunnanensis* populations sampled in this study. All sampled sites are in Yunnan province, China. Population abbreviations are described in Table 1.

height and diameter. Seedling height and diameter were significantly negatively correlated with latitude and altitude, and superior provenances for height and diameter were identified (Shu et al. 1992). Six seed zones were proposed based on ecology, provenance trials, and administrative division (Chen and Wu 1987). Two seed orchards (54.9 ha) were established in the early 1990s based on superior selections from different regions (He 1997), but seed yields were variable and generally not sufficient for reforestation needs.

At the molecular level, Yu et al. (2000) investigated genetic variation and differentiation of 10 natural populations sampled from the core range of Yunnan pine using 33 loci from 14 enzyme systems. They found relatively high levels of genetic variability and moderate differentiation among populations ($G_{ST} = 0.134$). The mean expected heterozygosity in *P. yunnanensis* was comparable to the means of other gymnosperm species. Recently, Wang et al. (2013) studied the intraspecific genetic diversity of *P. yunnanensis* among 16 populations based on three polymorphisms in uniparentally inherited maternal genomes (mtDNA) and five polymorphisms in paternal genomes (cpDNA). By combining these data with geographical and environmental variables, seven population groups were detected based on variations in mtDNA. They found no population-level differentiation based on cpDNA. The mode of inheritance (maternally, paternally, or biparentally inherited markers) has a major effect on the partitioning of genetic diversity (Petit et al. 2005). In pines, mtDNA is nonrecombinant and has a low mutation rate, making it appropriate for the detection of ancient genetic structure and genetic diversity (Powell et al. 1995, Provan et al. 2001). Nuclear microsatellites, in contrast, are biparentally inherited and recombinant, so they generally reveal contemporary genetic diversity and genetic structure (André et al. 2011).

Previous studies of the genetic diversity and spatial genetic structure of *P. yunnanensis* sampled a relatively small number (3–10) of populations (Yu et al. 2000, Wang et al. 2003, 2004), were based on uniparentally inherited genetic markers only (Wang et al. 2013), or were focused on morphological characteristics (Wang et al. 2003, 2004, Xu et al. 2015). The main objectives of this research were to use a sample of 20 natural populations of *P. yunnanensis* taken from across the species' natural range. Our goal was to complement previous studies by determining the level and structure of the genetic diversity of *P. yunnanensis* using nuclear microsatellites and to explore the relationship between genetic diversity and environmental factors. The findings will be useful for the conservation and managements of this species.

Methods

Sampling

To avoid sampling from closely related individuals, fresh needles were collected from 20 to 30 randomly selected trees separated by at least 100 m at 18 of the 20 sampled sites in Yunnan province, China (Figure 1; Table 1). Populations were selected on the basis of accessibility and to maximize regional representation for the species (Lemes et al. 2003). Trees were sampled without regard to phenotype. The needles of two populations (LJ and GS) were from seedlings growing in a nursery managed by Beijing Forestry University in 2011. Seeds from populations LJ and GS were collected from 20 to 30 cones from the middle part of the crown of each of 20 healthy appearing, apparently even-aged (older than 35 years), open-pollinated trees. Each sampled tree was at least 100 m from any other sampled tree (Mao et al. 2009). Seeds were mixed by population. Three hundred seeds were selected randomly from each population. The needles from populations LJ and GS were collected randomly from each of 30 2-year seedlings. For all samples, needles were collected, enclosed in plastic bags, and stored at −20° C before DNA extraction.

Table 1. Location and geographic characteristics of 20 populations of *Pinus yunnanensis* sampled.

Population name	Population abbreviation	Longitude	Latitude	Altitude (m)	T_{mean}	T_{max}	T_{min}	Prec. (mm)
		.....(°).....			.....(° C).....			
Baihe (Xinping 1)	BH	101.97	24.12	1,834	19	28	5	1,008
Chuxiong	CX	101.35	24.68	1,997	16	25	2	1,015
Eryuan	EY	99.98	26.06	2,290	13	22	−1	1,051
Funing	FN	105.33	23.42	1,240	17	27	5	1,362
Guangnan	GN	104.98	24.08	1,287	17	27	3	1,104
Gongshan	GS	98.82	25.97	1,689	17	25	2	1,436
Heqing	HQ	100.17	26.29	2,227	14	23	0	1,013
Jianshui	JS	102.75	23.70	1,609	18	27	5	1,074
Kunming	KM	102.60	25.07	2,192	14	23	0	1,031
Lijiang	LJ	100.23	26.89	2,449	13	23	−1	972
Luliang	LL	104.00	25.07	1,890	15	25	1	1,042
Linxiang	LX	100.15	23.90	1,875	16	25	3	1,210
Midu	MD	100.59	25.27	2,099	16	26	3	968
Shuangjiang	SJ	99.72	23.40	1,222	21	31	7	1,360
Shiping	SP	102.22	23.87	1,504	18	27	5	1,096
Tengchong	TC	98.32	25.36	2,024	14	22	0	1,541
Tala (Xinping 2)	TL	101.97	24.02	1,639	18	27	5	1,062
Xuanwei	XW	104.05	26.32	2,222	12	23	−1	969
Yuanjiang	YJ	101.81	23.68	1,654	18	28	5	1,188
Yongren	YR	101.62	26.37	2,132	14	25	0	1,013

Table 2. Primer sequence, annealing temperature, genetic diversity characteristics, and source for each of the SSR markers used in the study.

