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Genetic Diversity and Conservation Implications of Four *Cupressus* Species in China as Revealed by Microsatellite Markers

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Abstract Understanding the extent and distribution of genetic diversity is crucial for the conservation and management of endangered species. *Cupressus chengiana*, *C. duclouxiana*, *C. gigantea,* and *C. funebris* are four ecologically and economically important species in China. We investigated their genetic diversity, population structure, and extant effective population size (35 populations, 484 individuals) employing six pairs of nuclear microsatellite markers (selected from 53). Their genetic diversity is moderate among conifers, and genetic differentiation among populations is much lower in *C. gigantea* than in the other three species; the estimated effective population size was largest for *C. chengiana*, at 1.70, 2.91, and 3.91 times the estimates for *C. duclouxiana*, *C. funebris,* and *C. gigantea*, respectively. According to Bayesian clustering analysis, the most plausible population subdivision scheme within species is two groups in *C. chengiana*, three groups in *C. duclouxiana*, and a single group for both *C. funebris* and *C. gigantea*. We propose a conservation strategy for these cypress species.

Keywords Simple sequence repeat (SSR) · Genetic structure · Genetic drift · Effective population size · Conservation unit

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Introduction

Genetic diversity plays an important role in the adaptation and survival of tree species under environmental changes. Evaluation of the level and distribution of genetic diversity is essential for their management and the development of effective conservation strategies, especially for endangered species (Hedrick 2004). Population size and gene flow among populations are two important factors influencing genetic diversity (Hamilton 2009; Freeland et al. 2011). Generally, populations of small size have low genetic diversity. Therefore, their capacity to adapt to environmental change may be compromised and their ability to survive long-term environmental changes may be diminished (Ellstrand and Elam 1993; Lande 1999; Hamilton 2009). Small populations are also prone to genetic drift and inbreeding (Ellstrand and Elam 1993; Karron 1997; Lande 1999; Hamilton 2009). Genetic drift is expected to randomly reduce genetic variation within small populations, and inbreeding usually reduces population fitness, since it leads to increased expression of recessive deleterious alleles as homozygosity increases (Lande 1999; Freeland et al. 2011). Gene flow among populations leads to a combination of the respective gene pools, which counteracts the effects of genetic drift and inbreeding. However, gene flow reduces genetic difference among populations (Hamilton 2009; Freeland et al. 2011).

Cypresses (*Cupressus* L.) are trees or shrubs that occur in fragmented habitats in temperate regions of the Northern Hemisphere. Owing to their ornamental value, plants in this group are important in horticulture and are widely cultivated in numerous countries worldwide (Farjon 2005). Recent phylogenetic research has suggested that *Cupressus* (sensu lato) species in the Old World and New World form two distinct lineages and should be treated as two genera, namely *Cupressus* sensu stricto and *Hesperocyparis* (equivalent to *Callitropsis* sensu lato excluding *Xanthocyparis vietnamensis* and *Callitropsis nootkatensis*), respectively (Little et al. 2004; Little 2006; Adams et al. 2009; Mao et al. 2010, 2012). As a result, the recircumscribed *Cupressus* (sensu stricto) include only the 12 Old World species, three of which are found in North Africa and Mediterranean regions and the other nine in Asia (Little 2006). Among the latter, *C. austrotibetica, C. cashmeriana, C. gigantea,* and *C. torulosa* grow in the high-altitude regions of the Qinghai-Tibetan Plateau and west Himalayas, and *C. chengiana*, *C. duclouxiana*, *C. funebris*, *C. jiangeensis,* and *C. tonkinensis* occur in the low-altitude regions of the eastern plateau, central China, and Vietnam (Farjon 2005; Little 2006). Most of these Asian species occur allopatrically in the Qinghai-Tibetan Plateau and adjacent regions and have fragmented distributions (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005; Little 2006).

Among the species endemic to China, *C. chengiana*, *C. duclouxiana*, *C. funebris,* and *C. gigantea* are ecologically significant (Farjon 2005). Usually, *C. chengiana* and *C. duclouxiana* occur on south-facing mountain slopes at moderate altitude (ca. 1,200–2,900 and 1,400–3,000 m, respectively). In contrast, *C. gigantea* grows along the dry, hot valley of the Tsangpo River at high altitudes of ca. 3,000–3,400 m (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005). These three species are all dominant or codominant in their distribution ranges (Farjon 2005). However, as a

consequence of human activities (e.g., overlogging) and global warming, wild populations of these species have declined due to habitat loss and have been listed as either vulnerable or endangered in the IUCN Red List of Threatened Species (IUCN 2012). The Chinese weeping cypress, *C. funebris*, which occurs below 2,000 m in vast areas of southwestern and central China, is widely cultivated in southern China owing to its suitability to a wide range of soil types. Notably, this cypress grows vigorously on limestone rocks (Fu et al. 1999; Farjon 2005). This species is a significant component of artificial forests in the southern part of China, especially in Sichuan, Hubei, and Guizhou provinces (Zheng and Fu 1978). Nevertheless, the long cultivation history of this species blurs the boundary between wild and cultivated populations in low-altitude areas (Zheng and Fu 1978; Farjon 2005). The products of all four species are economically important to local residents, especially the wood, which has versatile uses including construction of buildings, ships, and furniture (Zheng and Fu 1978).

Despite their clear importance, the genetic diversity and population structure of these species have not been studied fully. A previous survey based on paternally inherited plastid markers suggested that limited gene flow among geographically isolated populations and population bottlenecks related to the Quaternary climate oscillations and human activities may have fixed local species-specific haplotypes and led to low haplotype diversity within each population (Xu et al. 2010). However, plastid markers, which are paternally inherited via pollen in Cupressaceae (Neale et al. 1989, 1991; Mogensen 1996; Kondo et al. 1998; Hwang et al. 2003; Sakaguchi et al. 2012), may have experienced a very different evolutionary history compared with nuclear markers. On the one hand, the effective population size of paternally inherited plastid markers is only a quarter that of the biparentally inherited nuclear markers when the sex ratio is equal to one (Freeland et al. 2011). On the other hand, pollen and seeds of *Cupressus* are dispersed by wind and gravity/water (Farjon 2005). Thus, gene flow of the plastid genome (mediated by pollen) may be more effective than for the nuclear genome (mediated half by pollen and half by seeds). Taken together, these factors may have resulted in a much faster rate of homogenization for the paternally inherited plastid genome than the bipaternally inherited nuclear genome in fragmented populations (Karron 1997; Fahrig 2003; Hamilton 2009; Freeland et al. 2011).

Therefore, in the present study, we employed the codominant biparentally inherited nuclear microsatellite markers (i.e., simple sequence repeat, SSR) to examine the genetic diversity of the four Chinese cypress species, *C. chengiana*, *C. duclouxiana*, *C. funebris,* and *C. gigantea*. We aimed to address the following questions:

(a) Is the genetic diversity level of each species related to the effective population size?
(b) How is the genetic diversity partitioned within and among populations within each species?
(c) Given the level and distribution pattern of genetic diversity in these species, what conservation strategies should be adopted?

