Biochemical Genetics, Vol. 38, Nos. 5/6, 2000

Allozyme Diversity and Population Genetic Structure of *Pinus densata* Master in Northwestern Yunnan, China

Hong Yu,[1,2] Song Ge,[1,3] and De-yuan Hong[1]

Received 27 Oct. 1999—Final 7 Feb. 2000

We investigated the levels and patterns of genetic diversity of Pinus densata *Master in Yunnan. Horizontal starch-gel electrophoresis was performed on macrogametophytes collected from nine populations in northwestern Yunnan, China. Compared with other gymnosperm species,* P. densata *has higher mean values for all measures of genetic diversity. Allozyme polymorphism (0.99 criterion) was 97.0% and 71.4% at the species and population levels, respectively. The average number of alleles per locus was 3.1 and 2.0 at the species and population levels. Mean expected heterozygosity was substantially higher in* P. densata *than average values investigated for other gymnosperms both at the population (*$H_{ep} = 0.174 \pm 0.031$*) and at the species (*$H_{es} = 0.190$*) levels. Of the total genetic variation, less than 12% was partitioned among populations (*$G_{ST} = 0.112$*). Our allozyme survey supports the suggestion that the observed higher diversity in* P. densata *may be attributed partly to its hybrid origin between two genetically distinct species,* P. yunnanensis *and* P. tabulaeformis. *In addition, we suggest that introgression would give rise to the increase in genetic diversity occurring in* P. densata.

KEY WORDS: *Pinus densata* Master; population; allozymes; diversity; differentiation.

INTRODUCTION

Pinus densata Master is distributed in the high mountains of southwestern China, including western Sichuan, southern Qinghai, eastern Xizang, and northwestern

[1] Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, P.R. China.
[2] College of Life Science and Chemistry, Yunnan University, Kunming 650091, P.R. China.
[3] To whom correspondence should be addressed. e-mail: song_ge@hotmail.com. Fax: 86-01-62590843.

0006-2928/00/0600-0138$18.00/0

Yunnan (Wu, 1956; Cheng and Fu, 1978). In Yunnan, the species is distributed mainly in the northwestern part, at altitudes from 2700 to 3700 m (Cheng and Fu, 1978; Yu, 1996). Because of the fact that it is absent at lower elevations and grows better than *P. yunnanensis* at higher elevations, Guan (1981) suggested that the two species adapted to specific habitats with different climate. Although the basic biology of this species, including its distribution, morphology, ecology, and cytology, has been investigated (Wu, 1956; Mirov, 1967; Farjon, 1984; Guan, 1981, Yu, 1996), almost nothing is known concerning its population genetics. Despite the fact that Wang *et al*. (1990a) surveyed the genetic variation of *P. densata* by allozymes in investigating its origin, only one population was analyzed in their work. Therefore, the genetic diversity and structure within and among populations of *P. densata* are still unknown.

P. densata is one of the important timber trees in southwestern China, and therefore it is important to investigate its genetic diversity and population genetic structure. The present work aims at determining the amount and pattern of genetic diversity within and among populations. We anticipated that the allozyme survey would provide insights into the population genetics of this species and help develop strategies for the genetic improvement of *P. densata*.

MATERIALS AND METHODS

We sampled 175 individuals from nine populations of *P. densata* in the high mountains of northwestern Yunnan (Fig. 1, Table I). Seeds were individually collected from about 20 trees in each population. Seeds were germinated for approximately 6–9 days before each megagametophyte was separated cleanly from its seed coat and embryo. For estimating parental genotypes, a minimum of eight megagametophytes per parent tree was assayed. This will correctly identify a heterozygous locus with a probability of $1 - (0.5)^7 = 0.992$. Crude extract was obtained by crushing two megagametophytes in 1 drop of 0.1 *M* Tris–HCl, pH 7.5, buffer containing 0.1% (v/v) 2-mercaptoethanol (Yu *et al*., 1999). The homogenates were absorbed on paper wicks, and electrophoresis generally followed the methods of Soltis *et al*. (1983). Two buffer systems were used for separating enzymes in 12% horizontal starch gels (Yu *et al*., 1999). System A was used to resolve shikimate dehydrogenase (SKD; EC 1.1.1.25), alcohol dehydrogenase (ADH; EC 1.1.1.1), glutamate dehydrogenase (GDH; EC 1.4.1.2), phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), phosphoglucomutase (PGM; EC 2.7.5.1), phosphoglucoisomerase (PGI; EC 5.3.1.9), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), isocitrate dehydrogenase (IDH; EC 1.1.1.42), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), diaphorase (DIA; EC 1.6.4.3), and triosephosphate isomerase (TPI; EC 5.3.1.1). Leucine aminopeptidase (LAP; EC 3.4.11.1) and aspartase aminotransferase (AAT; EC 2.6.1.1) were resolved using a modification of buffer