Locus	Primers: forward (5′ → 3′) and reverse (5′ → 3′)	T_m (° C)	H_o	H_e	Reference
PtTX2123	GAAGAACCCACAAACACAAG GGGCAAGAATTCAATGATAA	54.0	0.402	0.348	Elsik et al. (2000)
PtTX2146	CCTGGGGATTTGGATTGGGTATTTG ATATTTTCCTTGCCCCTTCCAGACA	54.0	0.787	0.752	Elsik et al. (2000)
PtTX3118	AACCATTTGCCCCTTCTT AAAAACAGTCTGCAATCAAATC	45.0	0.558	0.513	Kutil and Williams (2001)
PtTX3127	ACCCTTACTTTCAGAAGAGGATA AATTGGGGTTCAACTATTCTATTA	48.0	0.132	0.128	Elsik and Williams (2001)
PTest1	CGATGTCGATTAGGGATTGG CCTGTTCTTCGTCGGATGTT	52.0	0.531	0.427	Wang (2006)
PyMR08	CCCGCCAATGCATTTTATAC TTGGTGTGTGTGTGGATGAT	50.0	0.324	0.315	Xu et al. (2013)
Pr001	CAAAGATTACATTAATTCACTCCACC ATTCTTCCATCCACTCTATGAATG	54.1	0.531	0.516	Devey et al. (2002)

T_m, annealing temperature; H_o, observed heterozygosity; H_e, expected heterozygosity.

DNA Extraction

Total genomic DNA was extracted from needles following a modified cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle 1990). To improve DNA purity, we used a higher concentration of CTAB (4 g/100 ml) (Khanuja et al. 1999, Bai et al. 2013). The extracted genomic DNA was dissolved in 50 μl of Tris-EDTA buffer and stored at −20° C.

Polymerase Chain Reaction (PCR) Amplification and Genotyping

Four samples from each of five populations were selected to screen simple sequence repeat (SSR) primers. Eighteen loci were selected after initial testing, but nine were discarded because of failure of amplification, low product yield, or lack of polymorphism, and two were excluded from the analysis because of the presence of null alleles. Finally, seven previously published loci (Table 2) were used to analyze 459 individuals from 20 populations. Details concerning the microsatellites we used to characterize the samples were published in Elsik et al. (2000), Elsik and Williams (2001), Kutil and Williams (2001), Wang (2006), Devey et al. (2002), and Xu et al. (2013).

One primer of each pair carried a 6-FAM, TET, or HEX (TAMRA) fluorescent tag at the 5′ end. PCRs for all loci were performed separately in a 10-μl reaction volume containing 30 ng of genomic DNA, 0.2 μM concentrations of each primer, 1.0 U of Taq polymerase (Takara, Dalian, Liaoning, China), 0.4 mM dNTP mixture, 2.0 mM Mg^{2+}, and 1× PCR buffer. The amplification protocol was the following: 94° C for 4 minutes followed by 30 cycles at 94° C for 45 seconds, annealing at a specific temperature for each locus for 30 seconds, 72° C for 30 seconds, and a final extension step at 72° C for 10 minutes. GS-500 LIZ (Applied Biosystems) was used as a size standard. Amplification products were resolved using capillary electrophoresis on an ABI3730xl DNA analyzer (Applied Biosystems).

Data Analysis

The electropherograms were analyzed using GeneMarker version 1.75 (Applied Biosystems). CONVERT version 1.3.1 (Glaubitz 2004) was used to convert input files for analysis in subsequent software. Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004) was used to check for the presence of null alleles, scoring error due to stuttering, and large allele dropout in each population across all loci. The genetic diversity parameters of polymorphic loci including the number of different alleles (N_a), the number of effective alleles (N_e), Shannon's information index (I), observed (H_o) and

Table 3. Genetic diversity in 20 natural populations of *Pinus yunnanensis* across seven SSR loci.