Materials and Methods

Species and Samples

Four Asian species, *C. chengiana*, *C. duclouxiana*, *C. funebris,* and *C. gigantea*, were investigated in this study. According to the IUCN Red List of Threatened Species (IUCN 2012), *C. gigantea* and *C. chengiana* are range restricted and vulnerable, whereas *C. duclouxiana* is threatened by habitat loss and endangered, and the widely distributed and cultivated *C. funebris* is currently not threatened. Leaf samples were collected from 149 trees in 9 populations of *C. chengiana* (Pop 1–9), 137 trees in 10 populations (10–19) of *C. duclouxiana*, 102 trees in 10 populations (20–29) of *C. funebris*, and 95 trees in 6 populations (30–35) of *C. gigantea*. In total, samples were collected from 483 trees in 35 populations (Table 1; Fig. 1), which covered all or most of the natural or cultivated distribution of the species examined. Note that many populations of *C. funebris* may have been mixtures of cultivated and wild individuals since they were all collected from locations that were not far from residences (Pop 20–27; Table 1; Fig. 1), except for two populations (28 and 29); these two groups of populations are therefore referred to as putative mixed populations and putative wild populations, respectively. One population of *C. duclouxiana* (10: Kunming, Yunnan) was composed entirely of cultivated trees, whereas the other five (12–16) may have included a few cultivated individuals. Most populations of *C. chengiana* and *C. gigantea* were natural, although a few individuals in the Lixian population (Pop 3) seemed to have been cultivated. In every population, leaf samples were taken from trees at least 50 m apart. The latitude, longitude, and altitude of the localities for most populations sampled (Table 1) were recorded using an Etrex GIS monitor (Garmin, Taiwan).

DNA Extraction and PCR Amplification

Genomic DNA was isolated from approximately 50–100 mg of silica-gel dried, leaf-needle material using a modified CTAB method (Doyle and Doyle 1987). Two to four individuals from 3 to 5 populations of each species were used in an initial screen of polymorphic microsatellite markers. In total, 53 pairs of microsatellite markers originally developed for *C. sempervirens* (Sebastiani et al. 2005), *C. chengiana* (Xu et al. 2008), and *C. funebris* (Li et al. 2013) were employed in the initial screen. Six primer pairs (Table 2) that revealed polymorphisms in all four species were adopted to survey the genetic variation of all 483 trees.

To facilitate the detection of microsatellite polymorphism using a DNA Analyzer, one primer of each polymorphic primer pair was labeled with 6-FAM fluorescent dye (Takara, Dalian, China). PCR amplifications were performed in a 20 μl PCR mixture containing about 10–40 ng diluted genomic DNA, 0.5 mM of each dNTP, 0.3 μl of each primer, 2.5 μl *Taq* buffer, and 0.75 U r*Taq* polymerase (Takara). Amplifications were carried out in an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA) using the following program: initial denaturation for 5 min at 94°C, followed by 36 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 45–60°C (Table 2), and 80 s at 72°C, and a final extension at 72°C for

Table 1 Provenance of population samples of four cypress species in China

Pop. code	*Cupressus* species	Location	Latitude (N)	Longitude (E)	Altitude (m)	Individuals (*n*)
1	*C. chengiana*	Danba, SC	30°07.84′	102°10.43′	1,680	12
2		Xiaojin, SC	30°32.00′	101°35.00′	3,780	15
3		Lixian, SC	31°24.54′	103°06.92′	1,954	16
4		Jinchuan, SC	31°47.46′	101°56.48′	2,400–2,470	21
5		Maerkang, SC	31°55.72′	102°02.02′	2,417	10
6		Wenxian, GS	32°44.47′	104°54.45′	888	16
7		Wenxian, GS	33°12.03′	105°02.13′	1,025	24
8		Wudu, GS	33°14.90′	104°59.15′	1,400	24
9		Zhouqu, GS	33°52.27′	104°08.59′	1,531	11
10	*C. duclouxiana*	Kunming, YN	25°15.17′	102°44.46′	1,957	5
11		Lufeng, YN	25°05.82′	101°48.26′	1,801	4
12		Eryuan, YN	26°14.68′	099°56.49′	2,100	11
13		Yongsheng, YN	26°44.39′	100°45.96′	2,170	24
14		Yulong, YN	26°56.19′	099°57.07′	1,830	25
15		Lijiang, YN	27°07.80′	100°14.40′	2,900	22
16		Xianggelila, YN	27°20.17′	099°57.82′	2,510	6
17		Bennzilan, YN	28°08.43′	099°26.92′	2,559	10
18		Daocheng, SC	28°23.05′	100°14.35′	2,752	11
19		Deqin, YN	28°22.27′	099°03.41′	2,870	19
20	*C. funebris*	Kangxian, GS	33°20.00′	105°32.00′	2,400	9
21		Jiange, SC	32°14.13′	105°33.17′	617	10
22		Guangyuan, SC	32°37.00′	105°52.36′	652	10
23		Zitong, SC	31°39.59′	105°14.21′	493	12
24		Mianyang, SC	31°32.57′	104°48.99′	523	12
25		Wenchuan, SC	31°03.71′	103°29.18′	1,100	16
26		Jinyan, SC	29°40.02′	104°03.50′	419	10
27		Chongqing, CQ	29°33.06′	106°27.00′	300	7
28		Shennongjia, HB	31°21.06′	110°18.06′	1,700	9
29		Ruyuan, GD	24°59.34′	113°09.03′	411	7
30	*C. gigantea*	Jiacha, XZ	29°02.29′	093°03.23′	3,130–3,430	11
31		Langxian, XZ	28°59.95′	093°14.11′	3,060	7
32		Langxian, XZ	29°08.63′	093°27.64′	3,020	14
33		Milin, XZ	29°07.59′	093°50.93′	3,050	23
34		Linzhi, XZ	29°40.00′	094°20.00′	3,040	7
35		Milin, XZ	29°20.40′	094°22.63′	2,950	13

7 min. The PCR products were run on an ABI 3100 DNA Analyzer (Applied Biosystems); microsatellite allele sizes were scored against an internal ROX-500 molecular size standard, and genotypes were identified using Genemapper 4.0 (Applied Biosystems).

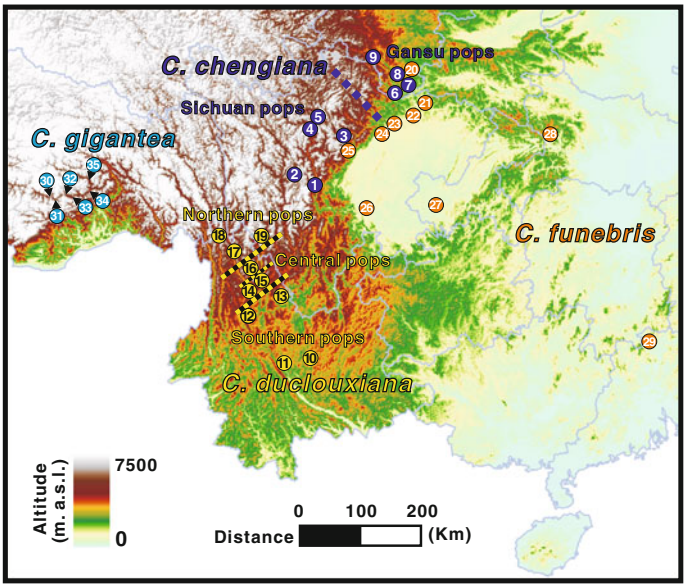

Fig. 1 Geographic distribution of 9 populations of *Cupressus chengiana* (Pops 1–9), 10 populations of *C. duclouxiana* (Pops 10–19), 10 populations of *C. funebris* (Pops 20–29), and 6 populations of *C. gigantea* (Pops 30–35) that were adopted in this study. Population numbers and species as in Table 1. *Dark blue dotted line* divides *C. chengiana* into Gansu and Sichuan populations; *thick yellow* and *black dotted lines* divide *C. duclouxiana* into northern, central, and southern populations; *thin yellow* and *black line* further divides central populations of this species into two management units (Color figure online)