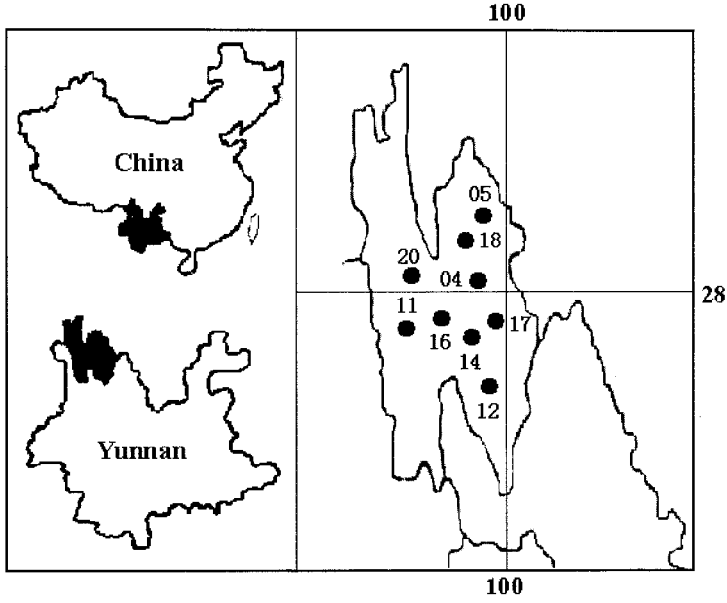

Fig. 1. Distribution of *Pinus densata* populations sampled in the present study. The relative locations of nine populations are shown at the right. Numbers correspond to populations in Table I.

system B. Staining procedures for all enzymes followed Soltis *et al*. (1983) and Wendel and Weeden (1989).

When more than one locus was observed for an enzyme, loci were numbered sequentially, with the most anodally migrating enzyme designated 1. Allelic variation at a locus was denoted alphabetically, with the most anodal as *a*, the next *b*, and so on. Interpretation of the genetic basis of enzyme banding patterns relied on knowledge of previously determined numbers of isozymes

Table I. Localities and Habitats of Nine Populations of *Pinus densata*

Pop. No.	Pop. name	Locality	Altitude (m)	Latitude	Longitude
YU04	Xiaoxieshan	Zhongdian	3600	28°05′N	99°49′E
YU05	Wumukuang	Zhongdian	3400	28°33′N	99°50′E
YU11	Cuobunaka	Zhongdian	3700	27°52′N	99°26′E
YU12	Songlinping	Zhongdian	3000	27°27′N	99°56′E
YU14	Qinkou	Zhongdian	3300	27°40′N	99°45′E
YU16	Napahai	Zhongdian	3400	27°53′N	99°38′E
YU17	Bitahai	Zhongdian	3500	27°48′N	99°57′E
YU18	Ongshui	Zhongdian	2900	28°27′N	99°48′E
YU20	Nixilinyezhan	Zhongdian	3200	28°07′N	99°27′E

expected and of one allele showing one band in the haploid megagametophyte of gymnosperms (Boyle and Morgenstern, 1987; Ge *et al*., 1997, 1998; Yu *et al*., 1999).

Statistics of allozyme diversity were calculated using the BIOSYS-1 program (Swofford and Selander, 1989). These measures included the percentage of polymorphism (P), mean number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e). Deviation from Hardy–Weinberg equilibrium (fixation index, F) (Wright, 1978) was calculated for each population. In addition, population differentiation was investigated using Nei's (1973) measures of genetic diversity. The total genetic diversity (H_T), genetic diversity within populations (H_S), genetic diversity among populations (D_{ST}), and proportion of genetic variation found among populations (G_{ST}) were calculated following the equations of Nei (1973). Population genetic structure was obtained by the G_{ST} value across all loci. A cluster analysis using UPGMA and Nei's (1978) unbiased genetic distance was also performed.