Population	N_a	N_e	I	H_o	H_e	UH_e	F	P/%	P_a	F_{IS}
BH	3.7	2.3	0.873	0.541	0.467	0.483	−0.165	100.00	0	−0.183
CX	4.4	2.1	0.824	0.437	0.421	0.431	−0.046	100.00	0	0.013
EY	4.0	2.0	0.834	0.440	0.440	0.450	0.004	100.00	0	0.046
FN	4.0	2.2	0.873	0.558	0.459	0.469	−0.195	100.00	1	−0.257
GN	3.7	2.0	0.750	0.434	0.372	0.382	−0.151	100.00	0	−0.265
GS	4.3	2.2	0.892	0.470	0.467	0.477	0.071	100.00	1	0.044
HQ	4.3	2.2	0.833	0.479	0.417	0.426	−0.135	100.00	0	−0.068
JS	4.7	2.1	0.871	0.463	0.429	0.438	−0.061	100.00	0	−0.010
KM	4.6	1.9	0.764	0.410	0.374	0.383	−0.088	100.00	0	−0.159
LJ	3.7	1.9	0.718	0.399	0.363	0.371	−0.068	85.71	0	−0.015
LL	4.9	2.2	0.888	0.489	0.441	0.451	−0.120	100.00	0	−0.174
LX	4.1	2.2	0.930	0.526	0.492	0.503	−0.071	85.71	0	−0.156
MD	3.9	2.0	0.795	0.469	0.424	0.435	−0.081	85.71	0	0.081
SJ	3.7	2.4	0.861	0.531	0.448	0.463	−0.182	85.71	0	−0.168
SP	4.7	2.2	0.941	0.482	0.467	0.477	−0.061	100.00	0	−0.026
TC	2.6	1.7	0.567	0.427	0.339	0.347	−0.216	85.71	0	−0.052
TL	4.4	2.0	0.883	0.442	0.453	0.463	0.018	100.00	0	−0.056
XW	3.3	1.8	0.607	0.329	0.312	0.319	0.071	100.00	0	−0.044
YJ	5.1	2.8	1.124	0.565	0.567	0.579	−0.006	100.00	1	0.044
YR	4.4	1.9	0.833	0.437	0.417	0.429	−0.053	100.00	2	−0.042
Mean	4.1	2.1	0.833	0.466	0.428	0.439	−0.075	96.43		−0.073
SE	0.2	0.1	0.039	0.021	0.019	0.019	0.016	1.42		

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; UH_e, unbiased expected heterozygosity; F, fixation index; P%, percentage of polymorphic loci; P_a, private alleles; F_{IS}, inbreeding coefficient. Population abbreviations are described in Table 1.

expected levels of heterozygosity (H_e), unbiased expected heterozygosity (UH_e), fixation index (F), private alleles (P_a), and genetic differentiation among pairwise populations (F_{ST}) were estimated using GenAlEx 6.4 (Peakall and Smouse 2006). The inbreeding coefficient (F_{IS}) for each population was estimated using Arlequin version 3.5 (Excoffier and Lischer 2010). The pairwise Nei's (1983) genetic distance was calculated using PowerMarker version 3.25 (Liu and Muse 2005), and Hardy-Weinberg equilibrium for each locus and pairwise loci linkage disequilibria (LDs) were evaluated for each population using POPGENE version 1.32 (Yeh et al. 1997). Genetic structure was analyzed using STRUCTURE version 2.3.3 (Pritchard et al. 2000, Falush et al. 2003, 2007). For each simulation, we used 10 independent runs for each K value (K = 1–10 populations) to obtain $L(K)$ with LOCPRIOR assigned (Hubisz et al. 2009) under the admixture model, allele frequencies correlated, with a burn-in of 100,000 steps, and Markov chain Monte Carlo for 200,000 steps. Initially, the number of genetic groups (K) was determined based on the number of natural populations. Then, the optimal number of clusters K was determined using the ΔK criteria following the procedure of Evanno et al. (2005). Bar plots of the proportional memberships of the individuals were generated and visualized using STRUCTURE version 2.3.3 (Pritchard et al. 2000, Falush et al. 2003, 2007). The degree of genetic divergence among populations was assessed by analysis of molecular variance (AMOVA) (1,000 permutations) using Arlequin version 3.5 (Excoffier and Lischer 2010). To test for isolation by distance, a Mantel test (Mantel 1967) was used to correlate pairwise Nei's (1983) genetic distance and geographical distance matrices using NTsys software (Rohlf 2004). The principal coordinates analysis (PCoA) was performed using GenAlEx 6.4 (Peakall and Smouse 2006).

Correlations among genetic diversity parameters and geoclimatic factors were determined using a Spearman nonparametric correlation coefficient matrix constructed with SPSS software version 18. The climatic data, i.e., annual mean temperature (T_{mean}), maximum temperature of the warmest month (T_{max}), minimum temperature of the coldest month (T_{min}), and annual precipitation (Prec) were extracted from Worldclim—Global Climate Data[1] (Hijmans et al. 2005) using each population's longitude and latitude with ArcGIS 9.3.

Results

Genetic Diversity

All loci displayed polymorphisms in *P. yunnanensis*. There were a total of 29 different alleles (N_a) across all seven loci, with an average of 4.1 alleles per locus. On a per-population basis, the average number of alleles per locus across all seven loci varied from 2.6 to 5.1, with an average of 4.1 (Table 3). Locus PtTX2146 detected the highest level of polymorphism, with an average of 6.7 alleles. Less polymorphism was observed in PtTX2123, with a mean of 2.1 alleles. The number of effective alleles (N_e) varied from 1.2 (PtTX3127) to 4.2 (PtTX2146) with an average of 2.1. On a per-locus basis, observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.132 to 0.787 and from 0.128 to 0.752, respectively. The overall observed heterozygosity (H_o) was 0.466, just slightly more than the expected heterozygosity (H_e) (0.428) (Table 3). We observed heterozygote excess in 17 of 20 populations sampled; FN had the largest heterozygote excess ($H_o = 0.558 > H_e = 0.459$). Mean observed heterozygosity was moderately higher than mean expected heterozygosity (8%). Values of H_e were not different from those for unbiased heterozygosity (UH_e), indicating that Hardy-Weinberg equilibrium assumptions applied to the sampled *P. yunnanensis* populations (Naydenov et al. 2011). There were no significant departures from Hardy-Weinberg equilibrium over all loci for any populations, but some populations deviated from Hardy-Weinberg equilibrium at up to four loci ($P < 0.05$). A significant LD ($P < 0.05$) was detected in a few pairs of loci in 10 of the 20 populations, but no locus pair showed significant LD in all populations. The number of loci in LD was highest (five locus pairs) in JS followed by KM (three locus pairs). Five private alleles were detected; two in YR, one in YJ, FN, and GS, and none in the remaining 16 populations.