Table 2 Microsatellite markers used in this study

Locus	Primer sequence (5′–3′)	Annealing temp (°C)	Repeat	Size (bp)
Cuc1	GACTTCATCCCTCTTATACATAGAC CTAGCTCCATTGACGTTTCATTCCC	55	$(CA)_{18}$	113–153
Cuc 6	ACTCCATGCCATTGCATGTTTTG ACAACCTACATAAAAGATGAGCA	52	$(TG)_{17}(GC)_4$	79–95
Cuc 7	CAACATACAAACATTAATGGTGTAG TGAGTGTATTTGAGCCAAGGTTC	52	$(TG)_{25}$	109–228
Cuc12	ACTGTCTCATGTTCTTGGTT GATGGAGATAATGATGGAAG	53	$(GT)_7$	108–132
Cuc13	TCCCATCAACATCTTCAA GGTGTCCACTTTCCCAAT	43	$(TC)_{13}(CA)_{16}$	129–208
Cuc14	CTCTTCTCAACTCTTCTCATCCTT ATTGGCCCAACCTAATAGTG	56	$(CA)_7$	118–136

Data Analysis

Chromatograms obtained from Genemapper 4.0 were scored into an original SSR dataset, where two alleles (of each SSR primer pair) of each individual were

encoded as the molecular size (in bp, base pairs) of the SSR-PCR products. Input files for different population genetic softwares were then prepared by transforming the original SSR dataset with DataTrans version 1.0 (Ge and Ren 2011). Analyses of genetic variability were performed with Popgene version 1.31 (Yeh et al. 1999) in terms of the average number of alleles per locus (A), effective number of alleles per locus (A_e), expected heterozygosity (H_e), observed heterozygosity (H_o), Shannon's information index (H_{pop}) (Lewontin 1972), Nei's (1973) expected heterozygosity and F-statistics (Wright 1965, 1978). Gene flow (N_m) was estimated using the equation $N_m = 0.25(1 - F_{ST})/F_{ST}$. These indices were calculated for each primer pair as well as mean values for all primer pairs in each species based on pooled population data (average over primer pairs). To facilitate comparison among populations within each species, A, A_e, H_o, H_e, H_{pop}, and Nei's expected heterozygosity were also estimated for each population and averaged over all populations in each species (average over populations).

Levels of genetic variation among species, within species among populations, and within populations were identified from cluster analysis, which involved estimating the allelic frequencies by analysis of molecular variance (AMOVA) using Arlequin version 3.01 (Excoffier et al. 2006), employing significance tests based on 1,000 permutations. Bayesian clustering analyses among and within species were determined using Structure version 2.3 (Hubisz et al. 2009) and the admixture model therein. The number of subpopulations (K) was set from 1 to 10, and for each K, 20 runs were carried out by fixing the burn-in period to 500,000 followed by 1,500,000 iterations. The number of population clusters (K) was estimated from the ΔK parameter (Evanno et al. 2005), and Distruct version 1.1 (Rosenberg 2007) was used to perform statistics and construct bar plots.

The effective population size (N_e) of the four species was estimated by Migrate version 3.2.1 (Beerli and Felsenstein 1999) based on the coalescent theory and maximum likelihood method, using pooled data for each species. Values of θ, which equals $4N_e\mu$ (where N_e is the effective population size and μ is the mutation rate for the microsatellite data set), were initially estimated. Assuming an average microsatellite mutation rate of 10^{-3} per generation, as assumed for other conifer species (e.g., Boys et al. 2005; Pandey and Rajora 2012), N_e was calculated as $\theta/4 \times 10^{-3}$. These estimations of θ were based on 20 short chains (10,000 trees) and three long chains (1,000,000) with 10,000 trees discarded as the initial burn-in.

Bottleneck tests for each species were estimated using the M-ratio method (Garza and Williamson 2001), which calculates the ratio of the total number of alleles to the overall range in allele size. We estimated the M-ratio assuming a microsatellite mutation rate of 10^{-3} and pre-bottleneck effective population size of 100 [$\theta = 4N_e\mu = 0.4$] and 400 ($\theta = 1.6$). The assumption of $\theta = 1.6$ was based on the estimated average effective population size for the four species examined in this study (average $N_e = 413.13$). As recommended by the developers (Garza and Williamson 2001), we set the base-step mutation and single-step mutation to 3.5 and 0.9, respectively. The M-ratio (M) and critical M-ratio (M_c) were estimated using the programs M_P_Val and Critical_M (Garza and Williamson 2001). We assumed that populations of a species experienced a significant bottleneck event if $M < M_c$ when $N_e = 400$, and a moderate bottleneck if $M < M_c$ when $N_e = 100$.

Results

Genetic Variation

The initial screening of 53 SSR primer pairs revealed that 6 primer pairs were polymorphic within all four species. These 6 primer pairs were then applied to survey the genetic variation of the four species across 483 trees from 35 populations. The results of statistical averaging over primer pairs (statistics for each of the six SSR primer pairs on pooled-population data for each species) showed that the mean observed heterozygosity (H_o) of *C. gigantea* was the highest (0.7954), *C. funebris* was the lowest (0.4920), and *C. chengiana* (0.5340) and *C. duclouxiana* (0.5425) were similar. However, the mean expected heterozygosity (H_e) and mean Shannon's Index (H_{pop}) of *C. gigantea* were the lowest (0.5987, 1.1978), *C. chengiana* was the highest (0.7636, 2.0083), and *C. funebris* (0.7109, 1.6706) and *C. duclouxiana* (0.7182, 1.7372) were close to each other (Table 3). Meanwhile, *C. gigantea* exhibited a higher mean H_o than mean H_e, and the other three exhibited a higher mean H_e than mean H_o (Table 3). When statistically averaging over populations (statistics of each population for a combination of all six SSR primer pairs), similar patterns were found when comparing the mean H_o, mean H_e, and mean H_{pop} among species (Table 4).

Genetic Structure Among and Within Species

AMOVA analyses (Table 5) revealed that total genetic variation was 17.24% among species, 8.67% within species among populations, and 74.09% within populations. For each of the four species, the proportion of intraspecific genetic variation among populations was similar for *C. chengiana* (11.00%), *C. duclouxiana* (12.76%), and *C. funebris* (11.85%) but much smaller for *C. gigantea* (3.02%). As shown in Table 3, the mean (average over primer pairs) fixation index (F_{ST}) of *C. duclouxiana* was the highest (0.1815), and *C. gigantea* was the lowest (0.0550), whereas the mean gene flow [N_m, equal to $0.25(1 - F_{ST})/F_{ST}$] of *C. duclouxiana* was the lowest (1.1272) and *C. gigantea* was the highest (4.2987).