RESULTS

Pinus densata maintains high levels of genetic diversity at both the population and the species levels. Of 33 isozymes surveyed 32 (97.0%) were polymorphic in at least one population and only *Tpi-4* was monomorphic in all populations. Two polymorphic loci, *Aat-1* and *Dia-3*, had five alleles; 8 loci, *Aat-3*, *Skd-3*, *Adh-2*, *Pgd-1*, *Pgd-2*, *Mdh-3*, *Idh-2*, and *Dia-2*, had four alleles; 20 loci, *Lap-2*, *Skd-1*, *Skd-2*, *Adh-1*, *Lap-2*, *Skd-1*, *Gdh*, *Pgm-1*, *Pgm-3*, *Pgi-1*, *Pgi-3*, *Mdh-1*, *Mdh-2*, *Me*, *G6pd*, *Dia-1*, *Tpi-1*, *Tpi-2*, *Tpi-3*, and *Tpi-4*, had three alleles; and the remaining 2 loci, *Adh-3* and *Dia-4*, had two alleles. Across all loci, 130 alleles were scored. The number of alleles per locus was 3.09, and 9 alleles (6.9%) were unique to a single population. At the species level, observed and expected heterozygosities were 0.171 and 0.190, respectively (Table II).

Within populations, the percentage of polymorphic loci ranged from 60.6 (YU17) to 81.8 (YU12), with a mean of 71.4 (Table III). The mean number of alleles per locus within populations ranged from 1.8 to 2.2. The mean observed heterozygosity ($H_o = 0.171$) within populations was slightly less than the mean expected heterozygosity ($H_e = 0.174$). In the Wumukuang, Cuobunaka, Songlinping, and Nixi populations (YU05, YU11, YU12, and YU20), the observed heterozygosity was higher than expected, with the fixation indices being negative (Table II).

To test the conformity of genotype frequencies to Hardy–Weinberg expectations, the fixation index, F, was calculated for each population. Negative fixation indices were found in four of nine populations, particularly in populations YU05 and YU12, with fixation indices of -0.157 and -0.112, respectively. However, five populations (YU04, YU14, YU16, YU17, and YU18) were

Table II. Genetic Variability at 33 Loci in Nine Populations of *Pinus densata* Masters (Standard Errors in Parentheses)

Population	*N*	*A*	*P*[a]	H_o	H_e[b]	*F*
YU04	19.9 (0.0)	2.2 (0.2)	78.8	0.219 (0.034)	0.228 (0.033)	0.039
YU05	19.9 (0.1)	1.9 (0.1)	63.6	0.214 (0.039)	0.185 (0.032)	−0.157
YU11	20.0 (0.0)	2.2 (0.2)	78.8	0.180 (0.032)	0.176 (0.028)	−0.023
YU12	16.0 (0.0)	2.2 (0.1)	81.8	0.229 (0.038)	0.206 (0.030)	−0.112
YU14	18.4 (0.3)	1.8 (0.1)	66.7	0.123 (0.022)	0.133 (0.024)	0.075
YU16	19.9 (0.1)	2.1 (0.1)	75.8	0.136 (0.024)	0.173 (0.030)	0.213
YU17	21.7 (0.2)	1.8 (0.1)	60.6	0.129 (0.032)	0.139 (0.033)	0.072
YU18	18.5 (0.2)	2.0 (0.1)	66.7	0.123 (0.030)	0.152 (0.030)	0.191
YU20	20.0 (0.0)	2.0 (0.1)	69.7	0.189 (0.036)	0.174 (0.031)	−0.086
Mean (SE)		2.0 (0.2)	71.4 (7.6)	0.171 (0.044)	0.174 (0.031)	0.024
Species		3.1	97.0	0.171	0.190	

[a] A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.99.
[b] Unbiased estimate (see Nei, 1978).

deficient in heterozygotes, particularly in deficient populations YU16 and YU18, with a fixation index higher than 0.190. The mean *F* value of 0.024 (Table II) indicates that *P. densata* populations approach random mating.