Table 4. F_{ST} values between pairwise populations across seven loci.

	BH	CX	EY	FN	GN	GS	HQ	JS	KM	LJ	LL	LX	MD	SJ	SP	TC	TL	XW	YJ	YR
BH	0.000																			
CX	0.012	0.000																		
EY	0.029	0.020	0.000																	
FN	0.021	0.020	0.027	0.000																
GN	0.041	0.032	0.040	0.033	0.000															
GS	0.029	0.018	0.018	0.020	0.050	0.000														
HQ	0.043	0.032	0.032	0.026	0.022	0.031	0.000													
JS	0.015	0.008	0.022	0.021	0.027	0.025	0.023	0.000												
KM	0.025	0.015	0.013	0.028	0.026	0.027	0.022	0.009	0.000											
LJ	0.063	0.042	0.032	0.050	0.048	0.047	0.037	0.036	0.028	0.000										
LL	0.023	0.008	0.014	0.021	0.023	0.019	0.020	0.012	0.014	0.027	0.000									
LX	0.017	0.019	0.042	0.036	0.051	0.040	0.044	0.021	0.036	0.070	0.026	0.000								
MD	0.029	0.021	0.033	0.038	0.041	0.031	0.026	0.020	0.024	0.052	0.022	0.030	0.000							
SJ	0.096	0.122	0.110	0.115	0.141	0.128	0.131	0.112	0.122	0.161	0.121	0.105	0.119	0.000						
SP	0.013	0.007	0.021	0.013	0.027	0.018	0.024	0.007	0.017	0.038	0.008	0.022	0.025	0.116	0.000					
TC	0.076	0.063	0.052	0.054	0.097	0.029	0.051	0.064	0.059	0.069	0.061	0.100	0.071	0.199	0.063	0.000				
TL	0.013	0.009	0.032	0.032	0.035	0.033	0.039	0.014	0.024	0.060	0.016	0.009	0.027	0.112	0.013	0.090	0.000			
XW	0.066	0.042	0.026	0.052	0.039	0.051	0.038	0.040	0.023	0.018	0.030	0.082	0.061	0.168	0.042	0.076	0.062	0.000		
YJ	0.046	0.058	0.062	0.065	0.086	0.067	0.077	0.052	0.073	0.090	0.055	0.038	0.055	0.042	0.050	0.116	0.050	0.108	0.000	
YR	0.022	0.013	0.022	0.019	0.014	0.029	0.016	0.010	0.013	0.031	0.007	0.028	0.026	0.122	0.007	0.068	0.018	0.030	0.059	0.000

Population abbreviations are defined in Table 1.

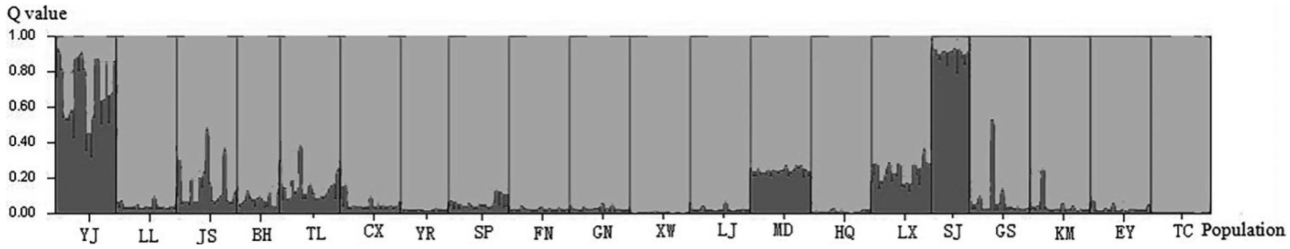

Figure 2. The proportions of cluster memberships at the individual level in 20 *P. yunnanensis* populations. Population abbreviations are described in Table 1.

Most of the genetic diversity was within populations. Per locus F_{ST} varied from 0.038 (PtTX3127) to 0.140 (Pr001) with an average of 0.086. The pairwise F_{ST} values ranged from 0.007 (LL versus YR, SP versus CX, SP versus JS, and SP versus YR) to 0.199 (SJ versus TC) (Table 4). All F_{ST} values between pairwise populations were >0, which indicated the presence of population structure in *P. yunnanensis*, but the overall value was relatively low (overall F_{ST} was 0.045 ± 0.035, indicating that the population structure was weak. Population SJ showed the highest mean pairwise F_{ST} with other populations (0.123 ± 0.031), and population YJ was the most genetically diverse (Table 4).