From the Structure analysis, plots of ΔK and the variability of likelihood suggested that $K = 6$ is the most likely group division scheme; although $K = 7$ had the smallest ΔK, its likelihood variability was larger than the former (data not shown). When K was increased from 2 to 7, the 35 populations of the four *Cupressus* species clustered into groups as follows: when $K = 2$, the populations of *C. gigantea* clustered into an independent group, and populations of *C. chengiana*, *C. funebris,* and *C. duclouxiana* clustered together; when $K = 3$, the populations of *C. duclouxiana* became an independent group; when $K = 4$, the populations belonging to each of the four species clustered together; when $K = 5$, the populations of *C. duclouxiana* clustered into two groups (Pop 10–15 in the southern range and 16–19 in the northern range); when $K = 6$, the populations of *C. chengiana* clustered into two groups (Pop 1–5 in Sichuan and 6–9 in Gansu); when $K = 7$, the southern (10–13) and northern (17–19) populations of *C. duclouxiana* formed two relatively pure clusters, but these two clusters and a third one formed

Table 3 Genetic diversity and gene flow estimated among cypress populations based on pooled data

Locus	A	A_e	H_o	H_e	H_{pop}	F_{IS}	F_{IT}	F_{ST}	N_m
C. chengiana									
Cuc1	24.0000	8.6251	0.4724	0.8876	2.5007	0.3216	0.4623	0.2074	0.9553
Cuc6	21.0000	7.5332	0.5693	0.8704	2.3675	0.2761	0.3563	0.1108	2.0059
Cuc7	36.0000	7.3535	0.3409	0.8673	2.6107	0.5301	0.5968	0.1419	1.5116
Cuc12	9.0000	1.7380	0.2074	0.4262	0.9354	0.3561	0.5557	0.3100	0.5563
Cuc13	16.0000	4.7087	0.8397	0.7906	1.8936	−0.1373	−0.0496	0.0772	2.9898
Cuc14	12.0000	3.7942	0.7742	0.7394	1.7420	−0.1882	−0.0954	0.0781	2.9530
Mean	19.6667	5.6255	0.5340	0.7636	2.0083	0.1810	0.2980	0.1429	1.5000
C. duclouxiana									
Cuc1	10.0000	4.3571	0.5809	0.7733	1.6912	0.0819	0.3102	0.2487	0.7551
Cuc6	11.0000	3.9953	0.3609	0.7525	1.7113	0.4077	0.5050	0.1643	1.2715
Cuc7	13.0000	1.8511	0.4724	0.4616	1.8511	−0.2517	−0.0435	0.1663	1.2529
Cuc12	14.0000	3.2283	0.2887	0.6927	1.6341	0.3451	0.5789	0.3570	0.4502
Cuc13	30.0000	5.2874	0.7945	0.8137	2.4521	−0.1147	−0.0294	0.0765	3.0179
Cuc14	12.0000	5.3358	0.7576	0.8157	1.8216	0.0373	0.1316	0.0980	2.3013
Mean	15.0000	4.0092	0.5425	0.7182	1.7372	0.0856	0.2516	0.1815	1.1272
C. funebris									
Cuc1	6.0000	2.7223	0.6951	0.6365	1.2944	−0.2420	−0.1314	0.0890	2.5592
Cuc6	9.0000	4.6621	0.2644	0.7900	1.7584	0.5654	0.6381	0.1673	1.2444
Cuc7	24.0000	10.298	0.8020	0.9074	2.6152	0.0324	0.1168	0.0872	2.6181
Cuc12	10.0000	1.8273	0.1340	0.4551	1.0190	0.5661	0.6796	0.2615	0.7061
Cuc13	9.0000	3.8552	0.6947	0.7445	1.7047	−0.0996	0.0561	0.1416	1.5158
Cuc14	10.0000	3.6717	0.3617	0.7315	1.6319	0.3309	0.4970	0.2482	0.7572
Mean	11.3333	4.5063	0.4920	0.7109	1.6706	0.1579	0.2910	0.1580	1.3322

Table 3 continued

Locus	A	A_e	H_o	H_e	H_{pop}	F_{IS}	F_{IT}	F_{ST}	N_m
C. gigantea									
Cuc1	5.0000	1.6983	0.4157	0.4135	0.8332	−0.1323	−0.0689	0.0561	4.2094
Cuc6	9.0000	4.7318	0.9213	0.7931	1.6949	−0.2019	−0.1377	0.0534	4.4291
Cuc7	14.0000	3.0625	0.8095	0.6775	1.5124	−0.4335	−0.3563	0.0539	4.3886
Cuc12	7.0000	1.8935	0.5532	0.4744	0.8289	−0.2796	−0.1678	0.0873	2.6126
Cuc13	6.0000	2.2797	0.9574	0.5643	0.9554	−0.6881	−0.6433	0.0265	9.1718
Cuc14	9.0000	2.9885	0.8989	0.6691	1.3620	−0.4972	−0.4101	0.0582	4.0454
Mean	8.3333	2.7757	0.7594	0.5987	1.1978	−0.3811	−0.3051	0.0550	4.2987

A observed number of alleles, A_e effective number of alleles, H_o observed heterozygosity, H_e expected heterozygosity, H_{pop} Shannon's information index, F_{IS} inbreeding coefficient at the population level, F_{IT} inbreeding coefficient at the total sample level, F_{ST} proportion of differentiation among populations, N_m gene flow estimated from $N_m = 0.25*(1 - F_{ST})/F_{ST}$

Table 4 Genetic variation within populations of four *Cupressus* species averaged over six SSR loci