A relatively high proportion of genetic variation was found among populations ($G_{ST} = 0.112$), suggesting that 89% of the genetic variation exists within populations of *P. densata* (Table IV). Nei's (1978) genetic identity ranged from 0.002 to 0.067, with the mean value being 0.023 for all pairwise comparisons of populations. The cluster phenogram constructed using genetic distance between populations revealed that population YU04 and other populations were the most genetically differentiated (Fig. 2). As a result, there is no trend that genetic distance increases with geographic separation. The regression tests further demonstrated that no correlation was found between genetic distance and linear geographic distance ($r = 0.342$, $P < 0.30$).

Table III. Matrix of Genetic Similarity and Distance Coefficients: Below Diagonal, Nei (1978) Unbiased Genetic Distance; Above Diagonal, Nei (1978) Unbiased Genetic Identity

Population	YU04	YU05	YU11	YU12	YU14	YU16	YU17	YU18	YU20
YU04	—	0.950	0.945	0.935	0.939	0.949	0.939	0.943	0.946
YU05	0.052	—	0.978	0.974	0.978	0.975	0.973	0.975	0.976
YU11	0.057	0.023	—	0.997	0.996	0.997	0.983	0.994	0.994
YU12	0.067	0.026	0.003	—	0.995	0.993	0.980	0.991	0.990
YU14	0.063	0.022	0.004	0.005	—	0.993	0.983	0.990	0.988
YU16	0.053	0.025	0.003	0.007	0.007	—	0.986	0.998	0.990
YU17	0.063	0.027	0.017	0.020	0.017	0.014	—	0.987	0.984
YU18	0.058	0.025	0.006	0.009	0.010	0.002	0.013	—	0.989
YU20	0.056	0.025	0.006	0.010	0.012	0.010	0.017	0.011	—

Table IV. Nei's (1978) Genetic Diversity Statistics for Nine Populations of *Pinus densata* at All Loci (Standard Errors in Parentheses)[a]

Locus	H_T	H_S	D_{ST}	G_{ST}
Lap-1	0.0303	0.0283	0.0020	0.0660
Lap-2	0.4832	0.4747	0.0085	0.0176
Aat-1	0.3602	0.3373	0.0229	0.0636
Aat-3	0.3864	0.3586	0.0278	0.0719
Skd-1	0.0396	0.0380	0.0002	0.0415
Skd-2	0.0488	0.0464	0.0024	0.0492
Skd-3	0.2758	0.2628	0.0130	0.0471
Adh-1	0.0971	0.0885	0.0086	0.0886
Adh-2	0.0991	0.0961	0.0030	0.0303
Adh-3	0.0058	0.0056	0.0002	0.0345
Gdh	0.0616	0.0571	0.0045	0.0731
Pgd-1	0.3502	0.3141	0.0361	0.1031
Pgd-2	0.3626	0.3426	0.0200	0.0552
Pgm-1	0.4713	0.3709	0.1004	0.2130
Pgm-3	0.2141	0.0777	0.1364	0.6371
Pgi-1	0.3745	0.3027	0.0718	0.1917
Pgi-3	0.2684	0.2608	0.0076	0.0283
Mdh-1	0.0895	0.0846	0.0049	0.0547
Mdh-2	0.1135	0.1078	0.0057	0.0502
Mdh-3	0.1402	0.1330	0.0072	0.0514
Mdh-4	0.1025	0.0976	0.0049	0.0478
Me	0.3108	0.2886	0.0222	0.0714
Idh-1	0.1388	0.1272	0.0116	0.0836
Idh-2	0.1581	0.1493	0.0088	0.0557
G6pd	0.2156	0.1770	0.0386	0.1790
Dia-1	0.4032	0.3270	0.0762	0.1890
Dia-2	0.2827	0.2562	0.0265	0.0937
Dia-3	0.2067	0.1929	0.0138	0.0668
Dia-4	0.0056	0.0054	0.0002	0.0357
Tpi-1	0.0667	0.0621	0.0046	0.0690
Tpi-2	0.0792	0.0746	0.0046	0.0581
Tpi-3	0.0332	0.0268	0.0064	0.1928
Mean	0.1902 (0.0257)	0.1689 (0.0231)	0.0213 (0.0056)	0.1118 (0.0198)

[a]Means for H_T, H_S, and D_{ST} are averages of all 33 loci; mean for G_{ST} is computed from the mean values for H_T, H_S, and D_{ST}.