Genetic Structure

Analyses of the genotypic data using the software STRUCTURE found the best partition to be two genotypic clusters ($K = 2$). The percentages of individuals in cluster I and cluster II were 81.48 and 5.23%, respectively, whereas 13.29% of the individuals had mixed membership, with Q scores of $0.8 > Q > 0.2$. Individuals with admixed population assignments were from eight populations, i.e., YJ, JS, TL, MD, LX, SJ, GS, and KM. The individuals in populations LL, BH, CX, YR, SP, FN, GN, XW, LJ, HQ, EY, and TC with Q values of >0.8 belonged to cluster I (Figure 2). No population contained individuals in both clusters. Individuals in population SJ were nearly all (14 of 15) assigned to cluster II; members of population YJ were either admixed or cluster II. All members of MD were admixed, and most members of LX were admixed. A few members of JS, TL, GS, and KM were admixed, possibly indicating gene flow from cluster II trees.

Further STRUCTURE analyses were performed to determine whether there was evidence of substructure within genetic cluster II (Pollegioni et al. 2014). We found no strongly supported genetic substructure within cluster II (data not shown), so although there was evidence for genetic separation of the two southern populations YJ and SJ (Figures 2 and 3), all of the other collection sites can be considered a single metapopulation.

Genetic Differentiation

AMOVA showed high within-population diversity (93% of variation) and low diversity among populations (7% of variation). Pairwise population Nei's (1983) genetic distance varied from 0.027 to 0.273, with a mean of 0.091 between populations. The highest Nei's (1983) genetic distance was between populations SJ and LJ. Cluster analysis (neighbor joining) based on Nei's (1983) genetic distance showed the presence of two major groups (Figure 3), which supported the results from STRUCTURE. Group I contained two populations from the southern periphery (YJ and SJ), whereas group II, spanned a large geographical area and was composed of the other 18 populations. No genetic substructure was strongly supported in group II. PCoA revealed two main groups of populations, much like STRUCTURE, but also provided support for the separation of the far-western population TC (Figure 4). The first component explained 51% of the total variance, and the second component explained 21% of the total variance. The correlation between matrices of Nei's (1983) genetic distance and geographical distance based on a Mantel test was nonsignificant ($r = 0.27$, $P > 0.05$), indicating little isolation by distance.

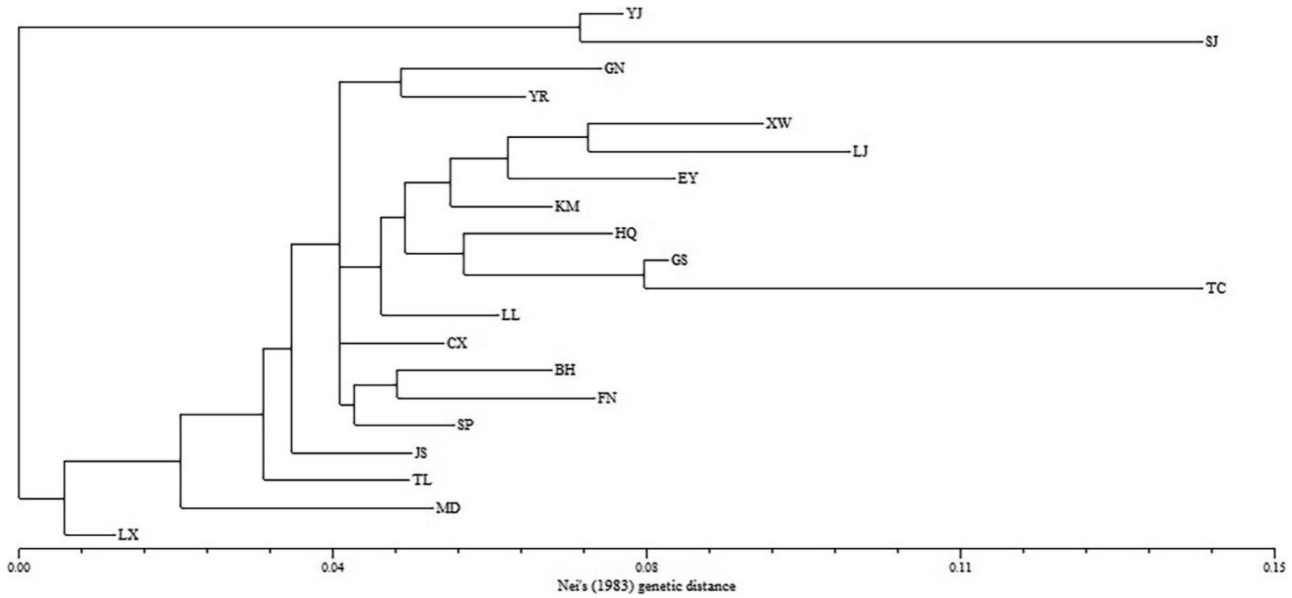

Figure 3. Neighbor joining dendrogram of *P. yunnanensis* populations based on Nei's (1983) genetic distance across seven SSR loci. Population abbreviations are described in Table 1.