Population	A	A_e	H_o	H_e	H_{pop}	Nei's
C. chengiana						
Pop 1	5.6667	3.9280	0.4995	0.6480	1.3609	0.6156
Pop 2	4.5000	2.8977	0.5114	0.5731	1.0970	0.5488
Pop 3	5.1667	3.0933	0.5468	0.6113	1.2048	0.5903
Pop 4	8.3333	4.8533	0.6099	0.7719	1.7321	0.7524
Pop 5	5.1667	3.4033	0.5667	0.6501	1.2423	0.6170
Pop 6	6.1667	3.7069	0.4313	0.6064	1.2855	0.5845
Pop 7	7.8333	4.0793	0.5417	0.7462	1.6168	0.7288
Pop 8	7.6667	4.3734	0.5121	0.7320	1.6186	0.7139
Pop 9	4.3333	3.3603	0.5329	0.6853	1.2383	0.6486
Mean	6.0926	3.7439	0.5280	0.6694	1.3774	0.6444
C. duclouxiana						
Pop 10	3.3333	2.4167	0.6667	0.5732	0.9398	0.5255
Pop 11	3.0000	2.3356	0.5139	0.6163	0.9083	0.5341
Pop 12	4.6667	2.8166	0.5611	0.6203	1.1750	0.5961
Pop 13	5.8333	3.1063	0.6465	0.6744	1.3053	0.6596
Pop 14	7.5000	3.9634	0.5022	0.7199	1.5281	0.7044
Pop 15	5.3333	2.6433	0.3621	0.5919	1.1583	0.5777
Pop 16	4.3333	3.5283	0.3667	0.7077	1.2535	0.6419
Pop 17	4.1667	3.3564	0.5423	0.5342	1.0465	0.5070
Pop 18	4.3333	2.8742	0.6136	0.5670	1.0565	0.5389
Pop 19	6.6667	3.8210	0.6106	0.6173	1.3180	0.6026
Mean	4.9167	3.0862	0.5386	0.6222	1.1689	0.5888
C. funebris						
Pop 20	4.0000	2.6576	0.4560	0.5441	0.9931	0.5116
Pop 21	4.6667	3.5991	0.4861	0.6374	1.2096	0.6028
Pop 22	4.8333	3.3529	0.3836	0.6304	1.1737	0.5956
Pop 23	4.6667	3.3310	0.4028	0.6180	1.1420	0.5900
Pop 24	5.0000	3.4750	0.3821	0.6848	1.2771	0.6543
Pop 25	4.5000	3.2170	0.4909	0.5848	1.0854	0.5620
Pop 26	4.0000	3.0406	0.5472	0.5826	1.0570	0.5501
Pop 27	4.0000	3.1910	0.5833	0.6786	1.1744	0.6235
Pop 28	4.6667	3.2540	0.6825	0.6480	1.2213	0.6081
Pop 29	4.3333	3.5851	0.6429	0.7637	1.3330	0.7092
Mean	4.4667	3.2700	0.5057	0.6372	1.1667	0.6007
C. gigantea						
Pop 30	4.0000	2.5596	0.8167	0.6044	1.0429	0.5742
Pop 31	3.0000	2.5550	0.7976	0.5828	0.9088	0.5404
Pop 32	4.8333	2.8846	0.8101	0.6434	1.1664	0.6187
Pop 33	4.5000	2.3572	0.6765	0.5466	0.9865	0.5340
Pop 34	5.1667	3.1811	0.7388	0.5911	1.1429	0.5794
Pop 35	3.0000	2.3345	0.8259	0.5562	0.8832	0.5335

Table 4 continued

Population	A	A_e	H_o	H_e	H_{pop}	Nei's
Mean	4.0833	2.6453	0.7776	0.5874	1.0218	0.5633

Population codes as in Table 1

A total number of alleles per population, A_e effective number of alleles per population, H_o observed heterozygosity, H_e expected heterozygosity, H_{pop} Shannon's information index, *Nei's* Nei's expected heterozygosity

All indices are averaged over six nuclear SSR loci

Table 5 Analysis of molecular variance (AMOVA) for populations of four cypress species based on SSR markers

Cupressus species grouping	Source of variation	df	SS	VC	%V	P value
Total	Among species	3	301.96	0.3878	17.24	<0.0010
	Among populations within species	31	214.72	0.1949	8.67	<0.0010
	Within populations	933	1554.56	1.6662	74.09	<0.0010
C. chengiana	Among populations	8	67.54	0.2138	11.00	<0.0010
	Within populations	277	479.10	1.7296	89.00	
C. chengiana	Among groups	1	14.26	0.0675	5.05	<0.0010
	Among populations within groups	7	29.76	0.0997	7.46	<0.0010
	Within populations	277	323.72	1.1687	87.49	<0.0010
C. duclouxiana	Among populations	9	77.96	0.2467	12.76	<0.0010
	Within populations	282	475.64	1.6867	87.24	
C. duclouxiana	Among groups	1	30.85	0.1877	10.16	<0.0010
	Among populations within groups	8	42.18	0.1361	7.37	<0.0010
	Within populations	282	429.77	1.5240	82.48	<0.0010
C. funebris	Among populations	9	53.83	0.2173	11.85	<0.0010
	Within populations	192	310.36	1.6165	88.15	
C. gigantea	Among populations	5	15.39	0.0495	3.02	<0.0010
	Within populations	182	289.46	1.5905	96.98	

df degrees of freedom, *SS* sum of squares, *VC* variance component, *%V* percentage of variance explained

P-value estimated by a permutation procedure based on 1,000 replicates

mixtures in the central populations (14–16). Bayesian clustering plots for all populations of the four species when $K = 6$ and $K = 7$ are shown in Fig. 2.

Estimation of Effective Population Size (N_e) and Bottleneck Test

The estimated effective population size of *C. chengiana* was the highest ($N_e = 756.23$), at approximately 1.70 times the estimate for *C. duclouxiana*

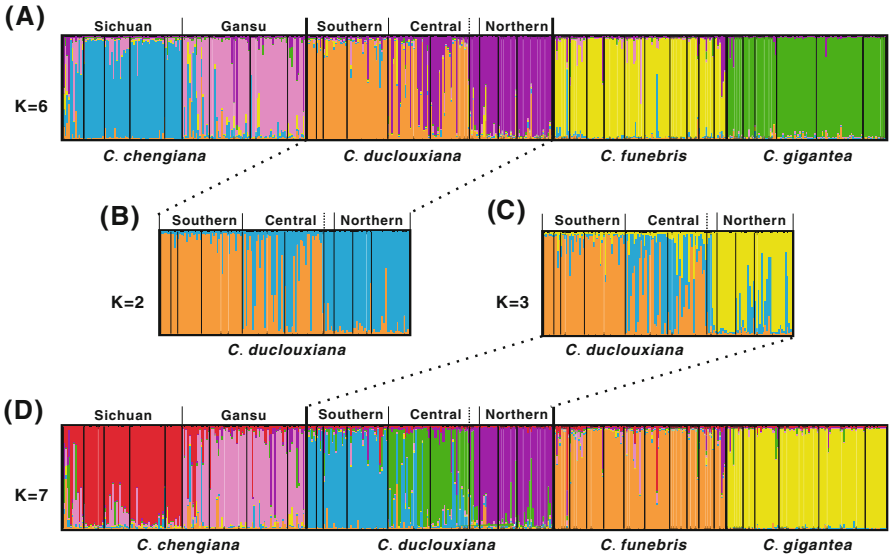

Fig. 2 Bayesian clustering plots of all populations of *Cupressus chengiana*, *C. duclouxiana*, *C. funebris,* and *C. gigantea* when $K = 6$ (*top*) and $K = 7$ (*bottom*), and populations of *C. duclouxiana* only (*center*) when $K = 2$ and $K = 3$. Each color represents one genetic lineage, and each vertical column represents one individual. *Thin vertical lines* divide populations, and *thick vertical lines* divide species. Subdivisions of each species are marked on the upper part of each plot, and *thin/dotted lines* divide *C. chengiana* and *C. duclouxiana* into two evolutionarily significant units and four management units (Color figure online)

($N_e = 445.25$), 2.91 times that of *C. chengiana* ($N_e = 259.62$), and 3.91 times that of *C. gigantea* ($N_e = 193.40$). The *M*-ratio test revealed significant bottlenecks in *C. gigantea* ($M < M_c$ when the pre-bottleneck *Ne* = 400), and moderate bottleneck signals were detected in *C. duclouxiana* and C. *funebris* ($M < M_c$ when the pre-bottleneck *Ne* = 100), but no significant bottleneck signal was detected in *C. chengiana*.