DISCUSSION

Compared with other gymnosperm species (Hamrick *et al*., 1992), *P. densata* has higher mean values for all measures of genetic diversity both at the species and at the population levels, with more variation residing among populations (Table V). In their studies on the origin of *P. densata*, Wang *et al*. (1990) detected a similar level of allozyme variation for one population of *P. densata* ($P = 84.6$ by the 0.99 criterion, $A = 2.5$, $H_o = 0.179$, $H_e = 0.210$).

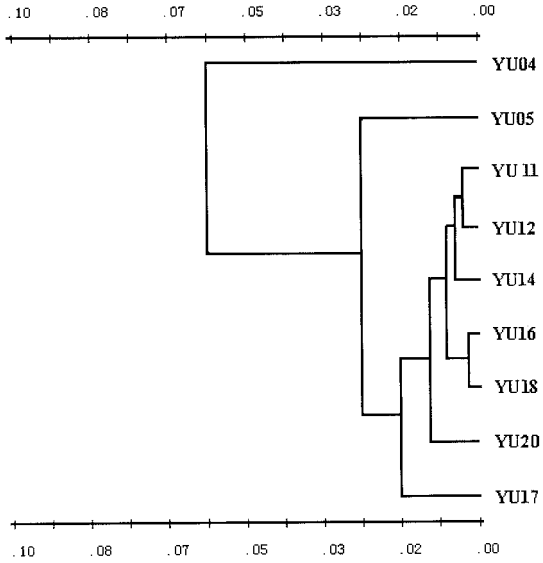

Fig. 2. Phenogram of UPGMA cluster analysis based on Nei's (1978) genetic distance between the nine populations of *Pinus densata* Master.

The high level of variability in *P. densata* most likely results from its hybrid origin (Wu, 1956; Wang *et al*., 1990; Wang and Szmidt, 1990). Based on morphological characters and geographic distribution, previous authors (Wu, 1956; Mirov, 1967; Farjon, 1984) suggested that *P. densata* was a natural hybrid species between *P. yunnanensis* and *P. tabulaeformis* during the Tertiary Period. Later, Wang *et al*. (1990); Wang and Szmidt, (1990) demonstrated the hybrid origin of *P. densata* with allozyme and chloroplast DNA evidence. It is well established that hybrid species maintain higher levels of genetic diversity than

Table 5. Mean Values for P_S, P_P, A_S, A_P, H_{ES}, H_{EP}, and G_{ST} for *Pinus densata* Compared to Mean Values for Other Gymnosperm Species[a]

Species	P_S	P_P	A_S	A_P	H_{ES}	H_{EP}	G_{ST}
P. densata	97.0	71.4	3.1	2.0	0.190	0.174	0.112
Gymnosperms (89–121)[b]	71.1	53.4	2.4	1.8	0.169	0.151	0.073
All plant species (662)[b]	51.3	34.6	2.0	1.5	0.150	0.113	0.228

[a] P_S and P_P are the percentage polymorphic loci within species and population, respectively; A_S and A_P, the mean number of alleles per locus within species and populations, respectively; H_{ES} and H_{EP}, the expected heterozygosity within species and populations, respectively; G_{ST}, the coefficient of gene differentiation.

[b] Data from Hamrick *et al*. (1992). Numbers in parentheses are the number of taxa reviewed.

their progenitors (Wheeler and Guries, 1987; Wang *et al*., 1990). In our case, *P. densata* ($H_{es} = 0.190$, $H_{ep} = 0.174$) shows a higher level of heterozygosity than does *P. yunnanensis* [$H_{es} = 0.164$, $H_{ep} = 0.145$ (Yu *et al*., 2000)]. This allozyme data conforms with Wang *et al*.'s (1990) suggestion that the observed higher diversity in *P. densata* compared to *P. yunnanensis* and *P. tabulaeformis* may be the result of hybridization between two genetically distinct parental populations.

Currently, the low-elevation populations of *P. densata* occur sympatrically with the high-elevation populations of *P. yunnanensis* in the Yunnan Plateau, where the two species could exchange genes to some extent (Wang *et al*., 1990; Yu, 1996). It is most likely that introgression increased the genetic diversity of *P. densata* in the overlapping area with *P. yunnanensis*. For instance, the Songlinping population (YU12), which overlaps with populations of *P. yunnanensis* at altitudes from 2800 to 3000 m, showed the highest values for all measures of genetic diversity ($A = 2.2$, $P = 81.8$, $H_o = 0.229$, $H_e = 0.206$) (Table II). Consequently, introgression is the second factor that led to the high variation occurring in *P. densata*.