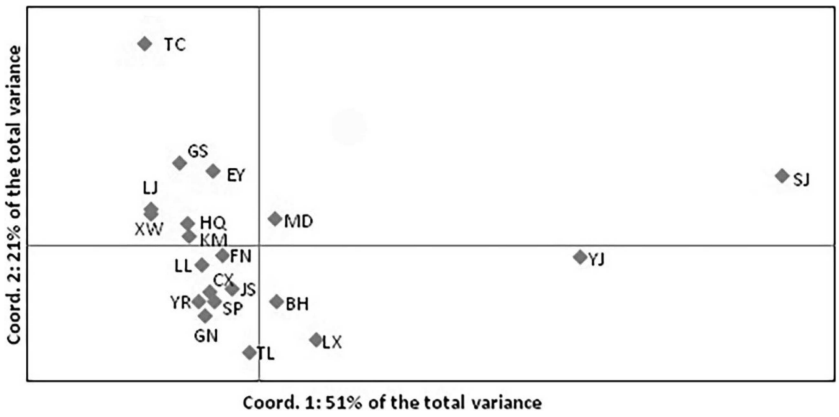

Figure 4. PCoA of *P. yunnanensis* populations based on variability at seven microsatellite loci. Population abbreviations are described in Table 1.

Table 5. Spearman correlation between genetic diversity parameters and geoclimatic variables.

Genetic diversity parameter	Longitude	Latitude	Altitude (m)	T_{mean}	T_{max}	T_{min}	Prec. (mm)
	.....(°).....			.....(° C).....			
N_a	0.26	−0.29	−0.17	0.24	0.21	0.24	0.06
N_e	−0.03	−0.64†	−0.57†	0.71†	0.67†	0.69†	0.33
I	0.04	−0.57†	−0.53*	0.62†	0.55*	0.61†	0.40
H_o	−0.01	−0.66†	−0.57†	0.68†	0.66†	0.72†	0.37
H_e	−0.07	−0.63†	−0.56*	0.69†	0.61†	0.69†	0.42
UH_e	−0.06	−0.63†	−0.56*	0.70†	0.63†	0.70†	0.40
F	−0.04	0.27	0.24	−0.16	−0.15	−0.23	−0.21
F_{IS}	−0.55*	0.43	0.43	−0.33	−0.40	−0.36	−0.15

The genetic parameter abbreviations are the same as in Table 3.
* $P < 0.05$.
† $P < 0.01$.

Correlation between Genetic Diversity Parameters and Geoclimatic Variables

We observed significant correlations between the calculated genetic diversity parameters and geoclimatic variables (Table 5). Genetic diversity indexes, i.e., number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), and unbiased expected heterozygosity (UH_e) exhibited significant and negative correlations ($r = \sim -0.5$ to ~ -0.7, $P < 0.01$) with latitude and altitude. Thus, higher genetic diversities were associated with lower altitudes and latitudes. These parameters were positively correlated with temperature variables ($P < 0.01$), which implied that there were higher genetic diversities

in populations that grow in warmer climates. Inbreeding coefficients (F_{IS}) displayed a negative correlation with longitude ($r = -0.55$, $P < 0.05$), so from west to east, the inbreeding coefficient decreased. The association between genetic diversity parameters and longitude as well as annual precipitation was insignificant ($P > 0.05$). Although the fixation index and the total number of different alleles differed among populations, they were not significantly correlated with any geoclimatic parameters.

Discussion

Genetic Diversity

Overall H_e (0.428) for Yunnan pine was inconsistent with a hypothesis of genetic erosion (Vendramin et al. 2008) and similar to the H_e reported for other *Pinus* species evaluated with SSRs, including *Pinus strobus* ($H_e = 0.531$) (Mandák et al. 2013) and *Pinus contorta* ($H_e = 0.543–0.708$) (Parchman et al. 2011). The high genetic diversity observed in *P. yunnanensis* may be associated with its long generation times, outcrossing mating system, wind pollination, high fecundity, and winged seed dispersal (Hamrick et al. 1979). Other factors that may have contributed to the high levels of diversity include the large spatial range of the species and the longevity of seeds in the soil seed bank (Sanchez et al. 2014). Further investigations of factors such as phenological variability within and among populations should provide new insights into the forces influencing the genetic diversity of Yunnan pine.