Discussion

Genetic Diversity

Surveys of genetic diversity in endangered and vulnerable species provide important information for evaluating the evolutionary and adaptation potential of these species, as well as developing conservation and sustainable management strategies to protect their populations from both short- and long-term climate changes (Hedrick 2004). In this study, we investigated the genetic diversity of *C. chengiana*, *C. duclouxiana*, *C. funebris,* and *C. gigantea* by employing nuclear microsatellite markers (nrSSRs) for the first time. Allelic diversity (A, A_e), expected heterozygosity (H_e), and Shannon's index (H_{pop}) are the three most important and commonly used measures of genetic diversity in natural populations (Hamilton 2009; Freeland

et al. 2011). Allelic diversity indices (A, A_e) are measures of allelic richness, H_e is a measure of allelic evenness, and H_{pop} is a measure of both allelic richness and evenness (Hamilton 2009; Freeland et al. 2011). Among these four species, H_e, H_{pop}, and the observed (A) and the effective number of alleles (A_e) were highest in *C. chengiana*, slightly lower for *C. duclouxiana* and *C. funebris*, and lowest in *C. gigantea* (Table 3). In contrast, the observed heterozygosity (H_o) was highest in *C. gigantea*, lower in *C. chengiana* and *C. duclouxiana*, and lowest in *C. funebris* (Table 3). Usually, the lowest value of H_o is around 25% less than the highest value (Table 3). However, a previous study has indicated that the distribution of plastid allele diversity is more uneven among these species (Xu et al. 2010), although the ranking of allele richness within each species was the same for both plastid and nuclear markers. Although abundant plastid alleles were found in *C. chengiana* (18 haplotypes) and *C. duclouxiana* (9 haplotypes), both *C. funebris* (2 haplotypes) and *C. gigantea* (1 haplotype) were shown to have an extremely poor plastid allele richness (Xu et al. 2010). The stark difference in allele richness allocation among species between nuclear and plastid markers may be due to the different effective population sizes (N_e) of these two types of markers. When the sex ratio is equal to one, N_e of biparental nuclear markers is four times that of uniparental markers (Hamilton 2009; Freeland et al. 2011). Higher gene flow of the plastid genome (via pollen) than nuclear genome (via both pollen and seeds) in wind-pollinated *Cupressus* may also have contributed to this pattern (Petit and Excoffier 2009).

Genetic drift depletes expected heterozygosity very slowly at a rate of half the N_e per generation. Similarly, bottleneck decreases H_e by 1/2 N per generation, where N is the effective population size during the bottleneck (Hamilton 2009; Freeland et al. 2011). This may explain the different patterns of allelic diversity and heterozygosity observed in our study, as both N_e estimations and bottleneck tests suggest that a species with smaller N_e and a stronger bottleneck signal usually has lower allelic diversity and expected heterozygosity (Tables 3, 4). According to Migrate estimates, N_e of *C. chengiana* is the largest, approximately 1.70, 2.91, and 3.91 times that of *C. duclouxiana*, *C. funebris,* and *C. gigantea,* respectively. Meanwhile, the bottleneck test revealed significant bottlenecks in *C. gigantea*, moderate bottleneck signals in *C. duclouxiana* and *C. funebris*, but no significant bottleneck signals in *C. chengiana*.

These findings of the population genetics from nuclear microsatellite markers agree well with the plastid allelic diversity based hypothesis (Xu et al. 2010). *Cupressus gigantea* occurs in the river valleys of the eastern Qinghai-Tibetan Plateau at altitudes around 3,000–3,400 m (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005), and because of the Quaternary glacial cycles and/or recent human activities, this species may have experienced severe bottlenecks. *Cupressus funebris* is found in central and southeastern China and adjacent regions (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005), where adverse climatic effects during glacial periods were probably less intense. However, due to the long history of exploitation and cultivation of this species, cultivated populations or mosaic populations (comprising both cultivated and wild individuals) are widespread (Zheng and Fu 1978); a few wild populations unaffected by cultivation are found in mountainous areas. Cultivation may have acted as a stronger bottleneck to plastid allelic richness

than nuclear allelic richness, since only two prevalent haplotypes were preserved, but the average observed and effective allele numbers (A and A_e) of nuclear markers were slightly lower or similar to those of *C. chengiana* and *C. duclouxiana*. The other two species, *C. duclouxiana* and *C. chengiana*, which occur at lower altitudes at the eastern margin of the Qinghai-Tibetan Plateau (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005), might have experienced relatively fewer bottlenecks and/or founder effects. However, given that *C. duclouxiana* has been cultivated relatively recently and only in North Yunnan but most populations of *C. chengiana* are natural, it seems likely that human activities as well as subsequent bottlenecks and/or founder effects may have had a stronger impact on *C. duclouxiana* than *C. chengiana*. This hypothesis is supported by our finding that a moderate bottleneck was inferred for *C. duclouxiana* and none for *C. chengiana*, and the effective population size of the former was only two thirds of the latter (445.25 vs. 756.23).

Although these four Asian cypresses may have experienced different demographic histories, their observed level of microsatellite genetic diversity was similar to or slightly lower than the congeneric *C. sempervirens* from the Mediterranean ($A_e = 4.285$, $H_e = 0.648$, $H_o = 0.494$; Bagnoli et al. 2009). When compared with other conifers, however, the microsatellite allelic diversity of these four Asian cypresses was lower than that reported for white spruce (*Picea glauca*, $A = 16.38$; Rajora et al. 2005), black spruce (*Picea mariana*, $A = 14.03$; Pandey and Rajora 2012), red cedar (*Thuja plicata*, $A = 10.33$; O'Connell et al. 2008), eastern white pine (*Pinus strobus*, $A = 9.44$; Rajora et al. 2000), eastern white cedar (*Thuja occidentalis*, $A = 7.32$; Pandey and Rajora 2012), and red spruce (*Picea rubens*, $A = 7.0$; Pandey and Rajora 2012). However, heterozygosity levels in the four Asian species were lower than in white spruce ($H_o = 0.649$, $H_e = 0.851$; Rajora et al. 2005) but similar to red cedar (H_o not reported, $H_e = 0.75$; O'Connell et al. 2008), eastern white cedar ($H_o = 0.601$, $H_e = 0.611$; Pandey and Rajora 2012), and eastern white pine ($H_o = 0.521$, $H_e = 0.607$; Rajora et al. 2000), and were higher than in red spruce ($H_o = 0.397$, $H_e = 0.528$; Pandey and Rajora 2012) and Sitka spruce (*Picea sitchensis*; $H_e = 0.580$; Gapare et al. 2005). As shown above, these four Chinese cypresses harbor moderate levels of genetic diversity among conifers.