ACKNOWLEDGMENTS

We thank Huang Rui-Fu and Zhang Can-Ming for assistance in wild investigation and the seed collection, and Qian Wei and Wang Ke-Qing for laboratory assistance. This research was supported by National Natural Science Foundation of China (Grant 39500024).

REFERENCES

Boyle, T. J. B. and Morgenstern, E. K. (1987). Some aspects of the population structure of black spruce in central New Brunswick. *Sil. Genet.* **36:**53.

Cheng, W. C., and Fu, L. K. (1978). *Flora Reipublicae Popularis Sinicae*, Science Press, Beijing, Vol. 7, pp. 255–259.

Farjon, A. (1984). *Pines: Drawings and Descriptions of the Genus*, E. Brill, Leiden, The Netherlands.

Ge, S., Wang, H. Q., Zhang, C. M., and Hong, D. Y. (1997). Genetic diversity and population differentiation of *Cathaya argyrophylla* in Bamian Mountain. *Acta Bot. Sinica* **39:**266.

Ge, S., Hong, D. Y., Wang, H. Q., Liu, Z. Y., and Zhang, C. M. (1998). Population genetic structure and conservation of an endangered conifer, *Cathaya argyrophylla* (Pinaceae). *Int. J. Plant Sci.* **159:**351.

Guan, C. T. (1981). Fundamental features of the distribution of Coniferae in Sichuan. *Acta Phytotaxon Sinica* **11:**393.

Hamrick, J. L., Godt, M. J. W., and Sherman-Broyles, S. L. (1992). Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6:**95.

Mirov, N. T. (1967). *The Genus Pinus*, Ronald Press, New York.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA* **70:**3321.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89:**583.

Soltis, D. E., Haufler, C. H., Darrow, D. C., and Gastony, G. J. (1983). Starch gel electrophoresis of ferns: A compilation of grinding buffers, gel and electrode buffers, and staining schedules. *Am. Fern J.* **73:**9.

Swofford, D. L., and Selander, R. B. (1989). *BIOSYS-1: A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics*, Release 1.7, Illinois Natural History Survey, Champaign.

Wang, X. R., Szmidt, A. E., Lewandowski, A., and Wang, Z. R. (1990). Evolutionary analysis of *Pinus densata* Masters, a putative Tertiary hybrid. 1. Allozyme variation. *Theor. Appl. Genet.* **80:**635.

Wang, X. R., and Szmidt, A. E. (1990). Evolutionary analysis of *Pinus densata* Masters, a putative Tertiary hybrid. 2. A study using species-specific chloroplast DNA markers. *Theor. Appl. Genet.* **80:**641.

Wendel, J. F., and Weeden, N. F. (1989). Visualization and interpretation of plant isozymes. In Soltis, D. E., and Soltis, P. S. (eds.), *Isozymes in Plant Biology*, Dioscorides Press, Portland, OR, pp. 5–45.

Wheeler, N. C., and Guries, R. P. (1987). A quantitative measure of the introgression between lodgepole and jack pines. *Can. J. Bot.* **65:**1876.

Wright, S. (1978). *Evolution and the Genetics of Populations, Vol 4. Variability Within and Among Natural Populations*, University of Chicago Press, Chicago.

Wu, C. L. (1956). The taxonomic revision and phytogeographical study of Chinese pines. *Acta Phytotaxon Sinica* **5:**131.

Yu, H. (1996). A Study on Genetic Diversity and Evolution of *Pinus yunnanensis* Franch, Ph.D. dissertation, Yunnan University, Kunming.

Yu, H., Qian, W., and Huang, R. F. (1999). Allozyme method in study on population genetics of *Pinus yunnanensis* Franch. *Acta Bot. Yunnanica* **21:**68.

Yu, H., Ge, S., Huang, R. F., and Jiang, H. Q. (2000). A preliminary study on genetic variation and close relationship of *Pinus yunnanensis* and its closely relative species. *Acta Bot. Sinica* **42**:107.