Although genetic diversity varied slightly (0.312–0.567) across all populations, no population had significantly lower allelic diversity than the average, which can be explained by high levels of gene flow. A similar pattern was observed for *P. contorta* (Parchman et al. 2011). In this study, the southernmost populations had higher genetic diversity than other populations. A similar trend was found by Yu et al. (2000), who studied *P. yunnanensis* using allozymes. The genetic diversity parameters of Yunnan pine, including number of effective alleles, Shannon's information index, observed heterozygosity, and expected heterozygosity were strongly and significantly negatively correlated with latitude, altitude, and climatic variables. The same genetic diversity parameters were significantly positively correlated with annual mean temperature, maximum temperature of the warmest period, and minimum temperature of the coldest period. Population YJ is located in a warmer environment than most of the other populations sampled, and it showed the highest genetic diversity. This finding corresponded with a recent study of needle traits, which showed that in YJ, the coefficients of variation for needle length and fascicle sheath length were higher than those for all other populations. Five other needle traits, i.e., needle width, fascicle width, ratio of length to width of needle, ratio of needle length to fascicle sheath length, and ratio of fascicle width to needle width also showed relatively high variability in YJ trees (Xu et al. 2015).

There were relatively few private alleles detected, and these were found in only four populations sampled. There was no trend indicating more private alleles in northern or southern populations. There was significant LD ($P < 0.05$) among 5 loci in 10 of 20 populations. No locus pair simultaneously revealed significant LD in all populations, implying that the loci were not closely linked to each other (Bai et al. 2014) and were suitable for use in Bayesian clustering methods (Naydenov et al. 2011). Gupta et al. (2005) reviewed factors that lead to an increase in LD, including inbreeding, small population size, genetic isolation between lineages, population subdivision, low recombination rate, population admixture, natural and artificial selection, and balancing selection. In this study, the LD emerged in 10 populations, i.e., LL, JS, BH, CX, YR, SP, XW, HQ, KM, and EY, which were all located in the north-central portion of the species' range. Selection may be the main cause for the LD in these regions, which are the most heavily logged (Nei 1967). Decreasing the population size by logging reduces the number of genotypes and results in genetic differences between the original population and the population at the end of the logging cycles (Degen et al. 2006). In this way, logging may also increase genetic structure and genetic differences among populations. Although the sites we sampled were natural populations, we were not able to determine their history of logging and regeneration.

Genetic Structure and Genetic Differentiation

The highest Nei's (1983) pairwise population genetic distance was between populations SJ and LJ (0.273), whereas the lowest value was between YR and SP (0.027). The large genetic distance between SJ (southwest) and LJ (northwest) may indicate strongly distinct patterns of genetic variation between the two regions. YJ was located at the lowest altitude and latitude; conversely, LJ was located at the highest altitude and latitude (Table 1). Wang et al. (2013) sampled 255 individuals from 16 populations of *P. yunnanensis*. Based on mtDNA, they detected seven population groups, and the differentiation between genetic groups in the western and eastern peripheries was consistent with the niche divergence and geographical isolation of these groups. Our results showed that the southernmost populations were separated from the other populations sampled, and both SJ and YJ had high pairwise genetic distance with the northwestern populations (i.e., LJ, TC, and GS). Population YJ was located in a unique, peripheral habitat. The biological basis for the genetic separation of YJ and SJ from the other populations is not clear, especially because the southern populations share chloroplast haplotype and mitochondrial haplotypes with the central and northern populations (Wang et al. 2013). Regrettably no other study has included the southernmost (SJ and YJ) populations, so direct comparison with the analysis of Wang et al. (2013) and Yu et al. (2000) was not possible.

The genetic separation of populations SJ and YJ could be explained by introgression of genes from. *Pinus kesiya* var. *langbianensis*, which occurs in the region and is capable of hybridization with *P. yunnanensis* (Yu and Huang 1998). More detailed studies are needed to evaluate the importance of gene flow from *P. kesiya* into *P. yunnanensis*. Surprisingly, gene flow from *Pinus densata* (at high latitudes and elevations) did not appear to increase the diversity of *P. yunnanensis.* There was some evidence for the divergence of the westernmost population (TC) from populations at the northern edge of the range (e.g., from XW, HQ, and YR), which may be affected by gene flow from *Pinus tabuliformis* through *P. densata*, but the importance of gene flow from *P. tabuliformis* into *P. yunnanensis* is far from certain. Wang et al. (2011) sampled 11 *P. yunnanensis* populations, 26 *P. densata* populations, and 17 *P. tabuliformis* populations and distinguished 29 mitotypes and 50 chlorotypes. The *P. densata* populations adjacent to *P. yunnanensis* presented the M24 mitotype of *P. yunnanensis*, but the *P. yunnanensis* populations adjacent to *P. densata* exhibited the M12 mitotype of *P. densata* at relatively low frequency. Similarly, the portion of the *P. densata* range adjacent to *P. yunnanensis* presented chlorotypes dominant in all *P. yunnanensis* populations, presumably because of long-distance gene flow from *P. yunnanensis*. It is possible that hybridization between these species is asymmetric, with genes flowing north

but not south. More sampling is needed to understand the pattern of diversity for *P. yunnanensis*.

In this study, cluster II, comprised of 18 populations, showed no clear genetic substructure, indicating minimal differentiation among western, central, and eastern populations, in contrast to the findings of Wang et al. (2013) based on mtDNA. The power of our analysis to identify substructure within cluster II was probably constrained by the relatively low number of loci we used, but contrasting results based on different marker systems are not surprising. Based on a literature review, Petit et al. (2005) concluded that studies based on maternally inherited markers presented considerably higher G_{ST} than those based on paternally or biparentally inherited markers for both gymnosperms and angiosperms. On the other hand, there was no significant difference between G_{ST} at biparentally inherited markers and at paternally inherited markers in gymnosperms. As an example, Meng et al. (2007) found population subdivision based on maternally inherited mitochondrial DNA that could not be detected using paternally inherited chloroplast DNA haplotypes.