Genetic Structure and Divergence Within Species

In agreement with Xu et al. (2010), who surveyed the phylogeographic pattern of Asian cypresses using plastid DNA sequence variations, AMOVA analyses of nuclear microsatellite data in the present study suggest that the genetic variation component among species was greater than that among populations within species. Whereas analysis of nuclear markers revealed that most components of total genetic variation existed within populations (74.1%, Table 5), analysis of plastid DNA sequence variations suggested that the major part of genetic variation existed among species (63.5%), and the ratio of the genetic variation component among species versus that among populations within species (Table 5) was much higher in plastid markers (63.5 vs. 7.7%) than in nuclear markers (17.2 vs. 8.7%). Such a sharp contrast may be explained by a faster lineage sorting speed of plastid markers than nuclear markers due to a smaller effective population size of the former (Freeland

et al. 2011). This result was also in line with a previous prediction that species delimitation should be more effective with markers experiencing high levels of gene flow (e.g., Petit and Excoffier 2009; Du et al. 2009). For the four *Cupressus* species considered here, plastid markers were found to be more efficient than nuclear markers in delimitating species, since gene flow of the paternally inherited plastid genome (mediated by pollen) is higher than the biparentally inherited nuclear genome (mediated by both pollen and seeds).

Within each of the four species, most components of the total genetic variation were found within populations; genetic variations among populations were similar for *C. duclouxiana* (12.76%), *C. chengiana* (11.00%), and *C. funebris* (11.85%), but much lower for *C. gigantea* (3.02%, Table 5). Smaller genetic differentiation among populations is usually mediated by strong and frequent gene flow. Because all six populations of *C. gigantea* were collected from the major river valley of Yarlung River (Fig. 1), gene flow among populations of this species is likely to be easier than for the other species, which occur either in isolated mountain valleys (*C. chengiana* and *C. duclouxiana*) or on a vast area of hill ranges (*C. funebris*). This is consistent with a previous survey based on plastid markers (Xu et al. 2010), where the allocation of genetic variation among populations was found to be higher in both *C. chengiana* (45.7%) and *C. duclouxiana* (23.3%), and a single plastid haplotype was detected across all sampled populations of *C. gigantea* (i.e., zero variation among and within populations). In contrast to the present study, only two haplotypes were identified in *C. funebris*, both of which were found in most populations across the whole distribution range of this species (Xu et al. 2010). Taken together with the findings on nuclear genetic structure, the most plausible explanation for such a genetic pattern is that *C. funebris* has been exploited and cultivated locally in different populations, and the ratio of cultivated to wild individuals became higher in these mosaic populations during the species' long history of cultivation (Bagnoli et al. 2009). During this process, some of the nuclear and plastid genetic variation was retained, but because of the founder effect, strong genetic drift caused by a smaller effective population size and strong wind mediated gene flow of the plastid genome (via pollen), these rare plastid haplotypes were completely lost and only two abundant plastid haplotypes were retained. In contrast, strong pollen-mediated gene flow of the nuclear genome affects only half of all nuclear alleles (Bagnoli et al. 2009; Freeland et al. 2011).

Bayesian clustering analysis of the four species showed that individuals of *C. chengiana* were clustered into two groups when $K = 6$ and $K = 7$ (Fig. 2). When considering only individuals of this species, $K = 2$ was the most likely subdivision scheme, since its likelihood variability was estimated to be the smallest. The division of this species into two groups confirmed previous results from plastid data (Xu et al. 2010), which showed that haplotypes from populations in Gansu and Sichuan clustered into two phylogenetic lineages. Similarly, a previous population genetic survey of this species using ISSR (inter-simple sequence repeat) markers also found that populations from Gansu and Sichuan are distinguishable in cluster analyses (Hao et al. 2006). Therefore, plastid data, nuclear microsatellite (SSR), and ISSR data support the proposal of Silba (1994, 1998) that plants from these two provinces should be treated as two independent varieties.

Notably, Bayesian clustering analysis showed that populations from *C. duclouxiana* could also be divided into two groups when $K = 5$, suggesting that the genetic differentiation between the two groups in *C. duclouxiana* may be even higher than that between the two putative varieties of *C. chengiana*. Independent Bayesian clustering analysis of *C. duclouxiana* populations also revealed that $K = 2$ was the best structure according to plots of ΔK and the variability of likelihood. As shown in Fig. 1, the Benzilan (17), Daocheng (18), and Deqin (19) populations were collected from valleys in the Hengduan mountains above 2,550 m (northern populations); populations from Yulong (14), Lijiang (15), and Xianggelila (16) were collected from the southern edge of the Hengduan (central populations); and populations from Kunming (10), Lufeng (11), Eryuan (12), and Yongsheng (13) were collected from the highlands to the south of the Hengduan at altitudes below 2,200 m (southern populations). When all sampled individuals of *C. duclouxiana* were clustered into two lineages ($K = 2$, Fig. 2), it was apparent that Pop 10–13 were a relatively pure stand of one lineage, 16–19 were a relatively pure stand of another lineage, and 14 and 15 were a mixture of both lineages. However, when $K = 3$, southern (10–13) and northern populations (17–19) were predicted to be relatively pure stands of the first and second lineages, respectively; Pop 14 and 15 were dominated by a third lineage; and Pop 16 was a mixture of the second and third lineages ($K = 3$, Fig. 2). Obviously, these results indicate an early divergence between the southern and northern populations in the evolutionary history of this species. This may have taken place during the Quaternary climate oscillations, with these two lineages surviving in two isolated refugia in the northern and southern ranges. Then, either during interglacial or glacial periods, populations of these two lineages may have met in the central range, producing mixed genotypes. The plastid haplotype distribution (Xu et al. 2010) fits with this hypothesis, since H23 and H21 are dominant in the northern populations and H22 and H24 are relatively dominant in the southern populations. All seven haplotypes, however, were found in the central populations, and the three low-frequency haplotypes (closer to H23) were found only in Pop 16 (Xianggelila). Higher plastid haplotype diversity in the central populations may be due to two possible scenarios: this area is either where southern and northern haplotypes met during the interglacial period, or a third refugium area where haplotypes from both northern and southern populations mixed well with each other during the glacial period. Regardless, the results suggest that *C. duclouxiana* may have a complex evolutionary history, involving cryptic divergence into southern and northern lineages and, afterward, a mixture of the two lineages in the central part of the distribution ranges. Notably, a similar intraspecific divergence pattern has been found in other species in the Qinghai-Tibetan Plateau and adjacent areas (e.g., Zheng et al. 2008; Wang et al. 2009; Tang et al. 2010; Jia et al. 2011).

Implications for Conservation

Understanding the extent and distribution pattern of genetic diversity is essential for the conservation and exploitation of tree species (Hedrick 2004). Heterozygosity and allelic richness are two popular indices for measuring genetic diversity (Hamilton 2009; Freeland et al. 2011). Usually, heterozygosity enables populations

and species to respond to immediate or short-term selection, whereas allelic richness is important for long-term survival and evolution of populations and species (Allendorf 1986; Freeland et al. 2011). Therefore, the conservation and management of tree species should prioritize populations with high allelic richness and heterozygosity. If genetic divergence among lineages in a species is strong, each lineage should be treated as an independent conservation unit, for example, management unit (MU) or evolutionarily significant unit (ESU) (Freeland et al. 2011). A MU is any population that exchanges so few migrants with others as to be genetically distinct from them (Avise 2000). Distinct MUs are often identified on the basis of significant differences in allele frequencies at multiple neutral loci. An ESU consists of one or more populations reproductively isolated for a considerable period of time, during which they have followed separate evolutionary pathways (Freeland et al. 2011). ESUs have often been characterized by reciprocal monophyly in phylogenetic trees based on uniparental inherited organelle DNA (i.e., mtDNA and cpDNA) and significant allele frequency differences at neutral nuclear loci (Moritz 1994; Crandall et al. 2000).