Cluster analysis based on Nei's (1983) genetic distance, PCoA, and STRUCTURE showed two major genetic groups (Figures 2, 3, and 4). The assignment of populations to two clusters was not completely consistent with their geographic origin. Cluster I was composed of two populations from the southern periphery of the *P. yunnanensis* range. These two populations were collected from regions that had similar climate and latitude, possibly reflecting an adaptational difference. Wang et al. (2013) implied that geographical and environmental factors together created stronger and more discrete genetic differentiation than isolation by distance alone. The differentiation of populations was probably explained by the ecological or environmental factors (i.e., ecoregion) described in Chen et al. (2014). However, the ecoregions described by Chen et al. (2014) did not correspond with ecotypes described by Wang et al. (2013).

Pairwise population differentiation was low (F_{ST} ranging from 0.007 to 0.199). AMOVA results showed that 7% of the total genetic variation was among populations, similar to the estimate published by Yu et al. (2000), who analyzed 10 populations of *P. yunnanensis* using 33 allozyme loci (G_{ST} = 13.4%). Conifers often show low levels of genetic differentiation among populations (Belletti et al. 2012, Iwaizumi et al. 2013, Mandák et al. 2013). High out-crossing rates in conifers maintain high intrapopulation genetic diversity (Rubio-Moraga et al. 2012), and wind-dispersed pollen usually results in high levels of gene flow (Petit et al. 2005) and low levels of genetic structure (Nybom 2004). *P. yunnanensis* covers large, continuous areas, which facilitates gene flow. As mentioned previously, there was a relatively high genetic differentiation between the southernmost populations and all other populations (Figure 3). For example, differentiation between SJ and other populations (average F_{ST} = 0.123) was 3 times higher than the overall value (F_{ST} = 0.045).

Mantel tests showed that genetic distance among the populations sampled was not significantly correlated with geographical distance. Therefore, isolation by distance was apparently not a mechanism shaping the present distribution of genetic variability (Belletti et al. 2012). Recently, Businský et al. (2014) divided *P. yunnanensis* into two clusters: one contained populations originating from high altitudes (mostly above 2,300 m) in the northern part of the species' range and the second represented lower altitudes (mostly below 1,900 m) in the southeastern part of the range. No significant morphological differences were found between these two clusters. In this study, the most southern populations (SJ and YJ) were genetically separated from the other populations, their differentiation not only was associated with altitude but also was correlated with climatic factors (i.e., T_{mean}, T_{max}, and T_{min}) (Table 5). It is clear that *P. yunnanensis* shows a trend of greater diversity in lower, warmer, and more southern populations. In *Pinus tecunumanii*, a pine species native to Central America and Mexico, high- and low-altitude populations are genetically distinct (Dvorak et al. 2009). But, as described earlier, the phenotypic and genetic characterization of the southernmost *P. yunnanensis* populations is poor compared with that of populations in the rest of the species' range, so it remains to be seen whether populations SJ and YJ represent an ecotype or distinct subgroup.

The power of our study to detect genetic differences was probably reduced by the relatively small number of loci we used and number of samples per population. Ruzzante (1998) showed that the number of loci needed for accurate estimation of population genetic parameters interacted strongly with sample size per population. Low numbers of loci and small sample sizes increase sample variance considerably.

Conservation

Our recommendations for populations that should be given priority for conservation are based primarily on the maintenance of genetic diversity, including rare and private alleles. Based on SSR data, a great majority of the total genetic variation in Yunnan pine was within populations (93%); results based on mtDNA and cpDNA (Wang et al. 2011) and allozyme data (Yu et al. 2000) were similar. This finding indicates that large numbers of samples from relatively few populations would be expected to contain most of the genetic diversity. Results from studies of DNA-based markers and of plant phenotypes have all indicated that southern populations of Yunnan pine were most diverse and should be a focus of in situ conservation efforts. Populations inhabiting distinct geophysical and climatological environments also deserve special consideration for conservation. Because of the importance of ecological factors in forming or maintaining genetic divergence (Wang et al. 2013), we recommend further research to determine whether populations such as YJ or YR contain adaptive traits that allow them to thrive in peripheral environments. Maintenance of important populations in situ will require fire suppression and better management of logging.

Additional provenance/progeny tests will be necessary to determine whether patterns of regional adaptation are important for improving the success of artificial regeneration. *Ex situ* conservation should focus on individuals selected from superior populations and maintenance of alleles with frequencies of 5% or greater. It is not clear whether the seed zones suggested by Chen and Wu (1987) were ever implemented, and they require additional testing. The deficiencies in the current Yunnan pine seed orchards (poor production and poor seedling form) limit their usefulness. These probably can be addressed by improved management and possibly roguing.

Endnote

1. For more information, see www.worldclim.org/.

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