Cupressus duclouxiana, the Yunnan cypress, is mainly distributed in central and northwest Yunnan and southwest Sichuan, at altitudes ranging from 1,400 to 3,300 m (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005). Due to habitat loss, this species has been listed as endangered in the Red List of Threatened Species (IUCN 2012). Genetic structure analyses of microsatellite data suggested that populations from the northern and southern (distribution) ranges formed two distinct genetic lineages, which mixed with each other in the central range (Fig. 1). The geographic distribution of plastid haplotypes showed a similar pattern of different dominant haplotypes in the northern and southern ranges, whereas all rare dominant haplotypes were found in the central range (Xu et al. 2010). Therefore, populations in the southern (Kunming, Lufeng, Yongsheng, and Eryuan; Pops 10–13) and northern (Daocheng, Deqin, and Benzilan; Pops 17–19) ranges should be considered as southern and northern MUs. In the central range, the allele frequencies of Lijiang and Yulong (Pops 14–15) were similar, but that of Xianggelila (Pop 16) was different (Fig. 2, $K = 3$). Therefore, two independent MUs should be considered. Since the plastid haplotype diversity and either allelic richness or expected heterozygosity of nuclear microsatellites was higher than the average value in the two central range MUs (Table 4), in situ protection (e.g., nature reserve area) should be seriously considered for them. Meanwhile, genetic diversity was highest in Deqin among populations within the northern MU and in Yongsheng within the southern MU. Therefore, we suggest establishing four nature reserves to preserve the majority of genetic diversity within Yunnan cypress (ideally all possible populations): one around Xianggelila (Pop 16), another around Lijiang and Yulong (Pops 14–15), a third in Deqin county (Pop 19), and the fourth in Yongsheng county (Pop 13).

The Minjiang cypress (*Cupressus chengiana*) grows in southern Gansu and the Minjiang watershed of Sichuan at altitudes ranging from 800 to 2,900 m (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005). This species has been listed as vulnerable in the Red List of Threatened Species (IUCN 2012) and as an endangered species in China (NAEP and IBCAS 1987). Previous work has suggested that plastid

haplotypes detected in populations from Gansu and Sichuan cluster into two distinct monophyletic lineages. Such a phylogeographic pattern conforms with the taxonomic treatment of this species as two varieties, *C. chengiana* var. *kansouensis* Silba and *C. chengiana* var. *wehchuanhsiensis* Silba (Silba 1994; Farjon 2005). The Bayesian clustering analysis of microsatellite data also supported this taxonomic treatment, as genotypes clustered into two lineages that are dominant in populations from either Gansu or Sichuan (Fig. 2). Although further morphological data at the population level is needed to confirm this taxonomic treatment, these populations from Gansu and Sichuan should at least be treated as two ESUs, particularly as they are reciprocally monophyletic in phylogenetic trees of plastid haplotypes (Xu et al. 2010). In addition, the allelic frequencies of the six microsatellite loci for the two groups were found to be very different (Fig. 2). Currently, this species has a restricted distribution. Therefore, we suggest establishing nature reserves (ideally for all possible populations) in Jinchuan (Pop 4) in Sichuan ESU, and in Wudu and Wenxian (Pop 7 and 8) in Gansu ESU, since the genetic diversity of populations in these areas is relatively high. For *C. chengiana*, as well as *C. duclouxiana*, the natural regeneration rate should be observed further, and if an artificial regeneration program in situ seems necessary, then as many populations as possible should be involved in local MUs or ESUs.

Cupressus gigantea, also known as the Tsangpo river cypress, occurs along the Yarlung Tsangpo river valley at altitudes generally above 3,000 m, and the distribution of this species is currently fragmented and highly disturbed (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005). This species has been listed as vulnerable in the Red List of Threatened Species (IUCN 2012) and as an endangered species in China (NAEP and IBCAS 1987). Our results revealed that this species has the lowest genetic diversity among the four species, and ideally, all populations should be protected so as to preserve as much genetic diversity as possible. Currently, only the largest relict population in Linzhi has been protected by the designation of a local natural protection region. Further, most material used in artificial regeneration programs conducted by the local governments has originated from this population (NAEP and IBCAS 1987). We suggest a new natural protection region should be established at least in Langxian (Pop 32), since based on microsatellite, ISSR, and RAPD data, allelic richness and heterozygosity of populations in this area are higher than average, and often higher than the Linzhi population (Xia et al. 2008). As a previous survey of genetic diversity using ISSR and RAPD data detected significant differentiation among different populations of this species (Xia et al. 2008), it is better to include as many populations as possible when conducting artificial recruitment and replanting programs in its original habitat and adjacent areas.

The Chinese weeping cypress, *C. funebris*, is widely distributed in southwestern and central China, as well as Vietnam. Owing to its high economic and ornamental value, this species is widely cultivated in southern China and other warm temperate and temperate regions (Zheng and Fu 1978; Fu et al. 1999; Farjon 2005). Because of its long history of cultivation and exploitation, the natural distribution of this species is uncertain. In this study, we examined two putative wild populations and eight putative cultivated populations and showed that genetic diversity detected by microsatellite markers was similar to that of the Mediterranean *C. sempervirens*

(A_e: 3.2700 vs. 3.2760; H_e: 0.6372 vs. 0.6480; H_o: 0.5057 vs. 0.4940), which has "a mosaic of recently introduced trees and remnants of ancient and depauperate populations in the central Mediterranean range" (Bagnoli et al. 2009). The average genetic diversity indices of *C. funebris* decreased only slightly when putative wild populations were excluded ($A_e = 3.2330$, $H_e = 0.6201$, $H_o = 0.4665$). The bottleneck test, however, showed that this species may have experienced a moderate bottleneck event when we assumed a pre-bottleneck value of $N_e = 100$. Therefore, it is reasonable to assume that these eight putative cultivated populations may have included remnants of wild populations and recently cultivated trees, which were collected from local populations. Such mosaic cultivation would be efficient in preserving nuclear alleles. This hypothesis needs to be further tested with a wider sampling coverage of wild populations of *C. funebris*. Nevertheless, the results showed that average genetic diversity indices of wild populations were higher than putative cultivated populations (A_e: 3.4196 vs. 3.2330, H_e: 0.7059 vs. 0.6201, H_o: 0.6627 vs. 0.4665). Therefore, to enhance the survival rate of this species to environmental changes, we strongly advise that wild populations, especially the core populations that dominate forest communities in mountain ranges, should be protected from exploitation.

In summary, the results suggested that the four Chinese cypresses may have experienced different demographic histories. Climate change, exploitation, and cultivation have probably had different impacts on these species. To better preserve their genetic diversity, we suggest that the two groups within *C. chengiana* and four groups within *C. duclouxiana* should be managed as two ESUs and four MUs, respectively. It is imperative to protect more natural distributions of *C. gigantea* and to avoid either exploitation or disturbance of the wild populations of *C. funebris*.

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